The impact of iron on the function and composition of the human gut microbiota

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Abstract

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Iron-supplements are widely consumed; however, most of the iron is not absorbed and enters the colon where potentially pathogenic bacteria can utilise it for growth. Assessing iron bioavailability and the effects on bacterial groups is an evolving subject area and forms the basis of the research presented in this thesis.

The growth of *Escherichia coli* and *Salmonella* Typhimurium was significantly impaired when cultured independently in iron-deficient media ($p<0.0001$). These observations positively correlated with a decrease in water-soluble iron concentrations present in the culture. However, depletion of iron did not affect the growth of the beneficial species, *Lactobacillus rhamnosus*.

Culturing human faecal microbiotas in an *in vitro* colon model identified changes in the growth of different bacterial taxa. 16S rDNA-based metataxonomics indicated that under conditions of iron depletion through BPDS, a chemical iron chelator, the relative abundance of several taxa decreased, including a 10% and 15% decrease in *Escherichia* and *Bifidobacterium,* respectively. This was supported by observations of lower viable counts of *Enterobacteriaceae* and bifidobacteria. Analysis using ^{1}H NMR indicated that the production of acetate, butyrate and propionate *in vitro* was reduced under iron-restricted conditions. Iron chelation through phytin, a dietary compound, illustrated similar results with the exception of a 33% increase in the relative abundance of *Bifidobacterium* and 225% increase in *Collinsella*. Furthermore, increases in propionate and formate concentrations were also observed when cultured with phytin.

A 6-week, crossover double-blinded randomised human dietary intervention trial was performed (n=14), where participants were asked to consume encapsulated phytin or placebo. Capsules were coated with a specialised formulation, Phloral®, designed to release phytin directly in the colon. No conclusions could be made regarding the iron chelating properties of phytin as analysis of stool samples collected revealed clumps of phytin and therefore, unsuccessful dispersal of phytin within the colonic lumen. This pilot human intervention study indicates that the form of phytin is an important factor and this should be considered for follow-up studies.

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Abbreviations

16S rDNA	16S ribosomal deoxyribonucleic acid
16S rRNA	16S ribosomal ribonucleic acid
^{1}H NMR	Proton nuclear magnetic resonance
22D	2,2-dipyridyl
ACD	Anaemia of chronic disease
Ahp	Alykylhydroperoxide reductase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
API	Active pharmaceutical ingredient
AST	Aspartate aminotransferase
ATCC	American Type Culture Collection
BCFA	Branched chain fatty acid
BHI	Brain heart infusion
BMI	Body mass index
BPDS	Bathophenanthroline disulphonic acid
$C_6H_{17}N_3O_7$	Ammonium citrate dibasic
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride anhydrous
$CaCl_2.2H_2O$	Calcium chloride dihydrate
$CaCl_2.6H_2O$	Calcium chloride hexahydrate
$CH_3COONa.3H_2O$	Sodium acetate trihydrate
CO_2	Carbon dioxide
CRP	C-reactive protein
D_2O	Deuterium oxide
DcytB	Duodenal cytochrome B
DES	DNA elution solution
dH_2O	Deionised water
DMT1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
DPS	DNA binding protein from starved cells

DSS	Dextran sulphate sodium
EPoM	The Effect of Phytin on the Human Gut Microbiome
FAAS	Flame atomic absorption spectrophotometry
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
$FeSO_4$	Ferrous sulphate
FLVCR	Feline leukaemia virus subgroup C receptor
FPN1	Ferroportin
Fur	Ferric uptake regulator
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GI	Gastrointestinal
HbA1c	Haemoglobin A1c
HCl	Hydrochloric acid
HFE	Human homeostatic iron regulator protein
HH	Hereditary haemochromatosis
HMP	Human Microbiota Project
HNO_3	Nitric acid
HO-1	Haem oxygenase 1
HPLC	High performance liquid chromatography
HPMC	Hypromellose cellulose
HrtAB	Haem regulated ABC transporter
IDA	Iron deficiency anaemia
IL-6	Interleukin 6
InsP	Inositol phosphate
$InsP_2$	Inositol bisphosphate
$InsP_3$	Inositol trikisphosphate
$InsP_4$	Inositol tetrakisphosphate
$InsP_5$	Inositol pentakisphosphate
$InsP_6$	Inositol hexakisphosphate
IRE	Iron responsive element
IRP	Iron regulatory protein
K_2HPO_4	Dipotassium phosphate

KH_2PO_4	Monopotassium phosphate
LB	Luria broth
Lf	Lactoferrin
LPI	Labile plasma iron
LPS	Lipopolysaccharide
M.Tb	Mycobacterium tuberculosis
M9	Minimal media
Mg^{2+}	Magnesium ion
$MgSO_4$	Magnesium sulphate
$MgSO_4.6H_2O$	Magnesium sulphate hexahydrate
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
MRS	Man Rogosa Sharpe
Na_2HPO_4	Disodium phosphate
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaN_3	Sodium azide
NaOH	Sodium hydroxide
NCTC	The National Collection of Type Cultures
NDO	Non-digestible oligosaccharides
NGAL	Neutrophil gelatinase associated lipocalin
NH_4Cl	Ammonium chloride
NMR	Nuclear magnetic resonance
NNUH	Norfolk and Norwich University Hospitals
NTBI	Non transferrin bound iron
OD_{600}	Optical density at 600 nm
OH^{-1}	Hydroxide
OUHT	Oxford University Hospitals
OUT	Operational taxonomic unit
PA	Phytic acid
PBS	Phosphate buffered saline
PCA	Principle component analysis
PCFT/HCP1	Proton-coupled folate transporter/haem carrier protein 1

PCoA	Principle co-ordinate analysis
PCR	Polymerase chain reaction
Phy	Phytin
PPS	Protein precipitation solution
QIB	Quadram Institute Bioscience
QIIME	Quantitative Insights Into Microbial Ecology
qPCR	Quantitative polymerase chain reaction
RBC	Red blood cells
RDP	Ribosomal database project
RES	Reticuloendothelial system
ROS	Reactive oxygen species
SCFA	Short chain fatty acid
SEM	Standard error of mean
SEWS-M	Salt/ethanol wash solution
SMF	Starch moisture factor
SodA	Superoxide dismutase
TA	Tannic acid
TBE	Tris/borate/ethylenediaminetetraacetic acid
Tf	Transferrin
Tfr1	Transferrin receptor 1
Tfr2	Transferrin receptor 2
TSP	Sodium 3-(Trimethylsilyl)-propionate- d4
UTR	Untranslated region
vs	Versus
WHO	World Health Organisation
Zn^{2+}	Zinc ion

Symbols

%	percentage
˚C	degrees Celsius
±	plus/minus
ΔW	difference in weight
µg/dL	microgram per decilitre
µg/L	microgram per litre
µg/mL	micrograms per millilitre
µL	microlitre
µM	micromole per litre
bp	base pairs
cm^2	square centimetre
g	Gram
g/d	gram per day
g/L	gram per litre
h	hours
Hz	hertz
IU/mL	International units per millilitre
kg	kilogram
Kg/cm^2	Kilogram per square metre
L	Litre
M	Moles per litre
mg	milligrams
mg/cm^2	milligram per square metre
mg/g	milligram per gram
mg/L	milligram per litre
mg/meal	milligrams per meal
mg/mL	milligrams per millilitre
min	minutes
mL	millilitres
mm	millimetre

mM	millimoles per litre
mm^2	square millimetre
mmol/mol	millimole per mole
ms	millisecond
ng/µL	nanogram per microlitre
ng/mL	nanograms per millilitre
nm	nanometre
nmol	nanomoles per litre
pH	potential of Hydrogen
ppm	parts per million
rpm	revolutions per minute
s	seconds
T0	pre inoculation
T24	24 hours post-inoculation
T8	8 hours post-inoculation
w/v	mass per volume
xg	times gravity
y	years
π	Pi

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CHAPTER ONE

1 An introduction to iron, the human gut microbiota and the relationship between the two

1.0 Introduction to iron

From ancient times, the special role of iron in health and disease has been recognised by man [1, 2] and has been used for its medicinal purposes by Greeks, Romans, Hindus and Egyptians [3]. During the 17th century, treatment of chlorosis (green disease), a condition arising due to iron deficiency, was treated with the administration of iron [4]. However, 1932 marked the year where the importance of iron was highlighted and it was proved that inorganic iron was essential for haemoglobin synthesis [5]. In humans, iron is incorporated into proteins as a component of haem (e.g. haemoglobin, cytochrome proteins, myoglobin, nitric oxide synthetases, myeloperoxidase), iron sulphur clusters (DNA primase, respiratory complexes I-III) or other functional groups [6]. Essential cellular and organismal functions are dependent on these iron-containing proteins, some of which include the transport of oxygen, mitochondrial respiration, nucleic acid replication and repair, cell signalling and host defence [7-12].

The oxygenation of the Earth's atmosphere over 2 billion years ago led to the oxidisation of the abundant soluble ferrous iron (Fe^{2+}) to insoluble ferric iron (Fe^{3+}), causing iron bioavailability to decrease [13]. Simultaneously, the potential toxic nature of iron also increased due to the redox cycling of iron under oxygenated environments. This leads to catalysis of free radicals by hydrogen peroxide, named the Fenton reaction, and subsequently causes damage to DNA, lipids and proteins [14-16]. Subsequently, humans as well as other organisms have acquired specialised proteins and strictly regulated homeostatic mechanisms for the uptake, transport, storage and export of iron to ensure iron availability for vital biological processes, but at the same time regulate the toxicity of excess iron.

1.1 Iron absorption, metabolism, regulation and homeostasis

1.1.1 Biochemistry and physiology

Iron is an abundant element on earth and is a biologically essential component of every living organism [17-19]. In response to iron scarcity, a variety of cellular mechanisms have developed to obtain iron from the surrounding environment in biologically useful forms. Such examples are siderophores (further details in section 2.2.2.1), iron-scavenging molecules secreted by microbes [20], or mechanisms that involve the reduction of Fe^{3+} to the soluble Fe^{2+} as in yeasts [21]. Analogous counterparts are also found in higher organisms, including humans, where iron is found mostly as complex forms bound to

various proteins (haemoprotein) as haem compounds (haemoglobin or myoglobin), haem enzymes or non-haem compounds (transferrin, ferritin) [3]. Iron is required for the production of oxygen transport proteins, especially haemoglobin and myoglobin, and is also necessary for the formation of haem enzymes and other iron-containing enzymes that are a part of the electron transfer process as well as oxidation-reductions [3, 22]. Approximately two-thirds of the iron within the body is found in haemoglobin present within circulating erythrocytes and is recycled in the process of erythrophagocytosis by reticuloendothelial macrophages. These iron-recycling macrophages are a major storage site of iron, along with liver hepatocytes. In comparison, all other cells in the body contain smaller amounts of iron for important cellular processes. A quarter of the iron is present within iron storage compartments and the remaining 15% is bound to myoglobin in muscle tissue and in a range of enzymes contributing to oxidative metabolism and other cellular functions [23].

1.1.2 Iron absorption and recycling

The absorption of iron by the epithelial cells of the small intestine is an extremely tightly regulated process and any disruption in this process could hinder the body's iron homeostasis [24]. The small intestine is responsible for iron uptake and transport into the systemic circulation, under the control of hepcidin (further detail on this protein can be found in section 1.1.5.1), and therefore absorption of iron correlates with the body's iron status or requirements when in normal physiological conditions [25]. For this reason, iron absorption is increased during hypoxia, iron depletion [25] and pregnancy [26], but is reduced in secondary iron-overload conditions. However, conditions such as anaemia of chronic disease (ACD) and mutations in genes involved in iron metabolism can have a large effect on iron absorption [27].

Dietary iron has broadly been classified into two types, non-haem and haem iron. Both these forms of dietary iron have a separate pathway of uptake by enterocytes. Haem iron is a lot more bioavailable and its bioavailability is less influenced by dietary constituents. Whilst the molecular mechanism of non-haem iron absorption is clear, the mechanism for haem iron absorption is still emerging.

The first step in the absorption process of non-haem iron is its uptake from the lumen of the intestine across the apical membrane and into the enterocyte. Divalent metal transporter 1 (DMT1), an iron transporter, is responsible for mediating this step, and transports iron as

Fe^{2+}. However, the majority of the iron that enters the duodenal lumen from the diet is Fe^{3+} and therefore the iron must first be reduced before it can be taken up by the enterocytes. Duodenal cytochrome B (DcytB), a brush border ferric reductase enzyme that is highly expressed in the duodenum, is responsible for the reduction of Fe^{3+} into Fe^{2+}. Once inside the enterocyte, the intracellular trafficking of iron from the brush border membrane to the basolateral membrane is poorly understood [28]. Intracellular iron may be bound to chaperone molecules to maintain its solubility, but to date none have been identified. In fact, many proteins have been proposed to be involved in absorption and transport of non-haem iron (Table 1.1). Iron that is not transported around the body is instead incorporated into ferritin, the iron storage molecule, and is lost when the cell is ultimately sloughed at the villus tip.

Ferroportin-1 facilitates the efflux of iron across the basolateral membrane and into the circulation. Ferroportin-1 also plays a role in the export of iron from other cell types, including monocytes and macrophages [29]. In addition to ferroportin-1, the basolateral efflux of iron from enterocytes requires the ferroxidase, hephaestin. Although the exact role of this protein has not been defined, it is thought that iron is exported as Fe^{2+}, oxidised to Fe^{3+} by hephaestin and ceruloplasmin, and loaded onto transferrin, the main plasma iron carrier [30-33].

As mentioned earlier, the recycling of iron through macrophages is the major source of iron for haemoglobin synthesis [34]. After a mean lifespan of 120 days, specialised macrophages phagocytose old and damaged red blood cells (RBCs). After lysis, iron is released from the haemoglobin of RBCs by haemoxygenase 1 and thereafter iron can be stored in ferritin and exported to the bloodstream by ferroportin through a similar process as described above for duodenal enterocytes. Ferroportin, the only known mammalian iron exporter, is therefore a “major gatekeeper controlling iron entry into the bloodstream” [35].

Table 1.1 - Proteins involved in intestinal non-haem iron absorption. *Adapted from Gulec* et al., *2014 [36]*.

Protein	Function
Duodenal cytochrome B (DcytB)	Ferric iron reduction for absorption via DMT1
Divalent metal transporter 1 (DMT1)	Ferrous iron transporter
Ferroportin (FPN1)	Ferrous iron exporter
Ferritin	Intracellular iron storage
Hephaestin	Ferroxidase
Hepcidin	Liver-derived, iron regulator

Currently, there are two prevailing hypotheses explaining the mechanisms of haem iron absorption; firstly, a well-known theory that haem is taken up by receptor mediated endocytosis; secondly, the recent discovery of a haem transporter that may have the capability of transferring haem from the small intestinal lumen directly into the cytoplasm [37]. These pathways are summarised in Figure 1.1 and discussed in detail below.

The hypothesis of haem uptake by receptor mediated endocytosis originated in 1979 from the discovery of a haem binding protein on the microvillus membrane of the upper small intestine of both pigs and humans [38]. Haem enters mucosal cells via the brush border membrane, possibly by endocytosis, as the intact iron-protoporphyrin complex [37]. Haem oxygenase 1 (HO-1) then initiates the release of iron which enters the same pathway as non-haem iron and subsequently is influenced by the same factors.

In recent years, two mammalian haem transporters have been discovered, namely proton-coupled folate transporter/haem carrier protein 1 (PCFT/HCP1) [39, 40] and feline leukaemia virus subgroup C receptor (FLVCR) [41]. These appear to function independently of the putative haem receptor and receptor mediated endocytosis in that they act as a direct transfer process across plasma membranes [37]. It is hypothesised that FLVCR transports intact haem across the basolateral membrane where it then binds haemopexin. Alternatively, haem may be catabolised to non-haem iron and biliverdin by HO-1 located on the endoplasmic reticulum. Any iron released from haem inside the enterocyte, regardless of the mode of uptake, ultimately joins the labile iron pool and is transferred to the bloodstream by FPN1 in the same fashion as non-haem iron.

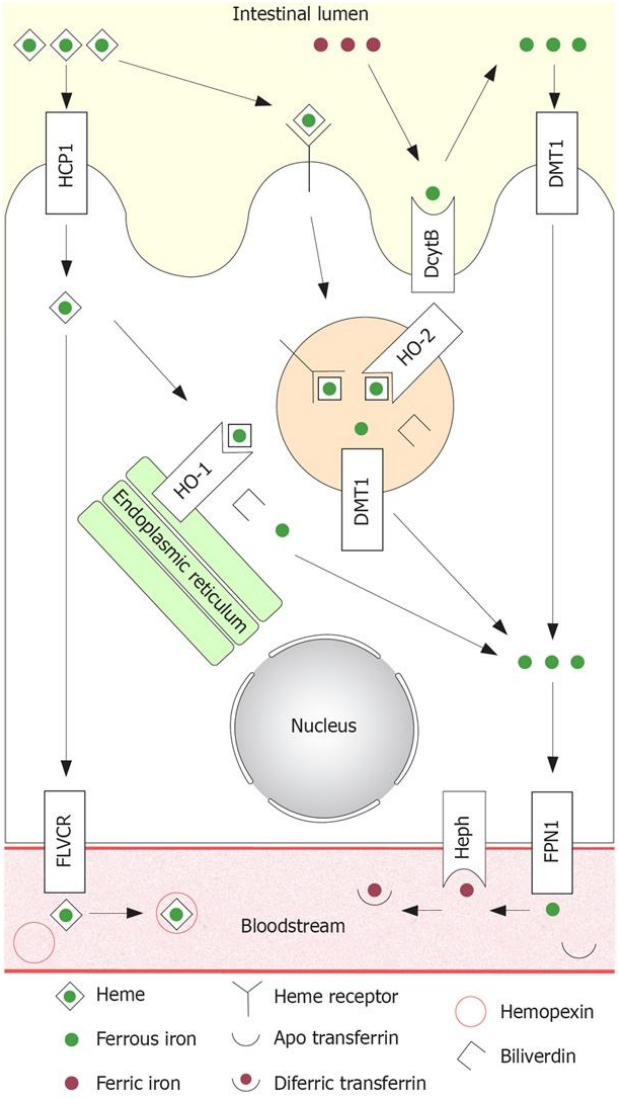


Figure 1.1 – Summary diagram of the hypothesised mechanisms of haem iron uptake. *DMT1 is responsible for the uptake of non-haem iron before joining the labile iron pool in the cytoplasm. Fe^{3+} is first reduced to Fe^{2+} by DcytB. Fe^{2+} is then transferred to the circulation by ferroportin, which requires hephaestin for oxidation to Fe^{3+} in order to bind to circulating apotransferrin. Haem iron is hypothesised to be taken up by receptor-mediated endocytosis. Internalised haem is degraded by haem oxygenase inside the vesicles, releasing non-haem iron and generating biliverdin. The non-haem iron is subsequently transported to the cytoplasm by DMT1. Haem iron may also be taken up by PCFT/HCP1 directly into the cytoplasm. Intact haem may be transported across the basolateral membrane by FLVCR where it binds circulating haemopexin. Alternatively, biliverdin may catabolise haem to non-haem iron by HO-1, which is located on the endoplasmic reticulum. Irrespective of how iron is taken up in to the cell, the iron which is released from haem within the enterocyte joins the labile iron pool. FPN1 then transfers this iron into the bloodstream in the same way non-haem iron is transported. Diagram and description taken from West and Oates, 2008 [37]*

1.1.3 Circulating iron

Under physiological conditions, transferrin-bound iron is the predominant form of iron circulating in the bloodstream [18]. Transferrin has the ability to carry up to two iron molecules and therefore ensures iron remains in an inert state. Transferrin saturation reflects the levels of iron occupation of the iron binding sites on transferrin, which typically ranges from 20-40%. Iron is delivered to tissues by transferrin for uptake by transferrin receptor 1 (TfR1), a ubiquitously expressed protein [42]. Transferrin receptor 2 (TfR2) is a homologue of TfR1 and has a much more limited expression. It has been thought that TfR2 may be involved in the sensing of transferrin-bound iron levels in other tissues, such as erythrocytes and the liver [34, 43]. In circumstances where transferrin is fully saturated, non-transferrin bound iron (NTBI) can circulate. This includes highly reactive labile plasma iron (LPI) that can lead to cellular damage if taken up by organs such as the pancreas, liver and heart [44].

Blood plasma also contains ferritin, which is mainly derived from macrophages [45]. Circulating ferritin is generally reflective of body iron stores, but as it is an acute phase protein its levels can be massively influenced by the presence of infection, inflammation, liver disease and also malignancy derived from other conditions [44].

Another protein, neutrophil gelatinase associated lipocalin (NGAL)/lipocalin-2, has also been reported to behave as an extracellular iron carrier by binding to siderophores (iron-binding compounds secreted by microorganisms). Furthermore, haemopexin and haptoglobin, which are haem and haemoglobin scavengers, respectively, are also proteins known to circulate within the bloodstream [46, 47].

1.1.4 Iron storage

Ferritin concentration, together with that of haemosiderin, reflects the body's iron stores. These proteins store iron in an insoluble form and are present primarily in the liver, spleen, and bone marrow [48]. The majority of iron is bound to the ubiquitous and highly conserved iron-binding protein, ferritin [49]. Haemosiderin is an iron storage complex that does not readily release iron. Under steady state conditions, serum ferritin concentrations correlate well with total body iron stores [50]. Thus, serum ferritin is the most convenient laboratory test to estimate iron stores.

1.1.5 Human iron homeostasis

The circulating pool of iron is relatively small (approximately 2-4 mg) and turns over every few hours to ensure the daily requirement of iron for erythropoiesis (RBC production) and other needs of the body (approximately 20-25 mg) are met [35]. On average, roughly 1-2 mg of iron is absorbed daily from the diet in the duodenum and proximal jejunum [51, 52]. This is balanced by the unregulated loss of iron through desquamation of skin, sloughing of intestinal epithelial cells and blood loss. Urinary iron excretion is minimal due to the largely protein bound form of circulating iron (transferrin-bound iron) and other mechanisms for iron retrieval in the kidney [53]. Since the human body has no controlled mechanism for the excretion of iron, the major avenues for regulating systemic iron balance are in the control of dietary iron uptake as well as the release of iron from recycling macrophages and hepatocytes [54]. An overview of systemic iron homeostasis is summarised in Figure 1.2.

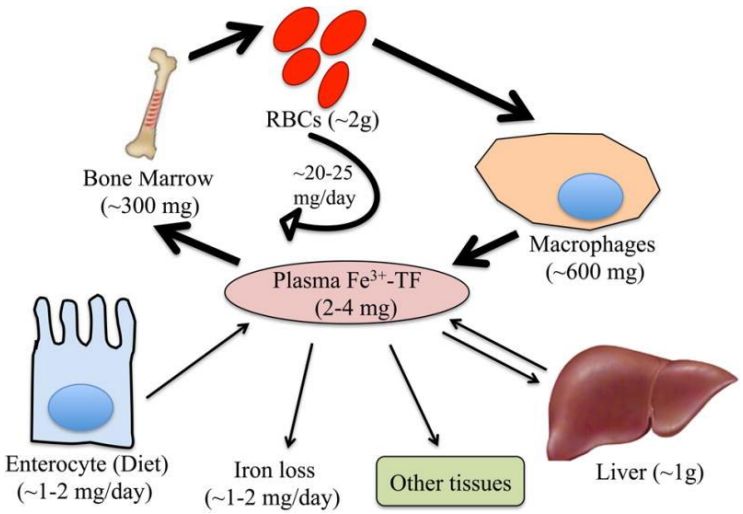


Figure 1.2 - Systemic iron homeostasis. *Transferrin (Tf) bound iron circulates in the bloodstream. Most of the iron is transported to the bone marrow for red blood cell synthesis, whilst smaller amounts are delivered to other tissues for essential cellular processes. Excess iron is transported to the liver for storage. Recycling of RBCs is the main process for ensuring iron homeostasis is maintained, whilst lesser levels of iron are provided from the diet through duodenal enterocytes. Diagram taken from Dev and Babitt, 2017 [35].*

Circulating iron is delivered to erythrocytes and other cells within the body through specific uptake mechanisms [42]. One key uptake mechanism is that of receptor-mediated endocytosis of transferrin-bound iron by TfR1 into clathrin coated pits. Clathrin is a protein that plays an important role in the formation of coated vesicles [55]. Once endocytosed, iron is then released into the endosome, an acidic environment, therefore

initiating the reduction of Fe^{3+} to Fe^{2+}. This reduction is mediated by the ferrireductase, STEAP3. DMT1 then exports Fe^{2+} out of the endosome and TfR1 undergoes recycling and returns to the surface of the cell to repeat this process. Once iron enters the labile-iron pool, also known as the 'chelatable' iron pool, it can be used immediately, stored in an inert form in cytosolic or mitochondrial ferritin, trafficked to the mitochondria for use in the iron-sulphur cluster pathways or exported out of the cell [35].

1.1.5.1 Systemic iron homeostasis

The successful functioning of cells and tissues is heavily dependent on maintaining optimal levels of iron in the circulation. For instance, too much iron could potentially lead to iron overload and related diseases, while too little systemic iron could lead to restricted erythropoiesis and consequent anaemia [54].

Another environment in which iron regulation is crucial is during infections and iron removal by the host is important in the innate immune response to pathogens. To ensure that the body's iron requirements are met, the human body has evolved many mechanisms to sense and adjust iron levels accordingly. Furthermore, these mechanism are also activated under the presence of inflammatory/infectious stimuli as well as hypoxia and erythropoietic signals [54]. Hepcidin and ferroportin, two molecules found in the liver, are the two major proteins involved in regulating and maintaining systemic iron homeostasis.

Ferroportin, the only known mammalian iron exporter, is found in various cells, with its highest expression being in macrophages, followed by duodenal enterocytes and hepatocytes. Ferroportin is also expressed in cells that are involved in iron recycling, absorption, storage and regulation [56]. Its expression is controlled at different levels, including transcriptional, translational and post-translational levels. The ferroportin mRNA contains a functional iron responsive element (IRE) in its 5′ untranslated region (UTR). Under low iron conditions, translation of ferroportin is repressed, leading to a decrease in cellular iron export [56]. Transcription of the macrophage ferroportin gene can be promoted by haem [57] and inhibited by inflammatory stimuli [58]. The complete loss of ferroportin expression in mouse models and zebrafish was demonstrated to be lethal due to the resultant inability of embryonic trophoblasts to transfer iron from the mother to the embryo [29]. New-born mice which lacked ferroportin developed severe iron-deficiency anaemia as a result of reduced dietary iron absorption and a faulty release of iron from hepatic storage and iron-recycling macrophages [29].

The hormone hepcidin is the major protein involved in regulating ferroportin levels at a systemic level. Hepcidin is a 25 amino acid polypeptide expressed in the liver and serves as an iron-regulatory hormone. It was first identified in urine and plasma as a disulphide bonded, liver-expressed antimicrobial peptide [59, 60]. Very soon after, it emerged that hepcidin also played a crucial role in iron homeostasis regulation [61-63]. Studies illustrated the binding of hepcidin to ferroportin, resulting in reduced cellular iron export activity and subsequently leading to the internalisation and degradation of ferroportin [64]. Through hepcidin-ferroportin binding, immediate ubiquitination and internalisation of this complex occurs and therefore lowers cell surface expression and export of iron [65, 66]. Since the discovery of hepcidin in 2000, it has become apparent that its interaction with ferroportin is critical for systemic iron regulation and that disturbances in this hepcidin-ferroportin complex are the foundation for several iron-related disorders [67].

When chronically overexpressed, hepcidin causes iron-deficient anaemia in both mice and humans, which generally presents as microcytic, hypochromic anaemia. On the contrary, reduced hepcidin levels in mice and humans results in iron overload with iron deposition in the liver and other parenchyma [64, 68]. When completely absent, hepcidin causes juvenile haemochromatosis, which is the most severe form of hereditary haemochromatosis.

Hepcidin homeostasis is regulated by iron and erythropoietic activity. When in excess, iron stimulates hepcidin production. Subsequently, dietary iron absorption is reduced and therefore prevents further loading. Under iron-deficient environments, hepcidin levels are repressed, which allows for increased iron absorption from the diet and subsequently leading to the replenishment of iron stores. When erythropoietic activity is heightened, hepcidin expression is, in turn, decreased. This allows for increased absorption as well as the quick release of macrophage and hepatocyte-stored iron, and therefore increasing the supply of iron for erythropoiesis.

Lastly, inflammation and infection also influence hepcidin. Under these conditions, hepcidin levels are upregulated and it is thought that this occurs as a host defence mechanism to limit iron availability to microorganisms [69]. Studies investigating this further examined the effect of administering IL-6 to human volunteers and showed an increase in urinary hepcidin excretion within 2 h of IL-6 administration [70]. Other cytokines have also been suggested to influence hepcidin expression and it was shown that IL-6 knockout mice still presented with elevated hepcidin mRNA levels compared to wild-type mice [70]. In this study, IL-1 was shown to upregulate hepcidin mRNA in mouse

hepatocytes independently of IL-6. An overview of iron regulation through hepcidin is shown in Figure 1.3.

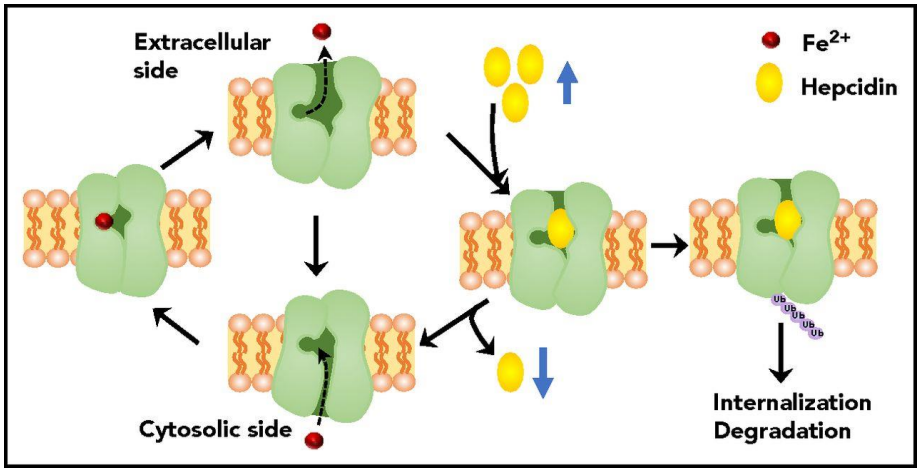


Figure 1.3 – Interaction of hepcidin and ferroportin complex. *Intracellular Fe^{2+} approach and bind to ferroportin in its inward-facing state, which leads to a change in conformation of ferroportin to the outward-facing state, allowing export of iron. Ferroportin then returns to its original inward-facing state to transport another intracellular iron molecule. Under high hepcidin levels, hepcidin binds ferroportin, which prevents the conformational transition and iron export. Conversely, when hepcidin levels are low under low iron environments, hepcidin-ferroportin binding does not occur, which in turn allows ferroportin to resume iron transportation. The binding of hepcidin also leads to a conformational change, which subsequently uncovers many ubiquitination sites. This then triggers the internalisation and degradation of ferroportin. Diagram and description adapted from Zhang and Rouault, [71]*

1.1.5.2 Cellular iron homeostasis

The iron regulatory protein (IRP) system is a major regulator of cellular iron homeostasis [72, 73]. In conditions where cellular iron levels are low, the expression of a number of iron homeostasis proteins are regulated by IRPs through the binding of these proteins to IREs. This occurs in the mRNA 5' UTR of the IREs to prevent translation of ferroportin and ferritin, for example. This could also occur in the mRNA 3'UTR of the IREs to inhibit the degradation of TfR1, for example [72]. Through the binding of the UTRs of the IREs, an increase in iron uptake is observed whilst iron storage decreases in environments of low cellular iron. The reverse is observed when cellular iron levels are high.

1.2 Iron bioavailability

Non-haem iron is derived from pulses, cereals, fruits, legumes and vegetables, whilst the primary sources for haem iron are haemoglobin and myoglobin from meat, fish and poultry

[74-76]. Compared to haem iron absorption, which represents roughly between 15-35% of total iron intake, non-haem iron absorption is considerably lower, between 2-20% of total iron intake [77]. As haem iron is complexed with globin, its absorption is largely unaffected by other dietary factors, whereas non-haem iron is strongly influenced by the presence of other food components [22, 77]. Despite its lower bioavailability, non-haem iron levels are much greater than that of haem iron in most meals and therefore, in general, non-haem iron plays a bigger part in iron nutrition compared to haem iron [78]. The most common enhancers of iron absorption are ascorbic acid, which could potentially reduce Fe^{3+} to Fe^{2+} and subsequently aid the binding of iron in soluble complexes to increase absorption, and muscle tissue from which digested peptides are believed to bind any free soluble iron [77]. Inhibitors of iron absorption are calcium, polyphenols and phytic acid [52, 77, 79, 80].

1.2.1 Factors enhancing iron absorption

Many dietary factors have a positive effect on iron absorption. Ascorbate behaves as a weak chelator of iron and subsequently increases iron uptake through increasing the solubility of iron in the duodenum [81-85]. The enhancing effect of ascorbate is largely attributable to its ability to reduce Fe^{3+} to Fe^{2+} as well as its ability to form a weak-chelate complex with iron. Hallberg *et al.*, (1989) [86] and Siegenberg *et al.*, (1991) [85] demonstrated that ascorbic acid is able to reverse the inhibitory effects on iron absorption by phytate and polyphenols. Another study reported that the addition of ascorbic acid at concentrations of 100 mg/L or higher in cow's milk supplemented with ferrous sulphate ($FeSO_4$) enhanced iron absorption and therefore bioavailability of iron [87]. In fruits and vegetables, the enhancing effects of ascorbic acid is often counteracted by the presence of polyphenols [88], and as ascorbic acid is the only enhancer of iron-absorption present in vegetarian diets, it is suggested that those following a vegetarian or vegan diet should include vegetables containing ascorbic acid to optimise iron absorption [84].

The enhancing effect of meat, fish, or poultry on iron absorption from non-vegetarian meals has been shown [89]. 30 g muscle tissue in meat is considered equivalent to 25 mg ascorbic acid [78]. One study reported that the addition of chicken, beef, or fish to a maize meal increased non-haem iron absorption 2-3-fold [90]. As with ascorbic acid, it has been somewhat more difficult to demonstrate the enhancing effect of meat in multiple meals and complete diet studies. One study demonstrated a small improvement in iron absorption (35%) in self-selected diets over 5 days when daily muscle tissue intake was increased to

300 g/day [91], although, in a similar 5-day study, 60 g pork meat added to a vegetarian diet increased iron absorption by 50% [92].

1.2.2 Factors inhibiting iron absorption

Phytate (myo-inositol hexakisphosphate) is the main inhibitor of iron absorption in plant-based diets [77, 93]. Low concentrations of phytate (2-10 mg/meal) have been demonstrated to have a dose-dependent negative effect on iron absorption [85, 94]. To significantly improve iron absorption in plant-based foods which do not contain any compounds to enhance iron absorption, the molar ratio of phytate to iron should be 1:1 or preferably 0.4:1 [95].

Various animal proteins have been shown to decrease iron absorption. These include proteins derived from animal milk and eggs, including albumin and egg whites [96, 97]. Whey and casein are the two major bovine milk proteins that have been illustrated to inhibit iron absorption in humans [97]. Soybean-derived proteins have also been demonstrated to inhibit iron absorption [98].

Compared to other inhibitors of iron absorption, which affect non-haem iron, calcium negatively affects both haem and non-haem iron [99]. Studies have shown that when doses of 75-300 mg of calcium is added to wheat bread rolls, a dose-dependent inhibitory effect is observed in iron absorption. This study also found that administering 165 mg calcium in the form of milk, cheese or calcium chloride also reduced iron absorption in humans by 50-60%. The same amount of calcium also significantly inhibited haem iron absorption, suggesting a role of calcium in the mucosal transfer of iron [100].

Polyphenols are dietary constituents obtained through plant-based foods, occurring in tea, coffee, wine, fruits, vegetables and some legumes and cereals. Black tea has been shown to reduce iron absorption, as have herbal teas but to a lesser extent [101, 102].

1.3 Iron disorders

1.3.1 Iron deficiency

1.3.1.1 Iron deficiency anaemia

Despite the high abundance of iron in the environment, iron deficiency is extremely common in humans. According to the World Health Organisation (WHO), approximately 30-50% of anaemia in children is due to iron deficiency and as such it is the most prevalent cause of anaemia worldwide [103]. It is estimated that two billion people worldwide who

are nutritionally iron deficient suffer from iron-deficiency anaemia (IDA) [104]. There are many health risks associated with IDA, including impaired cognitive and physical development in children, cognitive decline in the elderly, increased risk of mother and child mortality as well as a decreased output of physical performance and work productivity in adults [104, 105].

IDA can be due to various factors, but the main reason is insufficient dietary iron absorption. Other reasons include malabsorption, increased blood loss (for instance, gastrointestinal losses from ulcers or malignancies) and increased iron requirements during pregnancy [105].

1.3.1.2 Anaemia of chronic disease

ACD, also known as anaemia of chronic inflammation, is the most common cause of anaemia reported in hospitalised patients [106] and is the second most prevalent cause of anaemia, after IDA [107-109].

ACD is characterised by an immune activation with an increase in inflammatory cytokines, such as IL-6, and subsequently leads to elevated hepcidin levels. Imbalanced erythropoietin levels and the lack of responsiveness to erythropoiesis further contributes to ACD. As mentioned previously in this chapter, hepcidin, being the central regulator of iron metabolism, plays a crucial role in the pathophysiology of ACD. Hepcidin binds to ferroportin present on macrophages, hepatocytes, and enterocytes which then leads to the degradation of ferroportin. This, in turn, leads to iron trapping within the macrophages and hepatocytes, resulting in functional iron deficiency. ACD is therefore characterised by reduced levels of circulating iron despite adequate or high stores of total body iron [108-110].

1.3.2 Iron overload

1.3.2.1 Hereditary haemochromatosis

Hereditary haemochromatosis (HH) is an autosomal recessive disorder that disrupts the body's regulation of iron [111]. HH occurs in approximately 1 in 200-250 individuals, with approximately 0.4% of people of northern European descent having the genetic mutation and thereby increasing the risk of developing haemochromatosis [112-115]. Men have a 24-fold increased rate of iron-overload disease compared with women, and occurs predominantly in Caucasians [116]. Persons who are homozygous for the HFE gene mutation C282Y comprise up to 90% of phenotypically affected persons. In an estimated

10% of individuals homozygous for C282Y, end-organ damage or clinical manifestations of HH are present. HH symptoms are generally non-specific and are not entirely apparent in the early stages of the disorder. Typical symptoms of HH are weakness, joint pain (also known as arthralgia) and lethargy [117]. Later stages of HH encompass individuals suffering from osteoporosis, cardiomyopathy, diabetes mellitus, cirrhosis, hepatocellular cancer, dysrhythmia and hypogonadism [111, 116, 117]. The appropriate interpretation of transferrin saturation and serum ferritin results is essential in the diagnosis of iron overload, where both transferrin saturation and serum ferritin levels are elevated [117, 118].

Phlebotomy (also known as venesection therapy) is the standard treatment for patients with HH, having been implemented for over 60 y. As well as reducing iron levels, phlebotomy is also effective in reducing morbidity and mortality of HH [118, 119]. Iron overload during HH generally results in tissue injury mainly through the production of reactive oxygen species. These molecules are toxic to cell membranes and organelles, which subsequently results in cellular death [118]. During venesection therapy, an estimated 250 mg of iron is removed per 500 mL of blood withdrawal. This iron is released as a means of a compensatory process from tissues overloaded with iron, such as the liver. Therefore, repeated venesection therapy is required to ensure the complete removal of excess iron in the individual [118].

Chelation therapy is also another avenue of treatment for HH individuals, however this is usually reserved for extreme cases, such as individuals who do not respond to the standard therapy of venesection, or when phlebotomies are medically contraindicated (such as poor vein conditions) [118].

In general, HH universal screening is not implemented, however first-degree relatives of HH patients, individuals with abnormally high iron levels and those with evidence of active liver disease are tested for potential HH. Only individuals with HH and cirrhosis are screened for hepatocellular carcinoma.

HH patients are recommended to consume a healthy varied diet, avoiding foods with iron fortification, such as breakfast cereals. HH individuals are also discouraged from consuming iron and vitamin C supplements, as well as reducing alcohol intake and red meat consumption [116].

1.4 Chelation therapy

The main aim of iron chelation therapy is to ensure a 'safe' iron status is sustained at all times. The main situations under which iron chelation therapy should be sought is when iron accumulation is to be prevented, and to lessen the extent of iron-related complications including hepatic, cardiac and endocrinological dysfunction.

In practice, chelation therapy is generally utilised to eradicate excess stored iron and subsequently correct any complications related to the iron overload. Deferoxamine is a chelator used clinically and its use is generally implemented after 2 to 3 y of patients undergoing transfusion or when an individual's ferritin levels exceed 1,000 ng/mL [120].

Iron chelation therapy is extremely useful in not only treating iron overload but also minimising any harmful effects that usually present with iron burden.

The direct capture of NTBI and LPI via effective chelation therapy may help to prevent the adverse consequences of iron overload. Many iron chelators have been used clinically, each of which have been designed to remove tissue iron by creating a complex with iron which are in turn excreted in the urine and/or faeces. Table 1.2 summarises these iron chelators.

Table 1.2 – An overview of different iron chelators. *Adapted from Poggiali* et al.*, (2012)* [120]

Property	Deferoxamine	Deferiprone	Deferasirox
Stoichiometry (chelator:iron)	Hexadentate (1:1)	Bidentate (2:1)	Tridentate (3:1)
Route	Subcutaneous, intravenous	Oral tablet/solution	Tablets for oral suspension
Excretion	Urinary, faecal	Mainly urinary	Faecal
Half-life	20-30 m	3-4 h	8-16 h
Adverse effects	Local skin reactions Ophthalmological Allergic reactions Growth retardation Bone abnormalities Pulmonary (high doses) Neurological (high doses)	Gastrointestinal Agranulocytosis/neutropenia Arthralgia Elevated liver enzymes	Gastrointestinal Rash Creatinine increase Proteinuria Ophthalmological Auditory Elevated liver enzymes
Status	Licensed	Licensed in USA and Europe	Licensed in USA and Europe

1.5 The human gut microbiota

Humans represent a framework upon which wide varieties of microbial ecosystems are established. Previously it was believed that all mammals are subjected to a life-long process of colonisation instantly after birth [121], however, recent research proposes that colonisation of the gastrointestinal tract begins *in utero* [122]. After many years of evolution, an environment of mutualism has been created whereby many host-bacterial associations have become relationships.

The latter part of the gut (colon) is home to our highest number of microbes (over 100 trillion bacteria) [123]. Symbiotic bacteria of the mammalian gut have long been recognised for the advantages they confer to the host. Some of these benefits include defence against opportunistic pathogen colonisation, metabolism of indigestible compounds, provision of necessary nutrients, such as iron, and involvement in the

development of the intestinal structure. The human gut microbiota also contributes towards the basic developmental features and functions of the immune system [124]. Conversely, perturbations (e.g. antibiotic treatment and/or overgrowth of pathogenic bacteria) in these symbiotic relationships, termed dysbiosis, can lead to a negative impact on the host's health [125]. Dysbiosis can lead to a range of human disease states, such as autoimmune disorders [126, 127], increased vulnerability to cancers [128], irritable bowel syndrome [129-132] and obesity [133]. Furthermore, the number of studies examining the relationship between diet and the gut microbiota has increased over the past years and they have shown that the gut microbiota composition and metabolic activity is affected by diet [134]. Currently, it is not clearly defined whether the type of diet significantly contributes to host health or disease through the shaping of the gut microbiota. Elucidating the relationships between human health and the associated microbiota presents us with a new challenge, and, if successful, will provide an invaluable tool for diagnostics and possible mechanisms for human therapeutic interventions [135].

1.6 Factors that affect the gut microbiota

After birth, factors like type of feeding (either breast-feeding or formula feeding) and hospitalisation continues to influence the gut microbiota [136]. Hygiene, diet and illness then play a role in later life [134]. Genetic factors also play a role in influencing the gut microbiota and have a contribution to an individual's microbial composition.

Historically, culture-based analysis has suggested that the gut microbiota of healthy people share bacterial species, which are common among the majority of individuals. More recently, large-scale studies for identifying and characterising different microbial communities have been carried out (Human Microbiome Project, HMP [137] and Metagenomics of the Human Intestinal Tract MetaHIT). These studies have highlighted that the vast diversity of the human gut microbiota poses a significant challenge when analysing human microbiota data. Each individual's microbiota is dynamic and ever changing in response to diet, environment and host behaviour [138] and therefore this hinders our ability to make generalisations that are relevant across human populations. It has, however, been established that there is a high overall temporal stability of the microbial community amongst unrelated individuals, whereby a large collection of microbial genes are shared, encoding metabolic traits that pose an advantage to the host.

1.6.1 The effects of nutritional and metabolic stress on gut pathogens

Unabsorbed iron from the diet travels to the colon, and there is accumulating evidence to suggest that this can facilitate the growth of intestinal pathogens [139]. The vast majority of bacteria in the gut require iron for growth and development, and they have formulated many strategies to acquire this nutrient, which in its more common state (Fe^{3+}) has low or zero solubility. Potentially pathogenic bacteria make use of a continuous supply of micronutrients, such as iron, for metabolism and replication. Thus, there is constant competition for iron between various bacteria, many of which have developed mechanisms, such as siderophore production, to acquire iron, particularly when availability is limited. Unlike most bacteria, members of the *Bifidobacteriaceae* and *Lactobacilliaceae* families (two families that are believed to be beneficial to the host [140-143]) have a very limited need for iron [135]. Lactobacilli do not produce siderophores to sequester iron, and their growth is similar in media with and without iron [144]. The human body has created many ways to promote the growth of these beneficial bacteria, such as the presence of lactoferrin in breast milk which travels intact to the large intestine [145-147], which with its high affinity for iron, renders the iron unavailable to potentially pathogenic bacteria [148]. Along with high amounts of dietary iron in the gut lumen, the oral administration of iron supplements also results in freely available 'unbound' iron in the colon [149-151]. This disturbs iron homeostasis consequently modifying the gut microbial composition [152-154].

An emerging link between the gut microbiota and iron availability has been observed, however several studies that have investigated the effects of iron on the human gut microbiota are mainly focussed on the infant microbiota [150-152, 155-158].

1.6.2 The effect of redox stress on bacteria

Iron's inherent redox cycling properties makes it a good metal for electron carrying and as a biocatalyst in proteins [20, 139]. Fe^{3+} is mostly present under aerobic conditions and is virtually insoluble. It is only soluble in water when it is complexed in a strong acidic solution, making its bioavailability very low despite it being present in such copious amounts on earth. Fe^{2+} is mainly found when oxygen levels are low as well as in acidic environments and is more soluble than Fe^{3+}. Through the Fenton Reaction, reactive oxygen species (ROS) can be formed in the presence of free redox active iron under aerobic conditions, which can prove to be extremely toxic. The hydroxyl ion OH^- is one of the

most reactive ROS species and can have detrimental effects on biological molecules and cells.

Only a very small number of bacterial species do not require iron, such as those within the *Lactobacillaceae* family, which have acquired alternative metabolic solutions via evolution [159]. *Lactobacillus plantarum* was the first identified iron-independent microbial strain, which contains a maximum of 2 iron atoms – "a level that is considered to be too low to provide iron with any conceivable function" [160]. This property further justifies their presence in the natural gut microbiota and milk, a highly iron-restricted environment due to the lactoferrin [161].

Borrelia burgdorferi is a well-known pathogen that causes Lyme disease, transmitted to humans through the bite of infected ticks of the genus, *Ixodes*. This pathogen has advanced in an environment rich in manganese but poor in iron through the replacement of iron with manganese in their metalloproteins. This substitution is a vital trigger for bacterial virulence as well as the activation of superoxide dismutase (SodA) [162]. The lack of iron requirement by this bacterial species may facilitate infection conditions that strictly restrict iron bioavailability within the host systemic compartment [163].

Bacteria that utilise iron for their metabolism face the problem of having to overcome the stresses of the toxicity derived from free redox active iron molecules. To tackle this problem, many bacterial species have developed mechanisms to directly detoxify oxidative stress or iron itself. Catalase is a well-known enzyme that has the ability to neutralise ROS. This enzyme catalyses the reaction of two hydrogen peroxide molecules into the non-toxic products, oxygen and water.

Another method to deal with oxidative stress is to directly counteract the oxidative stress molecules. SodA is an enzyme produced by many bacterial species and is involved in catalysing the dismutation of superoxide into a less toxic hydrogen peroxide and oxygen. Interestingly, different members of the SodA family utilise different metals as cofactors. For example, SodA members that use manganese and zinc are unable to perform the dismutation process if these metals are sequestered by the host under inflammatory conditions, consequently making the bacteria more susceptible to oxidative stress. Finally, alkylhydroperoxide reductase (Ahp) and glutathione peroxidase are peroxidases that can rapidly detoxify hydroperoxides, hydrogen peroxides or peroxynitrite [164].

1.6.3 Iron detoxification

When iron is present in very high amounts, bacterial species are able to export iron to the outside of the cell. One example of this is a haem export mechanism (HrtAB), which reduces the haem-based iron stress in certain bacterial species, such as *Staphylococcus aureus*. Orthologues of the HrtAB system have been identified in *Bacillus anthracis, Listeria monocytogenes, Enterococcus faecalis,* and *Streptococcus agalactiae* [165].

Bifidobacteria have the ability to bind iron to their surface, preventing the formation of radicals in the surrounding environment as well as sequestering iron inside the colonic lumen, preventing pathogenic bacteria from acquiring iron [166].

Ferric iron can be bound to three types of bacterial high-affinity storage proteins [139]. These are ferritin (similar to eukaryotic ferritin), DNA binding protein from starved cells (DPS) and bacterioferritin. DPS has a dual role; it acts as a detoxification/iron storage protein but also binds DNA. For this reason, it is involved in the protection of bacterial DNA from redox stress [20]. By lowering the amount of intracellular free iron through the storage of iron in a non-toxic and soluble form, bacterial species are able to protect themselves from redox stress. When residing in the mammalian blood stream or host cells, for instance, iron levels are scarce and under these conditions, bacterial species are able to release the stored iron for utilisation.

1.7 Iron exploitation by gut bacteria

1.7.1 Bacterial uptake of iron

Many bacterial species have developed mechanisms to acquire iron, even when availability is sparse. On average, bacteria need 10^{-7} – 10^{-5} M iron for optimal growth [139]. *E. coli*, for example is able to take up both forms of iron. One study describes the Feo-uptake system that *E. coli* uses to achieve this [20], which is involved in the transport of Fe^{2+} (under anaerobic conditions). Alternatively, most bacterial species are able to acquire iron through the reduction of Fe^{3+} into Fe^{2+} with the help of an extracellular reductase, and this can then be transported into the cell [167]. Furthermore, bacterial siderophores act as iron chelators with a very high affinity for iron [168]. Haemophores are another specialised method in which haem iron is directly taken up.

Pathogenic bacteria using iron to thrive [169, 170] and conversely, decreasing in numbers when iron is limited, is a long-known concept. *In vivo* knockout studies on mice have

shown that particular iron uptake mechanisms, such as the Feo-uptake system, are essential in the survival and virulence of some bacterial species, such as *E. coli* [171, 172]. Bacteria upregulate or downregulate their virulence genes depending on the amount of iron that is available in the surrounding environment. For example, adhesion of *Salmonella* Typhimurium to enterocytes is increased in the presence of high amounts of iron [173]. Conversely, under iron-limiting conditions, bacterial toxins are decreased while bacterial siderophores are increased [174]. Either way, whether the iron availability is low or high, bacteria are able to alter different aspects of their virulence to adapt to the environment.

1.7.2 The battle for iron

One of the main reasons why free-iron concentrations are extremely low in the gut (approximately 10^{-24} M) is due to the toxic properties of iron, and for this reason iron is bound to high-affinity host proteins, preventing ROS formation [175]. These high-affinity proteins include transferrin, ferritin, lactoferrin and haemoproteins, such as haemosiderin. With bacterial species always competing for iron, the high-affinity binding of host proteins to iron is a form of innate defence against these microorganisms. Infection further boosts this defence mechanism. Under inflammatory conditions, the pro-inflammatory cytokine IL-6 is upregulated, as described earlier in this chapter. Once induced, hepcidin binds to ferroportin, and subsequently degrades and internalises it [69, 176, 177]. This leads to a reduced intestinal iron uptake and a concurrent increase in the iron stores belonging to the reticulo-endothelial system (RES). Consequently, extracellular pathogens have reduced access to iron. Therefore, being able to overcome these host iron-withdrawal mechanisms is an extremely important virulence trait that bacteria must possess or else they fail to thrive.

1.7.2.1 Siderophores

The more established method of bacterial iron acquisition is the secretion of iron-scavenging siderophores [20, 178]. Siderophores are small organic molecules produced by microorganisms under iron-limiting conditions which enhance the uptake of iron to the microorganisms. Siderophores can be split into three key classes depending on the chemical nature of the moieties that donate the oxygen ligands for Fe^{3+} co-ordination [168]. These are hydroxamates, catecholates or carboxylates. However, new ‘mixed-type’ siderophores identified recently leads to more complex classifications (Figure 1.4).

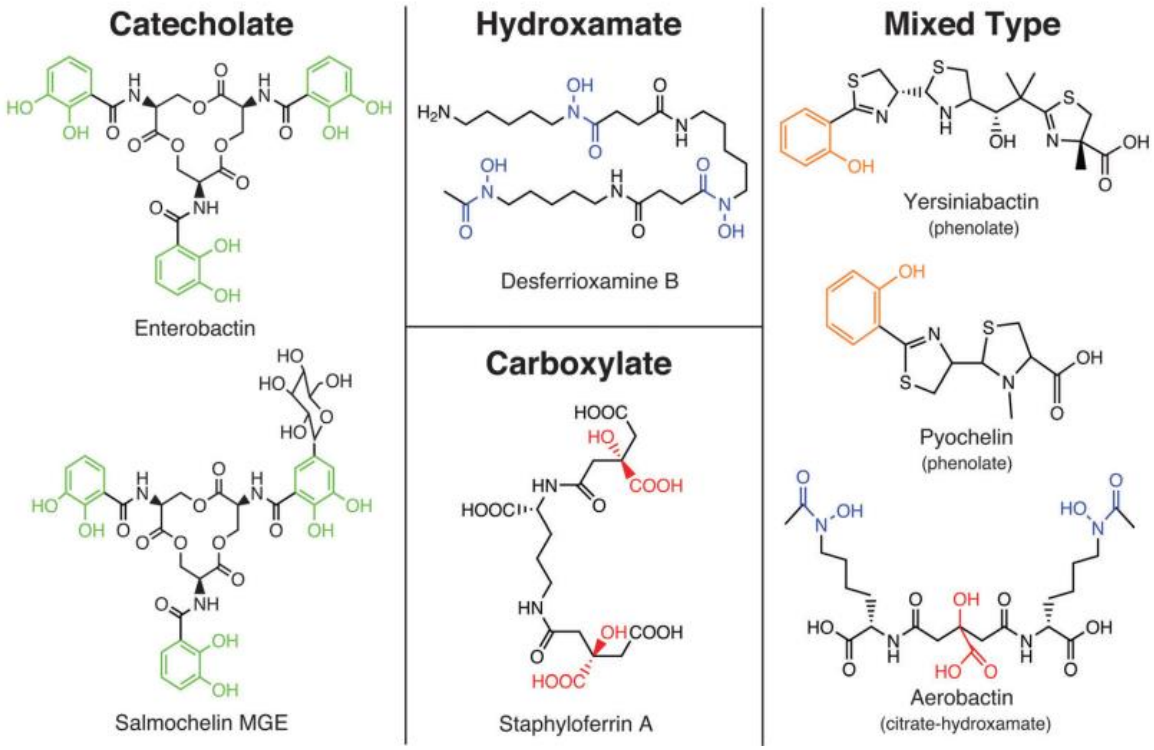


Figure 1.4 - Different structural families of siderophores. *Siderophores can be classified into three main structural families. These are catecholates, hydroxamates and carboxylates. The binding moieties are shown in green (catecholate), blue (hydroxamate) and red (carboxylate). Illustration taken from Holden and Bachman, 2015 [179].*

Siderophore production is the most common mechanism used by bacterial families such as *Enterobacteriaceae*, *Streptomycetaceae*, and *Bacillaceae*, in order to scavenge inorganic iron from the environment [180]. They are produced in high amounts by bacteria exposed to iron-limiting environments, due to their high ferric ion-specific chelating capacities [20, 181].

Pathogenic bacteria need to proliferate once inside the host for them to survive. Bacteria such as *E. coli*, *S.* Typhimurium and *Klebsiella pneumoniae* synthesise enterochelin [182, 183], which binds to Fe^{3+} very strongly (association constant of 10^{52} M^{-1}), unlike the host protein transferrin, which has an association constant that is much lower at 10^{22} M^{-1} [184]. The strong binding between enterochelin and iron can be exploited to scavenge even very low concentrations of iron in the surrounding environment. The iron is released from the siderophore once it is transported inside the bacterial cell through the action of reductases, which reduce siderophore-bound Fe^{3+} into Fe^{2+}, thereby enabling it to be incorporated directly into metallo-enzymes. If there is an excess of iron, it can then be stored as bacterioferritin, or in the related DPS proteins [185]. The ferric uptake regulator (Fur)

repressor protein is then responsible for the shutdown of iron uptake once the bacteria have accumulated enough iron, by preventing the biosynthesis of the iron transport system (Figure 1.5). Moreover, some bacterial species have evolved even further and are able to extract Fe^{3+} directly from transferrin or even use haem as an iron source [186, 187].

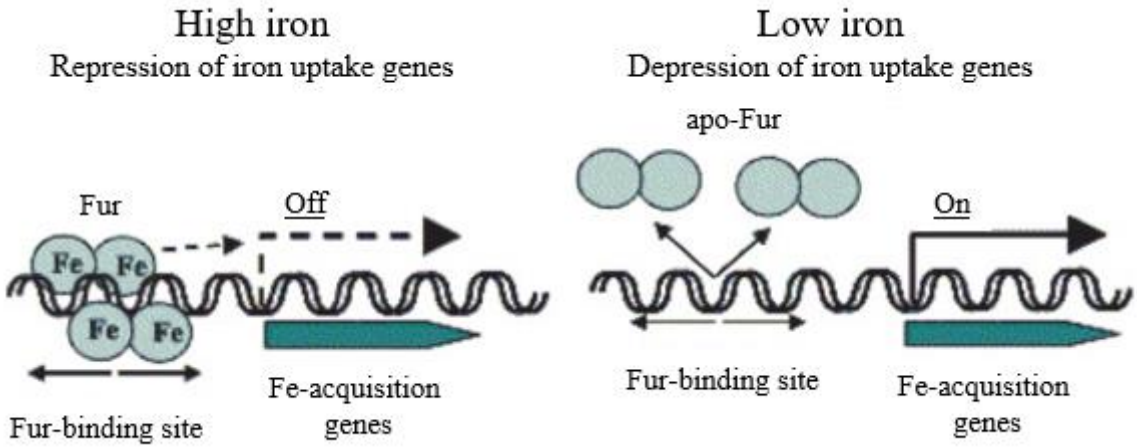


Figure 1.5 – Schematic representation of Fur-mediated repression. *Taken from Crichton, 2008 [188].*

An example of haem utilisation can be observed in *Bacteroides fragilis*. These bacteria are able to take up haem by either expressing high-affinity haem outer membrane transporters or producing haemophores [189]. Under conditions of iron-depletion, the availability of iron is likely to be limited in the gastrointestinal tract [190] and therefore, the bioavailability of iron greatly influences the composition of the gut microbiota.

1.7.2.2 Host counteraction of iron acquisition by bacteria

The host has developed another counteracting mechanism to protect iron levels via the sequestration of some bacterial siderophores with lipocalin-2, an innate defence peptide [191, 192]. Lipocalin-2 binds bacterial siderophores, preventing the bacteria from taking up the Fe^{3+}-enterobactin complex. However, some bacterial species overcome even this host response via 'stealth siderophores' [193, 194]. To illustrate, *E. coli* and *S.* Typhimurium can produce a C-glucosylated form of enterobactin, and salmochelin, which escapes the binding of lipocalin-2 [195]. Aerobactin is another example of a 'stealth siderophore' that also evades the binding of lipocalin-2. *Shigella* and *Klebsiella* are some bacterial genera that produce aerobactin. Some pathogenic *E. coli* strains and *Salmonella* serovars also produce this siderophore [196].

1.8 Iron speciation and mechanisms of iron uptake

1.8.1 Iron speciation

Most of the iron that reaches the intestine is in the form of Fe^{3+}. This is due to the acidic and high oxygen environment in the stomach, as well as the nature of foods itself. However, in the small intestine, the pH rises, rendering Fe^{3+} insoluble. This increase in pH facilitates the oxidation of any Fe^{2+} into Fe^{3+}. However, in the colon, Fe^{3+} could be potentially reduced into Fe^{2+} by certain bacterial species, most of which are acidophiles [197]. Studies looking at the effect of pH on siderophore activity have illustrated that mice with a higher colonic pH have increased siderophore production. This suggests that the iron availability is scarce in high pH environments and therefore bacteria upregulate their iron-scavenging mechanisms [198]. However, this study used phosphate buffer at different pHs to induce the colonic pH and iron is prone to form complexes with phosphate, which could have influenced their results. Another study carried out by Salovaara *et al.,* (2003) [199] supported the finding that Fe^{2+} uptake was increased by a lowering of the pH.

Many factors can influence iron speciation and solubility and it is notoriously difficult to measure the different species of iron in the gut lumen. This therefore makes it difficult to predict how much iron is readily available for potentially pathogenic as well as commensal bacteria to use in the colon. As well as pH and oxygen, dietary components also have a huge influence on iron speciation and, in turn, its availability, as described earlier in this chapter.

Other species of iron, including iron carbonates, iron hydroxides, iron phosphates and iron oxides, can also be found in the intestinal lumen, as the pH of the lumen enables precipitation with hydroxides as well as formation of complexes with proteins, amino acids and food components [139]. However, it not known to what extent bacteria are able to use these forms of insoluble iron. Several ways in which bacteria could possibly make these forms of iron more accessible is by reducing the pH of the surroundings through the production of different acids, such as lactic acid, binding to siderophores or directly reducing Fe^{3+} [200].

1.8.2 Interaction between phytates and bacteria

Phytates (the salt form of phytic acid) are derived from cereals and legumes. This compound has a very high iron-binding capacity and certain gut microorganisms (*Bifidobacteriaceae* and coliforms), are able to break down phytates. This mechanism

could have evolved as a way by which bacterial species can free the phytate-bound iron and use it for their growth [201]. One study showed that the highest phytate degrading activity belonged to *Lactobacillus reuteri, Lactobacillus salivarius and Bifidobacterium dentium* [201], which suggests that phytate-bound iron could potentially be a relevant source of iron for colonic microorganisms. Notably, phytate-bound irons found in the colon are present in the insoluble form making it difficult to degrade [52, 202]. A study that strengthens the idea of phytate degradation in the colon was carried out by Schlemmer *et al*., 2009 [203] who showed that degradation products of phytate were only seen in conventional rats when compared to germ-free rats, suggesting that phytate degradation occurs in the colon by the microbiota.

1.8.3 Interaction between polyphenols and bacteria

Tea and coffee are commonly consumed drinks that are high in polyphenolic compounds, including catechols and tannins. Polyphenols are known to reduce iron absorption in the small intestine due to their extremely strong iron-binding capacity. Strong binding between polyphenols and iron stops both the host and bacterial species from absorbing iron. However, bacterial species may have an advantage in these situations through siderophore production to compensate for low iron availability. Certain bacteria (*Staphylococcus lugdunensis* or *Streptococcus gallolyticus)*, have been shown to be able to degrade tannate and in turn free the iron from the tannate-iron complex [204].

1.9 Prebiotics

1.9.1 Induction of beneficial bacterial growth through prebiotics

According to the International Scientific Association for Probiotics and Prebiotics, prebiotics are defined as "a substrate that is selectively utilised by host microorganisms conferring a health benefit", such as bifidobacteria and lactobacilli, which have the potential to improve host health. Prebiotics are, simply speaking, the 'food' for beneficial bacteria.

Prebiotics have been shown to alter the microbial composition and metabolism, and in the process, potentially create an environment that favours iron bioavailability [205]. Common prebiotics include non-digestible oligosaccharides (NDOs) such as inulin and its partial hydrolysate fructo-oligosaccharides. Other identified prebiotics include galacto-oligosaccharides and lactulose [206]. Tako *et al.*, (2008) demonstrated that there was

significant variation in relative amounts of bifidobacteria and lactobacilli in the intestinal content between the treatment groups, with generally more bifidobacteria being present with increased prebiotic content [207].

Also known as, 'colonic foods', prebiotics resist digestion by gastric acid and pancreatic enzymes *in vivo* but are preferentially fermented by beneficial intestinal bacteria once they reach the colon. Studies have shown that provision of diets with inulin enhanced surface counts of bifidobacteria and lactobacilli in biopsy samples taken from the cecum, transverse and descending colon, and rectum of human subjects during colonoscopy [208]. Some reported benefits of beneficial bacteria include a decrease in toxic metabolites and detrimental enzymes secreted by pathogenic bacteria, strengthening the intestinal flora's ability to defend against invasion by potentially pathogenic bacteria and repressing the onset of constipation. It should be noted that both inulin and fructo-oligosaccharides are 'generally-regarded-as-safe' and their acceptable intake levels depend on the sensitivity of the consumer, ranging from <10 g/d to >30 g/d [209].

As an effect of prebiotics, a change of microbial composition can lead to a change in the production of short chain fatty acids (SCFAs). SCFAs have also been shown to induce the proliferation of epithelial cells, therefore increasing the absorptive surface, whereas prebiotics or their fermentation products may enhance the presence of iron regulatory genes such as DMT1 in both the duodenum and the colon [207, 209]. One study illustrated that iron absorption genes in the colon of mice were highly expressed in iron-deficient mice compared with healthy controls [210]. It also showed that Dcytb was present in low amounts in the colon of iron-deficient mice. As mentioned earlier, Dcytb facilitates the reduction of Fe^{3+}, which is necessary for the uptake of iron. Combined, this may indicate that it is not necessary for colonocytes to be able to produce Dcytb because the microbiota have other ways to elicit reductase activity that already contributes to the reduction of Fe^{3+} [210]. Another study examined the effects of iron absorption in gnotobiotic rats compared to conventionally raised rats [211]. It was found that the gnotobiotic rats had a much lower iron uptake activity in comparison to the conventionally raised rats, once again suggesting that microbial communities influence the ability of the host to absorb iron.

1.9.2 Short chain fatty acids

Humans lack the enzymes to degrade the bulk of dietary fibres which therefore pass the upper gastrointestinal tract unaffected and are fermented in the cecum and the large

intestine by the colonic bacteria. Fermentation of these indigestible fibres leads to the production of multiple groups of metabolites [212] of which SCFAs are the major group [213]. To the microbial community, SCFAs are an essential waste product, as they are needed to balance redox equivalent production in the anaerobic environment of the gut [214]. Also, SCFAs have been shown to exert multiple beneficial effects on mammalian energy metabolism. SCFAs are saturated aliphatic organic acids that consist of one to six carbons of which acetate (C2), propionate (C3), and butyrate (C4) are the most abundant (≥95%) [156]. Acetate is mainly used as an energy source in colonocytes [215]. Recently, a study suggested that the protection from enteropathogenic infection by bifidobacteria is partially attributed to the production of acetate [216]. The impact of butyrate on gut health has been examined comprehensively and has been associated with anti-cancerogenic effects and anti-inflammatory properties [217-219]. Furthermore, it has been shown that butyrate acts as an energy source for intestinal cells [220]. Lastly, propionate has been shown to be involved in cholesterol- and lipid-lowering mechanisms [221]. However, not all metabolites have beneficial effects on gut health. The accumulation of lactate in faeces has been correlated with inflammatory bowel disease and ulcerative colitis, and lactate concentrations increased during low iron availability conditions [156].

One study, with the use of *in vitro* colonic fermentations, carried out investigations looking at the relationship between iron availability and the effect it had on colonic metabolites [156]. A significant decrease of acetate, butyrate and propionate was observed under iron-restricted conditions. These results could potentially be attributed to the fact that many iron-dependent enzymes are required for the production of these SCFAs, mainly acetate and butyrate. An estimated one third of acetate production is through the acetyl-CoA pathway, a pathway that depends on iron behaving as a co-factor for many enzymes. In the same manner, the production of butyrate also requires iron-dependent pathways at certain stages. It is therefore justified to assume that SCFA production is consequently lowered in iron-limiting environments. However, it is important to note that as the host can very quickly absorb SCFAs, caecal, colonic or faecal metabolite levels do not automatically equate to the levels produced by bacteria.

1.10 Animal and human studies in the context of iron and the gut microbiota

A number of studies in animals and humans have investigated the effect of iron deficiency and/or supplementation on the composition of the gut microbiota, in animals and in humans. These studies have illustrated a fairly well-defined pattern of microbial alterations in the gut which correlate with conditions of either iron-limited or iron-supplemented diets.

1.10.1 Animal studies

Experimental animal studies provide a good foundation upon which hypotheses can then be tested in humans. Benoni *et al.,* (1993) administered iron at different doses to rats and found that *Clostridium difficile* enterotoxin increased after 24 h along with the number of *Clostridium spp*. after 4 w, with the high-iron dose [222]. *E. coli* and *Lactobacillaceae* numbers were also increased after 2 weeks of iron administration, with numbers of *Bacteroides spp*. and enterococci reduced. Another study reported that total anaerobes, *Enterococcus spp*. as well as lactobacilli were elevated in iron-deprived mice and that iron supplementation generally perturbed the gut microbiota [223]. Lee *et al.,* (2008) investigated weanling pigs that were put on an iron-supplemented diet, which resulted in no effect on clostridia, *Lactobacillaceae, Bifidobacteriaceae* and total anaerobic bacteria; however, increased numbers of coliform bacteria were observed [224].

Another mouse study which encompassed a genetic modification of iron metabolism in mice illustrated that the relative abundance of five lactic acid bacteria were significantly different among the mouse lines, suggesting that the deletion of iron metabolism-related genes in the host can affect the intestinal gut composition [225].

Constante *et al.,* (2017) performed a study investigating the effects of iron supplementation in dextran sulphate sodium (DSS) -induced mice illustrated that iron supplementation at different doses induced shifts in the gut microbial communities and inferred metabolic pathways. The most noticeable taxonomic changes included an increase in the relative abundance of Proteobacteria, and a decrease in the abundance of Firmicutes [226].

Dostal *et al.,* (2012) observed the effect of iron deficiency and subsequent iron repletion on the gut microbiota composition and metabolic activity in young Sprague-Dawley rats [151]. Iron deficiency led to an increase in *Enterobacteriaceae* and *Lactobacillus* but reduced counts of *Bacteroides spp*. and *Roseburia spp./Eubacterium rectale* members. Moreover, changes in metabolites were also seen, with decreases in butyrate and

propionate during iron-deficient conditions. Iron supplementation with $FeSO_4$ and electrolytic iron re-established the original gut microbiota composition to an extent and led to a full recovery of metabolic activity in the rats, suggesting that iron is required for these populations to survive adequately. Similarly, another *in vivo* study which investigated the influence of SCFAs on iron absorption in the proximal colon in rats displayed low levels of butyrate and propionate during environments of luminal iron deficiency [227].

Unsurprisingly, iron can promote the replication and virulence of gut enteric pathogens, including *Shigella, Campylobacter* and *Salmonella* [228]. Generally, the amount of iron in the gut can influence the infection cycle of a pathogen. The increased luminal iron and intracellular iron in enterocytes could potentially exaggerate or attenuate the virulence of enteric pathogens. However, thus far, relatively little is known about a potential link between iron and intestinal infection and this merits more research.

1.10.2 Human studies

Numerous studies have looked at the effect of iron fortification and iron depletion on the human gut microbiota. One of the oldest studies dating back to 1985, provided infants with an iron-fortified cow's milk preparation and investigated the changes in the gut microbial composition using culture-based methods [148]. These children had reduced counts of bifidobacteria but higher counts of *E. coli* and *Bacteroides spp*. compared to the infants receiving an unfortified cow's milk preparation.

Zimmermann *et al.,* (2010) examined the gut microbiota of schoolchildren from Côte d'Ivoire using molecular methods, such as quantitative polymerase chain reaction (qPCR) [229]. These children were given iron-fortified biscuits for a period of 6 months and they found that compared to the control group, which were receiving unfortified biscuits, isolation frequencies of lactobacilli were lower and *Enterobacteriaceae* were higher in their faecal samples. Conflicting results were seen in a study of iron-deficient women in India [230] where low levels of lactobacilli were observed.

Jaeggi *et al.,* (2015) examined the effects of low and high doses of in-home iron supplementation on the gut microbiota of Kenyan children [157]. In this setting, provision of iron-fortified porridge led to an increase in pathogen abundance, with numbers of enterobacteria, clostridia and pathogenic *E. coli* increasing whilst numbers of bifidobacteria decreased. Furthermore, in comparison to the control group (receiving

unfortified porridge), the children with iron-fortified porridge had elevated levels of faecal calprotectin, a marker of gut inflammation.

A lack of knowledge of host factors such as diet fluctuation, the immune system and iron status in the gut might be drawbacks to studying iron and the microbiota. Nevertheless, *in vitro* studies on microbial metabolism in the presence of iron and nutrients support the findings of *in vivo* studies. Dostal *et al.,* (2013) examined the effects of reduced iron availability in continuous *in vitro* colonic fermentations [156]. During very low iron conditions, a reduction in the counts of *Roseburia spp./E. rectale*, *Clostridium cluster IV* members and *Bacteroides spp*. were observed while *Lactobacillus spp*. and *Enterobacteriaceae* increased. Decreases in the main metabolites (propionate and butyrate) were also observed during iron-deficient conditions.

The role of iron and its influence on the replication and virulence of gut enteric pathogens has also been investigated. Olakanmi *et al.,* (2007) investigated the infectivity of *Mycobacterium tuberculosis* (M.Tb) in subjects with HH. This study showed that there was a reduced growth of M.Tb in HH subjects compared to control subjects as M.Tb acquisition of iron was much lower in the former, suggesting that cellular iron concentration is one of the critical determinants for infectivity [231].

In vivo studies have produced varying outcomes when studying the effect of iron on specific bacterial groups of the human gut microbiota. This could potentially be in part due to the intricate interactions between the host iron status, the response the host has to differing dietary iron levels, or the iron concentration in the gut lumen. Furthermore, other factors such as intestinal immune function, environmental changes, host physiology and dietary habits can also influence the gut microbial composition. *In vitro* gut fermentation models allow the gut microbiota to be examined without any influence from the host, as well as other environmental factors, through tightly controlled parameters [232]. The *in vitro* continuous colonic fermentation model [233] utilising immobilised child gut microbiota represents a good technological platform to investigate the impact of dietary changes on the gut microbiota [232].

1.11 Thesis aims

1. To grow pure cultures of bacteria in order to investigate:
   i. The effects of iron addition on bacterial growth
   ii. The effects of iron chelation on bacterial growth

2. To use an *in vitro* batch fermentation model to culture human faecal microbiota in a nutritive media simulating the colon, to investigate:
   i. Changes in viable counts of common bacterial groups under iron-supplemented and iron-chelated conditions
   ii. Changes in bacterial composition caused by iron-chelated conditions
   iii. Changes in bacterial metabolites caused by iron-chelated conditions

3. To optimise a delivery system to enable release of an iron chelator in the colon

4. To implement the colonic delivery system in a human trial investigating the effects of an iron chelator on the human gut microbiota

1.12 Hypotheses

The hypotheses to be tested in relation to the aims outlined in the previous section are:

1. Growth of pure cultures of bacteria in iron chelated media will lead to the decrease of bacterial growth, whilst addition of iron will encourage bacterial growth

2. *In vitro* batch fermentation models will indicate:
   i. A reduction in viable counts of common bacterial groups under iron-chelated conditions
   ii. Bacterial composition of bacteria that have the potential to display pathogenic phenotypes will reduce under iron-restricted conditions
   iii. A change in different bacterial taxa will subsequently alter the production of SCFAs

3. An optimised coating formulation will allow for successful delivery of an iron chelator to the colon

4. The consumption of an encapsulated chelator by healthy participants will reduce the relative abundance of potentially pathogenic groups of bacteria through the reduction in water-soluble iron concentrations

CHAPTER TWO

2 Materials and Methods

2.1 General Reagents

Unless stated otherwise, all chemicals were purchased from Sigma (UK) and were of the purest grade available. A 20% nitric acid (HNO_3) solution was generated by adding 714 mL deionised water to 286 mL 70% HNO_3, to achieve a total volume of 1 L. All glassware and equipment were acid-washed with 20% HNO_3 before iron quantification analysis.

A 1 M hydrochloric acid (HCl) solution was prepared by diluting 36.46 mL 37% HCl (Fischer Scientific, UK) with distilled water to a total volume of 1 L.

A 1 M sodium hydroxide (NaOH) solution was generated by dissolving 40 g NaOH pellets in 250 mL distilled water and then diluting the solution further to reach a total volume of 1 L.

The following chemicals were diluted to the desired concentration using ultrapure water and then subsequently added to pure or/and mixed bacterial cultures to assess the impact of either iron addition or chelation on bacterial growth: ammonium iron (II) sulphate hexahydrate; bathophenanthroline disulphonic acid; 2,2 Dipyridyl; phytic acid salt sodium hydrate; tannic acid; lactoferrin from human milk and sodium alginate (provided by Chris Tselepis, Birmingham).

Phytin mineral salt (TSUNO Rice Fine Chemicals, Japan) was diluted to the desired concentration using 0.1 M HCl and then subsequently added to mixed bacterial cultures to assess the impact of phytin dependent iron chelation on bacterial growth.

2.2 Bacterial strains and growth conditions

2.2.1 Media used for bacterial growth

The following bacterial strains were used: *Escherichia coli* 1BO4 (isolated from human faeces), *Bifidobacterium longum* B78 F110564 (isolated from human faeces), *Lactobacillus rhamnosus* GG F111027, *Salmonella* Typhimurium (ATCC SL1344), *Clostridium perfringens* (NCTC 3110) and *Bacteroides thetaiotaomicron* VP1-5482 (ATCC 29148). All bacteria were grown anaerobically at 37°C.
Table 2.1 outlines the composition of media used for the growth of the bacterial strains mentioned above.

Table 2.1 - Composition of bacterial culture media

Media	Compositions in 1 L H_2O	pH	Bacteria
BHI	12.5 g brain infusion solids, 5 g beef heart infusion solids, 10 g protease peptone, 5 g sodium chloride, 2 g glucose, 2.5 g disodium phosphate	7.4 ± 0.2	*B. longum*
BHI + haemin (BHI-H)	Same as BHI + 0.5% w/v haemin	7.4 ± 0.2	*B. thetaiotaomicron*
BHI + complement (BHI-C)	Same as BHI + 0.01 mL vitamin K [10 mM], 0.01 mL haemin [0.5% w/v], 4 mL resazurin [0.02 %], 0.5 g L -cysteine	7.4 ± 0.2	*C. perfringens*
LB	10 g tryptone, 5 g yeast extract, 10 g NaCl	7.0 ± 0.2	*E. coli and S.* Typhimurium
MRS + glucose	8 g peptone, 5 g yeast extract, 5 g $CH_3COONa.3H_2O$, 2 g K_2HPO_4, 2 g $C_6H_{17}N_3O_7$, 5 mL salt solution (0.2 g $CaCl_2$ anhydrous, 0.2 g $MgSO_4$, 1 g K_2HPO_4, 1 g KH_2PO_4, 10 g $NaHCO_3$, 2 g NaCl in 1 L), 1 mL Tween 80, 20 g glucose	6.5 ± 0.2	*L. rhamnosus*
M9	Solution A: 6 g Na_2HPO_4 [40 mM], 3 g KH_2PO_4 [20 mM], 0.5 g NaCl [8 mM], 1 g NH_4Cl [20 mM], 790 mL dH_2O; Solution B: 0.147 g $CaCl_2.2H_2O$ [10 mM], 100 mL dH_2O; Solution C: 0.246 g $MgSO_4.7H_2O$ [10 mM], 100 mL dH_2O; Solution D: 11 mL leucine, 6.5 mL histidine, 1.7 mL thiamine, 1.5 mL threonine, 0.23 g proline, 0.13 g arginine, 2 g glucose, 100 mL dH_2O	7.3 ± 0.2	*E. coli*, *S.* Typhimurium
Nutritive media	2 g peptone water, 2 g yeast extract, 0.01 g NaCl, 0.04 g K_2HPO_4, 0.04 g KH_2PO_4, 0.01 g $MgSO_4.6H_2O$, 0.01 g $CaCl_2.6H_2O$, 2 g $NaHCO_3$, 0.5 g cysteine.$HCl.H_2O$, 0.5 g bile salts, 2 mL Tween80 and 10 μL vitamin K1	7.0 ± 0.2	*in vitro* colonic fermentations

2.2.2 Selective agar plates used for enumeration of bacterial groups

Table 2.2 outlines the different agar plates used to visualise viable counts of different bacterial groups analysed during *in vitro* colonic fermentation studies. All media, except

bifidobacteria agar plates, were first autoclaved and cooled before pouring into petri dishes. All media were purchased from Oxoid, except Brucella, which was purchased from Difco.

Antibiotics were purchased from Sigma and diluted to required stock concentration using ultrapure water.

Table 2.2 – Composition of agar plates used for enumeration of different bacterial groups

Bacterial group	Compositions in 1 L H_2O	Antibiotic
Total anaerobes	43 g Wilkin chalgren	-
Enterobacteriaceae	51.5 g MacConkey #3	-
Lactobacilli	15 g agar, 52 g MRS broth powder	-
Clostridia	43 g Wilkin chalgren	8 mL novobiocin and colistin (1 mg/mL)
Bacteroides	28 g Brucella, 15 g agar, 10 mL haemin solution (0.5 mg/mL), 200 µL vitamin K solution and 50 mL laked horse blood (Oxoid)	3 mL kanamycin (25 mg/mL) and 7.5 mL vancomycin (1 mg/mL)
Bifidobacteria	39 g Columbia agar, 5 g glucose, 0.5 g cysteine.HCl and 5 g agar. 5 mL propionic acid to be added after cooling and adjusted to pH 5 with 6 M NaOH	-

2.3 Quantification of iron from stool samples

2.3.1 Measurement of total iron

Flame atomic absorption spectrophotometry (FAAS) was used to determine the concentration of iron in faecal samples. All glassware and equipment used were acid-washed with 20% 16 M HNO_3. Fresh faecal samples were weighed and then dried at 110^{o}C in an oven. The sample was re-weighed to calculate water content, transferred into glass crucibles and ashed in a muffle furnace for 48 h at 600^{o}C. The ashed sample was dissolved in 20% 16 M HNO_3 and crucibles were then placed on a hot plate until almost dry. The residue was dissolved with 1 M HCl and then diluted further to a final volume of 25 mL of 1 M HCL. The spectrophotometer (Perkin Elmer Atomic Absorption Spectrophotometer Model 3300) was calibrated against a range of iron standards (0-6 ppm) and samples were measured at an absorption wavelength of 248 nm.

2.3.2 Measurement of water-soluble iron in stool samples

A 0.2 g aliquot of a fresh faecal sample was homogenised with a 0.2 g of ultrapure water, mixed on a rotator stirrer (300 rpm) for 30 mins at room temperature, and centrifuged at 3,200 xg for 15 mins at 4ºC. Supernatants were then analysed using the Ferrozine assay (Abcam, Cambridge, UK), where ferric iron in the sample is reduced to ferrous iron using an iron reducer provided in the kit, after which the iron reacts with Ferene S (an iron chromogen) to produce a stable coloured complex with an absorbance at 593 nm.

2.4 Assessing the impact of either iron chelation or addition on pure bacterial cultures

A range of pure bacterial cultures were grown overnight in the desired media at 1% inoculation. Cultures were seeded in 100-well honeycomb plates, and the cells were then exposed to the chemical of interest. Samples were analysed using a Bioscreen C, which monitors the growth of microorganisms by measuring the turbidity (optical density, OD) of the liquid growth medium. The experiments were run for 24 h – 48 h, with measurements at OD_{600}, taken every 10 mins. The temperature of all experiments was set at 37ºC. Depending on the type of organism, these studies were carried out under either aerobic or anaerobic conditions.

2.5 Culturing human faecal microbiota

2.5.1 Donor recruitment for in vitro *colonic fermentation experiments*

Faecal samples used in the colon model experiments were obtained from participants recruited onto the Quadram Institute Bioscience (QIB) Colon Model study. Men and women aged 18 y or older who live or work within 10 miles of the Norwich Research Park were recruited onto the QIB Colon Model study if they satisfied the following criteria. Participants who were assessed to have a normal bowel habit, regular defecation between three times a day and three times a week, with an average stool type of 3 – 5 on the Bristol Stool Chart, and not diagnosed with chronic gastrointestinal health problems, such as irritable bowel syndrome, inflammatory bowel disease, or coeliac disease were eligible to enrol onto the study. Demographic information was collected, and a brief health questionnaire was completed during the eligibility screening. Participants were asked additional questions immediately prior to donating a stool sample to confirm that they had not taken antibiotics or probiotics within the last four weeks, had not experienced a

gastrointestinal complaint, such as vomiting or diarrhoea, within the last 72 h, were not currently pregnant or breast-feeding, had not recently had an operation requiring general anaesthetic, and were not taking iron or multivitamin supplements. The study was approved by the Quadram Institute Bioscience (formally Institute of Food Research) Human Research Governance committee (IFR01/2015), and London - Westminster Research Ethics Committee (15/LO/2169). The informed consent of all participating subjects was obtained, and the trial is registered at http://www.clinicaltrials.gov (NCT02653001).

2.5.2 Culturing human faecal microbiota in nutritive media

All samples were processed within 4 h of stool collection. A 10 g portion of faecal material was placed into a Seward stomacher bag using a sterilised spatula. Deoxygenated 1x PBS was added to the stomacher bag to obtain a mass of 100 g. The stomacher bag was placed into the Stomacher 400 circulator and the faecal matter was homogenised at 230 rpm, for 45 seconds. A 15 mL aliquot of the homogenised faecal suspension was transferred into a 150 mL sterile vessel. The working volume of each vessel was set at 150 mL of which 135 mL was nutritive media (composition of nutritive media can be found in Table 2.1). The pH was maintained between 6.6 – 7.0 and the temperature of the vessels were kept at 37ºC.

Conditions tested were either nutritive media with faecal inocula only (control), or with faecal inocula supplemented with the reagent of interest. For each donor, one vessel was used for each condition. Samples were taken at 0, 4, 8, and 24 h and spun at 3200 xg, 4ºC for 15 mins. A 100 µL aliquot of the supernatant suspension was serially diluted in PBS (900 µL) and enumerated on selective agar plates (Table 2.2). The remaining supernatant was aliquoted and used for ^{1}H nuclear magnetic resonance (NMR) analysis (section 2.7). The resulting pellet was frozen and later used for bacterial DNA extraction (section 2.6).

2.6 Phylogenetic analysis of cultured human faecal microbiota

2.6.1 Extraction of bacterial DNA

The bacterial pellets were thawed at room temperature and the DNA was extracted using a FastDNA SPIN Kit for Soil (MP Biomedicals, UK) with an additional modification incorporated into this method [234]. Sodium phosphate buffer (978 µL) and MT buffer (122 µL) were added to the thawed bacterial pellets and vortexed until the pellet was completely homogenised. This mixture was then incubated at 4˚C for 1 h, with the sample

being mixed using a vortex every 15 mins. After vortexing, samples were transferred to Lysing Matrix E tubes, and mechanically disrupted three times with a FastPrep instrument (MP Biomedicals, UK) at 6.5 ms^{-1} for 60 s. The supernatant was transferred to a sterile tube containing 250 µL protein precipitation solution (PPS), inverted 10 times by hand and centrifuged at 14,800 xg for 5 mins. The supernatant was transferred to a sterile tube containing 1 mL Binding Matrix suspension, inverted by hand for 2 mins, then incubated at room temperature for 3 mins to allow for the binding matrix to settle. Carefully, 1 mL of the supernatant was discarded, and samples were vortexed, prior to 600 µL of the mixture being added to a SPIN filter tube. Samples were centrifuged at 14,800 xg for 1 min, then underwent three 500 µL DNase-free salt/ethanol wash solution (SEWS-M) wash steps, with centrifugation at 14,800 xg for 1 min between each step. Samples were spun for a further 2 mins to allow for the removal of any remaining ethanol. DNA was eluted with 50 µL of DNase/Pyrogen free DNA elution solution (DES) and stored at -20˚C.

2.6.2 Assessing bacterial DNA extraction

A 1% (w/v) agarose solution was generated by adding 0.5 g agarose powder to 500 mL 0.5 mM Tris/borate/ethylenediaminetetraacetic acid (TBE) buffer, then dissolved by heating in a microwave at 800 watts for approximately 3 mins at full power until clear. The 1% (w/v) agarose solution was added to an electrophoresis gel tray in a horizontal gel electrophoresis system (Life Technologies, Inc.), with a toothed comb fitted (to a depth of ~8 mm) and left to set at room temperature (approx. 40 m). 2 µL Hyperladder I (Bioline) was added to the outer well to act as a molecular weight marker, and 1 µL DNA were mixed with 1 µL 10x loading dye before being added to the remaining wells. The gel was run at 100 volts (PPV 300/200.4, Northumbria Biologicals Ltd) in 5mM TBE until the samples had migrated towards the end of the gel. The gels were stained in an ethidium bromide solution (5 µg-10 µg/mL ethidium bromide in water) for 30 mins and rinsed with water. DNA fragments were visualised and photographed using the AlphaImager HP system (Alpha Innotech) under UV trans-illumination. Total DNA was quantified using the NanoDrop ND-1000 UV/vis spectrophotometer (NanoDrop Technologies, Inc., USA) and the ND-1000 computer software. The nucleic acid, DNA-50 settings were selected, DNase/Pyrogen free water (1 µL) was used as a blank, and 1 µL of each sample was analysed.

2.6.3 Bioinformatic analysis of 16S rDNA

DNA extracted from *in vitro* batch fermentation models were sent to Novogene for sequencing. Sequencing reads were analysed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline and Ribosomal Database Project (RDP) classifier. All sequences were filtered to meet the following criteria: read length within 200 and 1,000 bp; a maximum of 6 ambiguous bases; a minimum average quality score of 25 within a 50 bp window; and exact match to primer sequences. ChimeraSlayer was used to filter trimmed reads for chimeric sequences, and RDP classifier (version 2.10) was used for bacterial taxonomy assignment with a confidence value threshold of 50%, with trimmed reads clustered into operational taxonomic units (OTUs) at 97% identity level. Observed species (number of unique OTUs) and the Shannon Index (species richness and evenness) were used to compute alpha (α)-diversity and rarefaction plots. Weighted and unweighted UniFrac distances were used to generate beta (β)-diversity principle co-ordinate analysis (PCoA) plots, which were visualised using the Emperor tool. Primers U515F (5'-GTGCCAGCMGCCGCGGTAA) and U806R (3'-GGACTACHVGGGTWTCTAAT) were used to amplify the V4 region of the 16S rDNA.

2.7 Short chain fatty acid quantification

Faecal water was prepared to quantify short chain fatty acids in stool. Briefly, samples (13 mL) taken from colonic batch fermentations were centrifuged at 3220 xg for 15 mins at 4ºC. 100 µL NMR buffer (0.26 g NaH_2PO_4 and 1.41 g K_2HPO_4 made up in 100 mL deuterium oxide (D_2O), containing 0.1% NaN_3 (100 mg), and 1 mM sodium 3-(Trimethylsilyl)-propionate-*d*4, (TSP) (17 mg) as a chemical shift reference) was added to 900 µL supernatant and analysed using ^{1}H NMR spectroscopy (this mixture is defined as 'faecal water'). The ^{1}H NMR spectra were recorded at 600 MHz on a Bruker Avance spectrometer (Bruker BioSpin GmbH, Germany) running Topspin 2.0 software and fitted with a cryoprobe and a 60-slot autosampler. Each ^{1}H NMR spectrum was acquired with 256 scans, a spectral width of 12,300 Hz, and an acquisition time of 2.67 s. The "noesypr1d" pre-saturation sequence was used to suppress the residual water signal with a low-power selective irradiation at the water frequency during the recycle delay and a mixing time of 10 ms. Spectra were transformed with a 0.3 Hz line broadening, and were manually phased, baseline corrected, and referenced by setting the TSP methyl signal to 0

ppm. The metabolites were quantified using the software Chenomx® NMR Suite 7.0™. NMR analysis was performed by Dr Gwenaelle Le Gall (UEA).

2.8 Effects of phytin on the human gut microbiota dietary intervention study

Full details can be found in the Appendix in the form of annexes. Briefly, the study method was as follows.

2.8.1 Study Recruitment

Fourteen participants were recruited onto the 'Effect of Phytin on the Human Gut Microbiome' (EPoM) human study (ClinicalTrials.gov: NCT03917693) and gave written informed consent for their biological samples to be used as described in the study protocol (Appendix). The EPoM study protocol was approved by the Human Research Governance Committee at Quadram Institute Bioscience and the East of England – Cambridge Central Research Ethics Committee (19/EE/0005). All study participants were assessed for eligibility on the basis of a screening health questionnaire and the results of clinical laboratory tests sent to Norfolk and Norwich University Hospital (NNUH). All participants produced a urine sample for urinalysis which was screened for protein, blood, leukocytes, nitrites, glucose and ketones via a dipstick urine test (Multistix® 8SG; Siemens). The following exclusions applied:

- Failing screening test
- Pregnant, or have been pregnant in the last year or are lactating and/or breast feeding
- Currently suffering from, or previously suffered from, any diagnosed gastrointestinal disease, gastrointestinal disorders including regular diarrhoea and constipation (excluding hiatus hernia unless symptomatic), and/or gastrointestinal surgery
- Diagnosed with any long-term medical condition that may affect the study outcome (e.g. cancer, diabetes, haemophilia, cardiovascular disease, glaucoma, anaemia), as assessed on an individual basis
- Diagnosed with any long-term medical condition requiring medication that may affect the study outcome
- Regularly taking over the counter medications for digestive/gastrointestinal conditions

- Long-term antibiotic therapy (at least 4 weeks since end of a course of antibiotics, assessed on an individual basis)
- Regular laxatives (once a month or more)
- User of dietary supplements or herbal remedies and unwilling to stop taking them for one month prior to and during study period (assessed on an individual basis)
- Consumer of pre- or pro-biotic drinks &/or yoghurts on an irregular basis.
- On a diet programme that may affect the study outcome (e.g. 5:2 fasting diet)
- Recently returned to the UK following a period abroad and suffered gastric symptoms during the period abroad or on return to the UK (assessed on an individual basis).
- Regular/recent (within 3 months) use of colonic irrigation or other bowel cleansing techniques
- Involvement in another research project that includes dietary intervention or blood sampling
- Blood seen in stools or two or more episodes of constipation or diarrhoea (type 1, 2, or 7 stools) during the study
- Unwilling to provide GPs contact details
- Unable to provide written informed consent
- Regularly consume more than 15 units (women) or 22 units (men) of alcohol a week
- Regularly taking iron supplements
- Unable to swallow capsules
- Abnormal blood pressure measurements (i.e. 160/100 or higher, or low blood pressure)
- Related to someone in the study (e.g. spouse, partner, immediate family member).

Details of the participants that were enrolled onto the study are displayed in Table 2.3.

Table 2.3 – Age, gender, Body Mass Index (BMI) of EPoM study participants. *All participants were non-smokers.*

Participant Code	Age (y)	Gender	BMI (kg/cm^2)
EPoM114	27	Male	19.7
EPoM120	32	Female	27.0
EPoM125	33	Female	19.5
EPoM129	27	Female	26.5
EPoM134	26	Male	25.9
EPoM139	25	Female	25.0
EPoM148	28	Female	28.3
EPoM150	23	Female	21.4
EPoM151	29	Male	28.5
EPoM155	24	Male	20.9
EPoM156	23	Male	21.4
EPoM163	18	Male	25.8
EPoM169	25	Male	24.9
EPoM191	30	Female	23.8

2.8.2 EPoM study design

Recruited participants (n=14) were asked to maintain their habitual diet throughout the length of the study. Following randomisation (see Appendix: Study Protocol, 'Randomisation process'), half of the participants (dependent on randomisation results) consumed two capsules, each containing 0.4 g phytin (test capsule), three times a day with a meal for a period of two weeks. The remaining participants consumed two placebo capsules, each containing 0.4 g microcrystalline cellulose, three times a day with a meal for a period of two weeks. Phase 1 was followed by a 2-week washout period, where all participants ceased capsule consumption. After the washout period, the alternative treatment was given (Figure 2.1).

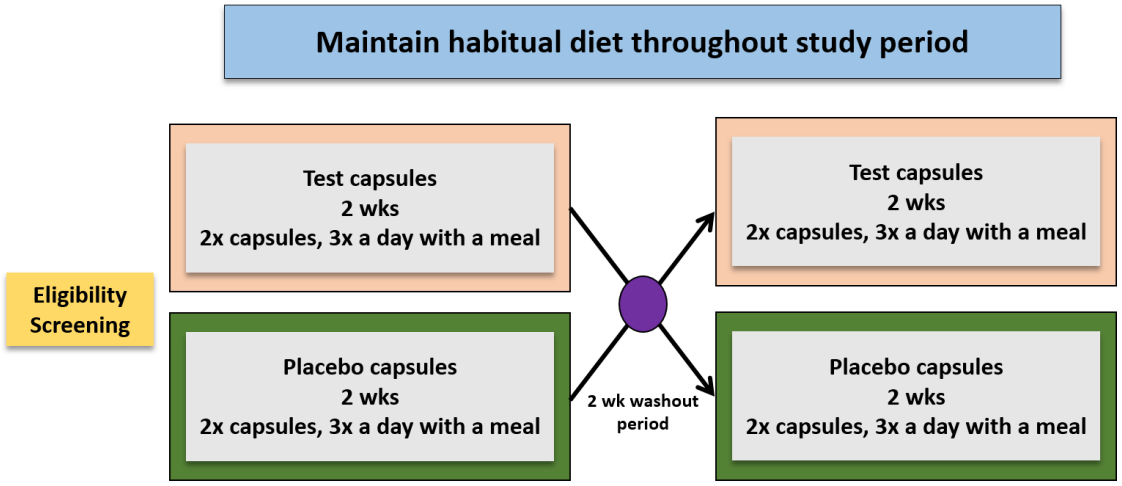


Figure 2.1 - Summary of EPoM study design. *The diagram represents a two-phase crossover, 6-week dietary intervention trial, where participants consumed a randomly allocated set of capsules during each phase. Phases were separated by a washout period, during which no capsule consumption took place. Habitual diet was maintained throughout the study period. Faecal samples were collected three times during each phase, at the start, middle and end. During each phase, stool charts, food frequency questionnaires and capsule checklists were completed for a consecutive period of time.*

Participants were asked to maintain their habitual diet during the entirety of the study period. Three faecal samples were collected per participant per phase, following the instructions provided, at the following stages in the study: before commencing the phase, seven days after starting the phase and upon completion of the phase. Faecal samples were used to analyse the composition of the human gut microbiota (detailed method in section 2.8.3.1) . Participants were asked to complete stool charts to assess any effects of the capsule content on gut function, and to complete a food frequency questionnaire. These were completed in consecutive 7-day periods during each intervention phase. Finally, all participants were asked to complete a capsule checklist throughout the entire study as a measure of compliance.

2.8.3 EPoM study sample analysis

2.8.3.1 Phylogenetic analysis of human faecal microbiota

Aliquots of faecal samples were collected from each sample, using sterilised spatulas and stored at -80˚C. Bacterial DNA was extracted, visualised, and the DNA concentration was quantified following the method described in section 2.6.1. Bacterial DNA concentration was normalised to 5 ng/µL by dilution with ultra-pure water to produce a final volume of

50 µL. Normalised DNA samples were sequenced in-house for 16S rRNA gene using paired-end Illumina sequencing (2x 250 bp) on the MiSeq platform. In-house sequencing was performed by Dave Baker (QIB).

Sequencing data were analysed by Dr Andrea Telatin (QIB), using the QIIME (version 1.9.1) pipeline and RDP 16S rDNA sequence database, as described in section 2.6.3.

2.8.3.2 Metabolite analysis of human faecal waters

Human faecal waters were prepared simultaneously with pellets for bacterial DNA extraction. Approximately 0.2 g of thawed or fresh faecal samples were added to sterile tubes. NMR buffer (2.4 mL) was added to the faecal sample and homogenised before centrifuging at 3,220 xg for 15 mins at 4^{o}C. The supernatant ('faecal water') was transferred into sterile tubes and analysed for metabolite profiling following the method outlined in section 2.7.

2.9 Statistical analysis

Data from pure culture experiments and *in vitro* colonic fermentations were expressed as means ± standard errors of the means (SEM). Pure culture data were analysed using one-way ANOVA followed by Bonferroni post-tests with GraphPad Prism software (Version 5.04), whilst *in vitro* colonic batch fermentations were analysed using unpaired t-tests assuming unequal variances on Microsoft Excel. $p \leq 0.05$ was considered statistically significant. PCA plots illustrated in Chapter 7 were generated using the XLSTAT package in Microsoft Excel.

CHAPTER THREE

3 Baseline iron concentrations in human faecal samples and the effects of iron addition on pure and mixed bacterial cultures

3.0 Summary

The requirement for iron in bacterial growth and survival has been observed for a wide range of bacterial species. This chapter focuses on the impact of iron addition to a range of independently cultured bacteria as well as mixed cultures derived from human faecal microbiota. The data outlined in this chapter highlights the positive effect that iron has on the growth of *E. coli* and *S.* Typhimurium when cultured independently, whilst varying results are observed in the gut microbial composition when iron is added to human faecal microbiota.

3.1 Introduction

Iron availability is typically very limited to the microorganisms due to host iron-withholding mechanisms and can therefore stop pathogenic organisms from growing [235]. An established method of iron acquisition is the secretion of iron-scavenging siderophores (also known as enterobactin and enterochelin) and haemophores, molecules that are synthesised by bacteria and released into the extracellular medium in order to scavenge inorganic iron or haem iron [20, 178].

Siderophores can be split into three key classes depending on the chemical nature of the moieties that donate the oxygen ligands for Fe^{3+} co-ordination [168]. These are hydroxamates, catecholates or carboxylates. Pathogenic bacteria need to proliferate once inside the host for them to survive. The high affinity of the bacterial siderophores helps them to compete with host proteins for iron; bacterial siderophores have an iron-association constant of approximately 10^{51} M^{-1} whilst the host protein transferrin has an association constant that is much lower, at 10^{22} M^{-1}. The iron is released from the siderophore once it is transported inside the bacterial cell through the help of reductases, which reduce siderophore-bound Fe^{3+} into Fe^{2+}. As a result, Fe^{2+} can now be incorporated directly into metallo-enzymes. If there is an excess of iron, it can then be stored as bacterioferritin, or in the related DPS proteins [185]. The Fur repressor protein is then responsible for the shutdown of iron uptake once the bacteria have accumulated enough iron, by preventing the biosynthesis of the iron transport system. Moreover, some bacterial species have evolved even further and are able to extract Fe^{3+} directly from transferrin or even use haem as an iron source [186, 187].

Haemophores are another specialised method in which haem-iron is directly taken up. However, haem iron is more readily available for humans, compared to non-haem iron [236,

237]. On average, iron in omnivorous diets consists of approximately 90% non-haem iron and 10% haem iron.

On average, bacteria need 10^{-7} – 10^{-5} M iron for optimal growth [139]. *E. coli*, for example is able to take up both forms of iron (ferrous and ferric). One study describes the Fe-uptake system that *E. coli* uses for the transport of Fe^{2+} under anaerobic conditions [20]. Most bacterial species are able to acquire iron through the reduction of Fe^{3+} into Fe^{2+} with the help of an extracellular reductase and this form can then be taken up into the cell [167].

Bacteria have also developed mechanisms to reduce iron-induced toxicity. One example of this is a haem export mechanism (HrtAB), which reduces the haem-based iron stress in certain bacterial species, such as *Staphylococcus aureus*. Orthologues of the HrtAB system have been identified in *Bacillus anthracis, Listeria monocytogenes, Enterococcus faecalis,* and *Streptococcus agalactiae* [165]. *Bifidobacteriaceae* have the ability to bind iron to their surface, thereby preventing the formation of free radicals in the surrounding environment as well as sequestering iron from the colonic lumen and preventing pathogenic bacteria from acquiring iron [166].

Animal studies have highlighted the importance of iron within the gut microbial community. One study administered iron at different doses to rats and found that *Clostridium difficile* enterotoxin increased after 24 h, as did the number of *Clostridium spp*. after 4 weeks, with the high-iron dose [222]. *E. coli* and *Lactobacillaceae* numbers also increased after 2 weeks of iron administration, with a reduction in the numbers of *Bacteroides spp*. and enterococci. Another study reported that the numbers of anaerobes, *Enterococcus spp*. and lactobacilli were elevated in iron-deprived mice, and that iron supplementation generally perturbed the gut microbiota [223]. This study examined the effects of an iron-deficient diet on healthy mice and found that numbers of *Lactobacillaceae* were higher in the mice fed with an iron-deficient diet compared to the standard diet control group. Moreover, the numbers of total anaerobes were also higher in the mice on an iron-deficient diet. A more recent study observed the effect of iron deficiency and subsequent iron repletion on the gut microbiota composition and metabolic activity in young Sprague-Dawley rats [151]. Iron deficiency led to an increase in *Enterobacteriaceae* and *Lactobacillus* but reduced counts of *Bacteroides spp.* and *Roseburia spp./E. rectale* members. Moreover, changes in metabolic profiles were also seen, with decreases in butyrate and propionate under iron-deficient conditions. Iron supplementation with $FeSO_4$ and electrolytic iron partially re-established the original gut

microbiota composition, suggesting that iron is required for these populations to survive adequately.

Given the importance of iron, the aim of the experiments described in this chapter was to investigate the effect of added iron on various bacterial species.

3.2 Objectives

Prior to the series of experiments to examine the effects of iron on various groups of bacteria (both independently and in mixed cultures), it was first important to determine baseline levels of iron present in human faeces. This would allow the administration of appropriate concentrations of iron (or chelator) to investigate any potential effects of iron on the bacteria tested. Next, pure and mixed cultures of bacteria were supplemented with iron and subsequently examined to see what impact iron had on bacterial growth.

3.3 Materials and Methods

3.3.1 Human faecal microbiota

Faecal samples used in the colon model experiments were obtained from participants recruited onto the QIB Colon Model study. Men and women aged 18 y or older who live or work within 10 miles of the Norwich Research Park were recruited onto the QIB Colon Model study if they satisfied the following criteria. Further details on donor recruitment can be found in chapter 2, section 2.5.1.

3.3.2 Measuring total iron concentrations in stool samples

FAAS was used to determine the concentration of iron in faecal samples. All glassware and equipment used were acid-washed with 20% 16 M HNO_3. Further detail on faecal sample preparation and processing can be found in chapter 2, section 2.3.1.

3.3.3 Measuring water-soluble iron in stool samples

A 0.2 g aliquot of a fresh faecal sample was homogenised with a known volume of ultrapure water, mixed on a rotator stirrer (300 rpm) for 30 mins at room temperature and centrifuged at 3,000 xg for 15 mins at 4^{o}C. Further detail on sample analysis can be found in chapter 2, section 2.3.2.

3.3.4 The effect of adding ferrous iron to pure cultures of bacteria

A range of pure bacterial cultures were grown overnight in M9 minimal media at 1% inoculation. Cultures were seeded in 100-well honeycomb plates and cells were then

exposed to 250 µM $FeSO_4$. Samples were analysed using a Bioscreen C machine, which monitors the growth of microorganisms by measuring the turbidity (OD) of the liquid growth medium. The experiments were run for 24 h, with measurements at OD_{600}, taken every 10 mins. The temperature of all experiments was set at 37°C. Depending on the type of organism, these studies were carried out under either aerobic or anaerobic conditions.

3.4 Results

3.4.1 Iron quantification from human faecal microbiota

Table 3.1 – Iron concentrations in healthy human faecal microbiota

Donor	Water-soluble iron (mg/g)	Total Iron (mg/g)
CM031	0.113	0.270
CM036	0.067	0.244
CM075	0.064	0.271

Table 3.1 shows the iron concentrations quantified from three healthy human microbiota. On average, the faeces of these three healthy humans contained an average of 0.081 ±0.02 mg/g water-soluble iron (unbound iron that is freely available for use in the colonic environment) and 0.258 ±0.03 mg/g (dry weight) total iron (both water-soluble and insoluble iron, including any iron within the bacterial structure). The total iron was very similar between the three donors, but the water-soluble iron varied, with one donor having a much higher level than the other two.

3.4.2 Iron addition in pure bacterial cultures

For pure culture experiments, a wide range of gut bacteria that are known to be affected or unaffected by iron were tested. The selection of bacteria to be tested was, to some extent dependent on availability in the laboratory.

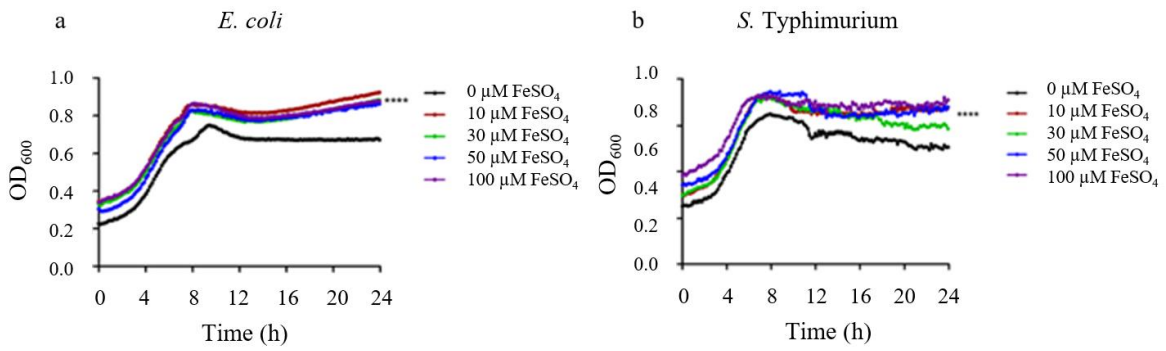


***Figure 3.1 - The impact of $FeSO_4$ on the growth of E. coli* and *S.* Typhimurium**. *$FeSO_4$ added at various concentrations, ranging from 0 – 100 µM to* E. coli *(a) and* S. *Typhimurium (b) grown anaerobically in minimal M9 media, at 1% inoculation, with a total working volume of 300 µL.*

Iron, in the form of $FeSO_4$, was added to pure cultures of *E. coli* (Figure 3.1a) and *S.* Typhimurium (Figure 3.1b) and left overnight to grow at 37ºC. When analysed, the addition of $FeSO_4$ from 10 – 100 µM resulted in a significant increase in the growth of both bacteria ($p<0.0001$) compared to the culture with no iron addition.

To confirm that the effects seen on these bacteria were iron-dependent, a reverse saturation assay was conducted, whereby bacteria were first grown in iron-chelated media and later supplemented with $FeSO_4$.

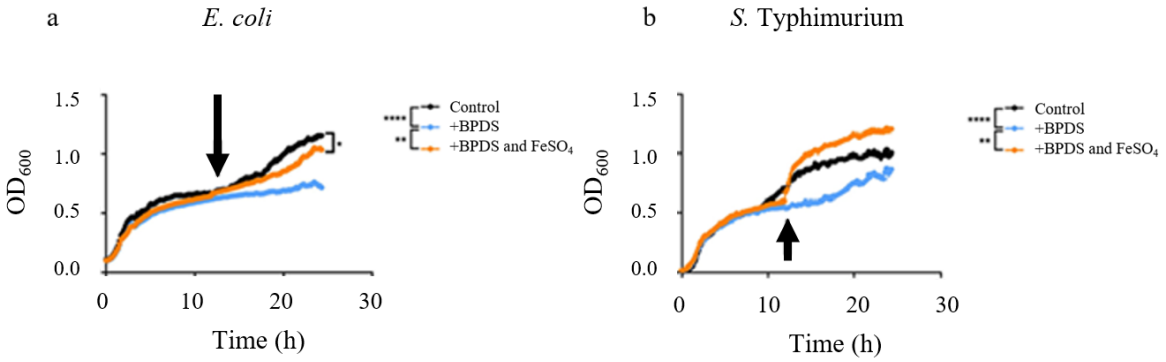


***Figure 3.2 – The impact of iron saturation on the growth of E. coli* and *S.* Typhimurium.** E. coli *(a) and* S. Typhimurium *(b) were cultured overnight under anaerobic conditions at 37ºC in iron-chelated media (achieved through BPDS) at 1% inoculation, with a total working volume of 300 µL. After a period of 12 h, $FeSO_4$ was added to the culture and growth was monitored for a further 12 h. *p<0.05, **p<0.01 and ****p<0.0001.*

E. coli (Figure 3.2a) and *S.* Typhimurium (Figure 3.2b) were cultured overnight in iron-chelated media, achieved with the chemical iron chelator, BPDS. At 12 h post-culture, $FeSO_4$ was added to the culture and the effects on growth were determined after a total period of 24 h (indicated by arrows on both graphs). For both bacterial species, growth was

significantly reduced in the presence of BPDS (blue line; $p<0.0001$ *E. coli* and $p< 0.001$ *S.* Typhimurium). However, when $FeSO_4$ was added to the culture at 12 h, the growth of both bacteria increased and was comparable to the growth of the control. The growth of *E. coli* was significantly higher when iron was added to the culture in comparison to the growth of *E. coli* under iron-chelated conditions ($p<0.01$), and the same was true for *S.* Typhimurium ($p<0.0001$). In the case of *E. coli*, although when supplemented with iron the growth was still significantly less than the control ($p<0.05$), it was still much higher compared to *E. coli* cultured under iron-chelated conditions.

3.4.3 Iron addition in in vitro *colonic fermentations*

Faecal samples used in the colon model experiments were obtained from participants recruited onto the QIB Colon Model study (refer to section 3.3.1 and chapter 2 for further details). Aliquots of fresh faecal samples obtained from the healthy volunteers were diluted in deoxygenated phosphate buffered saline (pH 7.7), and homogenised using a Stomacher 400 (Seward, United Kingdom) at 230 rpm for 45 s. The pH was maintained between 6.6 – 7.0 and temperature of vessels were kept at 37ºC. All samples were processed within 4 h of stool collection. Details of the donors (denoted by a code number in the format of CM0xx) are outlined in Table 3.2. All donors were non-smokers.

Table 3.2 - Age, gender and BMI status of faecal donors

Donor ID	Age (y)	Gender	BMI (kg/m^2)
CM011	51	M	25.7
CM031	33	M	22.4
CM065	34	M	21
CM075	70	M	18

Conditions tested were either nutritive media with faecal inocula only (Control), or with faecal inocula supplemented with $FeSO_4$ (0 – 250 µM). For each donor, 1 vessel was used for each condition. Samples were taken at 0, 4, 8, and 24 h from each vessel, serially diluted in PBS and enumerated on selective agar plates.

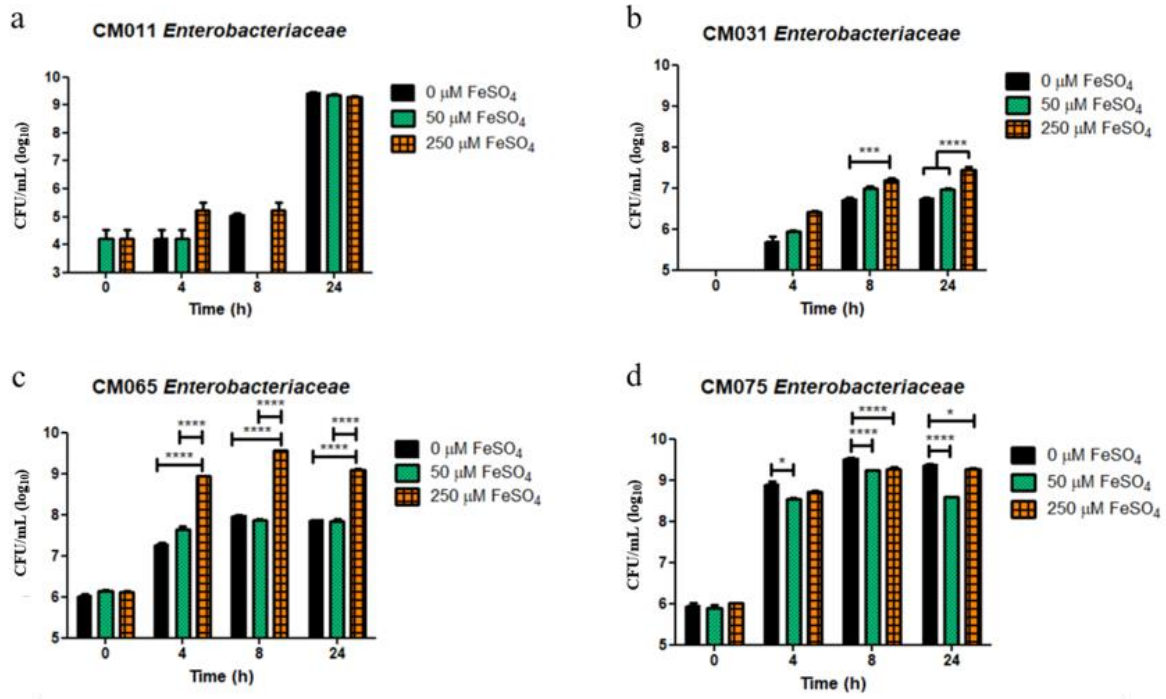


Figure 3.3 – The effect of iron addition on* Enterobacteriaceae *viable counts from* in vitro *colonic fermentations. *Iron was added to* in vitro *colonic fermentations, at concentrations of 50 µM and 250 µM. Significant differences were seen between the control and treatments at the respective time-points, as analysed by one-way ANOVA analysis. *p<0.05, ***p<0.001 and ****p<0.0001.*

Results vary between individual donors when investigating the effect of iron addition on the growth of *Enterobacteriaceae* (Figure 3.3), a group which contains bacterial species with the potential to display pathogenic phenotypes. 2 out of the 4 donors (CM031 and CM065, Figure 3.3b and 3.3c, respectively) had displayed higher counts of *Enterobacteriaceae* in the presence of additional iron (250 µM $FeSO_4$ - CM031 8 h and 24 h $p<0.001$ and $p<0.0001$, respectively, and CM065 8 h and 24 h $p<0.0001$). *Enterobacteriaceae* counts from donor CM011 (Figure 3.3a) remained unaffected by the presence of additional iron, whilst donor CM075 (Figure 3.3d) displayed significant decreases under iron-supplemented conditions (50 µM $FeSO_4$ – $p<0.05$ 4h, $p<0.0001$ 8 h and 24 h; 250 µM $FeSO_4$ – 8 h $p<0.0001$ and 24 h $p<0.05$).

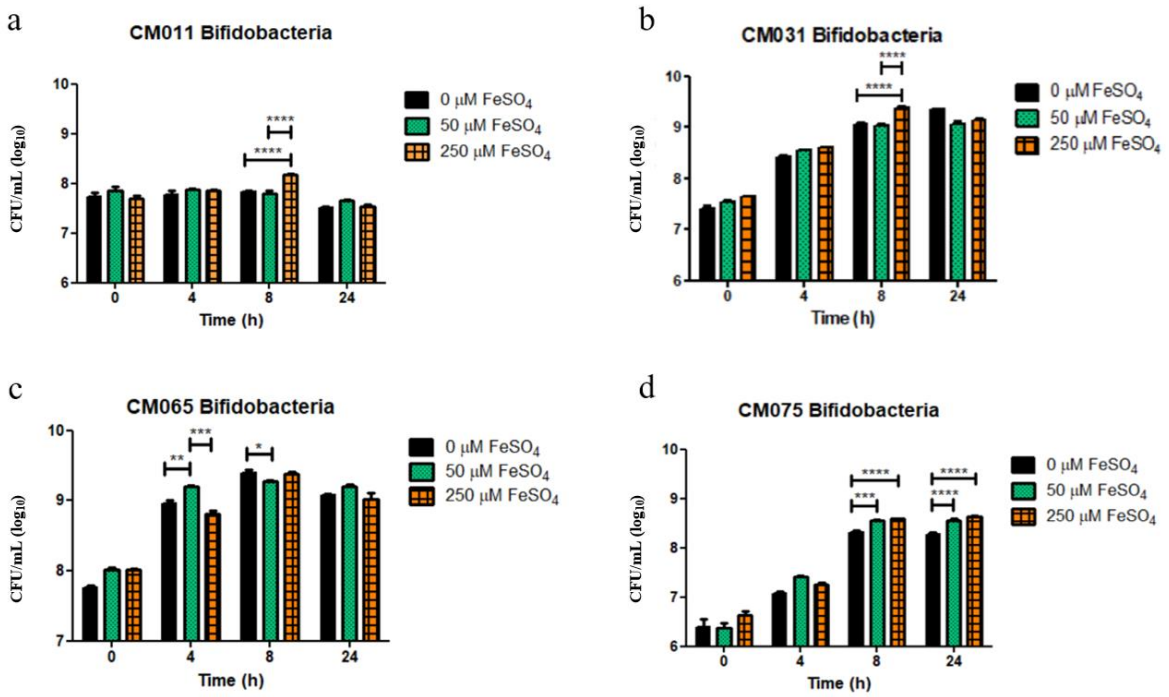


Figure 3.4 – The effect of iron addition on bifidobacteria viable counts enumerated from* in vitro *colonic fermentations. *Iron was added to* in vitro *colonic fermentations, at concentrations of 50 µM and 250 µM. Significant differences were seen between the control and treatments at the respective time-points, as analysed by one-way ANOVA analysis. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.*

As with *Enterobacteriaceae*, the viable counts for bifidobacteria varied amongst individuals (Figure 3.4). From these observations, the most common finding amongst majority of the donors was that of the viable counts at 8 h. For 3 out of the 4 donors, the addition of 250 µM $FeSO_4$ was accompanied with a significant increase in the counts of bifidobacteria at 8 h ($p<0.0001$ for donors CM011, CM031 and CM075, Figure 3.4a, 3.4b and 3.4d, respectively). The faecal microbiota of donor CM075 (Figure 3.4d) was the only community which exhibited higher counts of bifidobacteria by the end of the 24 h fermentation, with significant increases seen at both concentrations of iron ($p<0.0001$ at 50 µM and 250 µM $FeSO_4$). No effect of iron addition, at either concentration, was observed for the remaining 3 donors at 24 h.

Bacteroides and *Clostridium*, another set of bacterial genera which can harbour potentially pathogenic bacteria, remained largely unaffected in the presence of additional iron, with results varying largely between individuals. Finally, *Lactobacillus* counts were also observed to fluctuate between individuals, leading to an inconclusive pattern in relation to iron addition.

3.5 Discussion

Any excess dietary iron that has not been absorbed in the duodenum passes through to the colon and is therefore available for colonic bacteria. This is indicated by the presence of iron in faecal samples of weaning infants as well as British adults on a standard Western diet; studies have shown that approximately 1.8 mM of iron is passed through to the colon, which is in excess of the iron requirements of the majority of the gut bacteria (10^{-7} – 10^{-5} M) [139].

Several animal studies have looked into the effects of iron on the bacterial communities. Physiologically, pigs have gastrointestinal tracts that are the most closely comparable to humans. Lee *et al.,* (2008) performed a study in which weanling pigs were given an iron-supplemented diet had no effect on clostridia, *Lactobacillaceae, Bifidobacteriaceae* and total anaerobic bacteria; however, increased numbers of coliform bacteria were observed [224]. Our findings are comparable to the results in the experiments performed in pigs, where clostridia and *Lactobacillaceae* remained largely unaffected by the presence of additional iron. Similarly, we also observed an increase in *Enterobacteriaceae,* which contains species such as *Escherichia,* a coliform member.

A few studies have examined the effect of iron fortification and iron depletion on the human gut microbiota. One study provided infants with an iron-fortified cow's milk preparation and investigated the changes in the gut microbial composition using culture-based methods [148]. These children had reduced counts of bifidobacteria but higher counts of *E. coli* and *Bacteroides spp*. compared to the infants receiving an unfortified cow's milk preparation. In the experiments detailed in this chapter, similar to the study mentioned above, a general increase in the viable counts of *Enterobacteriaceae* was also observed, the group to which *E. coli* belongs. Additionally, an increase in viable counts of bifidobacteria was observed, which is contrary to the study above, suggesting the differences in bacterial composition seen between children and adults. It is also worth noting that the study carried out by Mevissen-Verhage *et al.,* (1985) was investigating the effects of iron on neonatal gut flora. It has long been known that neonatal microbiota are vastly different to the adult microbiota as the gut microbiota is not fully established until the later years of life and is instead dominantly populated by bifidobacteria [238-240].

Zimmermann *et al.,* (2010) examined the gut microbiota of school children (aged 6-14 y) from Côte d'Ivoire using molecular methods, including faecal DNA extractions and classification, PCR and qPCR [229]. These children, who belonged a rural area of Côte

d'Ivoire with a high infectious disease burden, were given iron-fortified biscuits for a period of 6 months and it was observed that compared to the control group, who were receiving unfortified biscuits, lactobacilli abundance was lower and *Enterobacteriaceae* was higher in their faecal samples. Conflicting results were seen in a study of iron-deficient women in India [230] where low levels of lactobacilli were observed. Our results are in line with the Côte d'Ivoire study as we also observed higher counts of *Enterobacteriaceae* under iron-supplemented conditions. The inconsistency in lactobacilli levels in the studies in Côte d'Ivoire and India further strengthen the notion that predicting how the gut microbial composition is altered when exposed to additional iron is a challenging task due to the high variability amongst individuals. This is also the case with our results, where an inconclusive pattern was observed amongst our donors when investigating the effects of additional iron on the relative abundance of lactobacilli.

Dostal *et al.,* (2013) examined the effects of reduced iron availability in continuous *in vitro* colonic fermentations [156]. Samples were acquired from three healthy 6-10 y old children. During very low iron conditions, a reduction in the counts of *Roseburia spp./E. rectale*, *Clostridium cluster IV* members and *Bacteroides spp*. were observed while *Lactobacillus spp*. and *Enterobacteriaceae* increased. Decreases in propionate and butyrate were also observed during iron-deficient conditions. Our study demonstrated higher counts of *Enterobacteriaceae*, whilst the study mentioned above displayed higher counts of this bacterial group under low iron conditions. This highlights the scavenging ability that this bacterial group may possess when iron availability is sparse. Interestingly, their study also showed a strong increase of previously subdominant families like *Bifidobacteriaceae* under low iron conditions. Contrary to their study, we observed high bifidobacteria counts from the faecal microbiota of 2 out of 3 donors when iron was added to the fermentation medium.

Jaeggi *et al.,* (2015) examined the effects of low and high doses of in-home iron supplementation on the gut microbiota of Kenyan children [157]. In this setting, provision of iron-fortified porridge led to an increase in pathogen abundance, with numbers of enterobacteria, *Clostridium* and pathogenic *E. coli* increasing whilst numbers of bifidobacteria decreased. Furthermore, in comparison to the control group (receiving unfortified porridge), the children with iron-fortified porridge had elevated levels of faecal calprotectin, a marker of gut inflammation.

These contradictory results suggest that changes in bacterial numbers might not only be due to iron concentration in the gut lumen but also the result of host responses to iron and a number of other environmental factors. For example, in the Côte d'Ivoire study, calprotectin, a marker of intestinal inflammation, was increased in children provided with iron-fortified biscuits, and mucosal inflammation can give *Enterobacteriaceae* a growth advantage [229]. In *in vitro* fermentations, however, environmental and host factors are excluded. Thus, the absence of host variables, in particular inflammatory factors, might also contribute towards the differences observed between *in vivo* and *in vitro* studies.

In vivo studies on the effect of iron on specific bacterial groups have produced inconsistent findings in both the human and animal gut microbiota. This could potentially be, in part, due to the intricate interactions between the host iron status, the host response to differing dietary iron levels, and/or the iron concentration in the gut lumen. Another factor is the inter-individual differences in the gut flora, which are host-specific. Furthermore, other factors such as intestinal immune function, environmental changes, host physiology and dietary habits can also influence the gut microbial composition. *In vitro* gut fermentation models allow the gut microbiota to be examined without the influence of the host, as well as other environmental factors, through tightly controlled parameters [232]. The *in vitro* continuous colonic fermentation model established in the 90's [241, 242] represents a good technological platform to investigate the impact of dietary changes on gut microbiota activity. The advantage of an *in vitro* colonic model is that it allows for long-term stability and biodiversity, making it possible to examine the effects of the compound of interest on different bacterial populations [232]. However, the inoculation and colonisation of *in vitro* fermentation systems influences the reproducibility of the studies and constitute a challenge of the models [232]. In order to facilitate the reproducibility of experiments, recent developments are addressing the inoculation of fermentation models with defined populations of human gut microorganisms represented by common saccharolytic and amino acid-fermenting populations in the large intestine [243].

3.6 Conclusions

Supplementing bacteria with iron confirmed the positive effect it had on the growth of the bacterial species, *E. coli* and *S.* Typhimurium. This was further strengthened by supplementing iron to *E. coli* and *S.* Typhimurium cultured in iron-depleted conditions, resulting in comparable growth as compared to the control. Results varied when iron was supplemented to mixed bacterial cultures derived from human faecal microbiota,

suggesting the influence of neighbouring bacterial taxa. To further investigate the effects of iron, the next chapter details the impact on pure and mixed bacterial cultures when iron is chelated from the environment.

CHAPTER FOUR

4 Iron chelators: The effect of iron chelation on the growth of specific groups of bacteria

4.0 Summary

This chapter investigates how a range of iron chelators, both chemical and those present in the diet, affects the growth of a range of independently cultured bacteria. Bacterial cultures that have the potential to display pathogenic phenotypes, such as *E. coli, S.* Typhimurium, *C. perfringens and B. thetaiotaomicron* declined in growth when cultured independently in media supplemented with iron chelators. When the beneficial species *B. longum* and *L. rhamnosus* were cultured in the presence of iron chelators, their growth remained largely unaffected.

4.1 Introduction

The microbial community relies on proteins, complex carbohydrate and micronutrients that pass through undigested from the small intestine into the colon for metabolism and replication and there is constant competition for micronutrients [139, 244-246]. Iron is one of the micronutrients that the majority of the gut bacterial species need for their growth and metabolism [20]. Previous studies have shown that on average, bacteria need 10^{-7} – 10^{-5} M iron for optimal growth, and total iron in the colon far exceeds this value [139].

Many bacterial species have developed mechanisms to acquire iron [247], even when iron-availability is sparse, suggesting the importance of this nutrient for successful growth and development. Therefore, despite iron bioavailability being low in the colon, with strong bacterial iron-uptake mechanisms, the ability of potentially pathogenic bacteria to exploit this nutrient is relatively high. Only a very small number of bacterial species do not require iron, such as *Lactobacillus*, a genus of bacteria known to have beneficial properties. Instead, bacterial species belonging to this genus depend on alternative metals such as manganese for its metabolism [144].

Food safety studies have demonstrated the requirement of iron by *Salmonella* species for successful growth in the tomato fruit [248]. To test whether iron acquisition was essential for *Salmonella* growth in tomatoes, a mutant, which lacked the ability to import iron-associated siderophores was investigated. Compared to the wild type, the growth of the mutant was significantly reduced within tomatoes. Furthermore, when exogenous iron was provided to the fruit, the defect of the mutant was fully reversed, demonstrating the requirement for bacterial iron scavenging. These studies also suggest a role for iron chelation in the farming industry.

Dietary compounds also have natural iron-chelating properties. For example, tannins are present in a wide variety of fruits and vegetables. Anti-microbial activity of tannins through iron deprivation has been suggested as it acts as a siderophore to chelate iron, rendering it unavailable for other micro-organisms [249]. Another example of a dietary iron chelator is phytic acid, also known as inositol hexakisphosphate and is the principle storage form of phosphorus in many plant tissues. Phytic acid is a very potent iron chelator and its iron-chelating properties have been found to be similar to desferrioxamine (a clinically used iron chelator). When iron binds to phytic acid, it forms an insoluble precipitate and is unavailable for absorption in the intestine [52, 79, 203, 250-252].

When examining the effects of iron on independently cultured bacteria, the bacterial media used in pure culture studies are designed to contain all the essential nutrients for bacterial growth. For example, *E. coli* and *S.* Typhimurium grow successfully in Luria broth, whilst *L. Rhamnosus* grows well in de Man, Rogosa and Sharpe (MRS) broth. Various types of bacteria require different compositions of media for optimal growth, and one of the key nutrients contributing towards successful bacterial growth is iron. Therefore, removing iron from the media with the addition of an iron chelator enables the effects on bacterial growth associated with a particular nutrient to be examined.

4.2 Objectives

The research included in this chapter reports the effect of chemical iron chelators on single cultures of bacteria that are known to require iron for growth. This chapter also examines the effects of iron chelators found in foods on bacteria cultured independently. The hypothesis being tested was that a reduction in iron availability through iron chelation would exert a negative impact on the growth of iron-dependent bacteria.

4.3 Materials and Methods

The bacterial strains and their growth conditions tested in this section are outlined in chapter 2, section 2.2.1. A range of pure bacterial cultures were grown overnight in selective rich media at 1% inoculation. Compositions of bacterial culture media can be found in chapter 2 (section 2.2.1, Table 2.1). No external source of iron was added to the cultures, and any iron present in the culture originated from the rich media. Cultures were seeded in 100-well honeycomb plates and cells were then exposed to a range of iron chelators. Bacterial growth was analysed using a Bioscreen C machine, which monitors the cell growth by measuring the turbidity (OD) of the liquid growth medium. The

experiments were run for 24 h – 48 h, depending on the bacteria of interest, with measurements at OD_{600}, taken every 10 mins. The temperature of all experiments was set at 37°C. Depending on the type of organism, these studies were carried out under either aerobic or anaerobic conditions. The final concentration of chelators was decided based on previously published literature.

Table 4.1 indicates which media was used for specific bacteria. Further details on media composition can be found in chapter 2.

Table 4.1 - Media used for different bacterial species

Bacterial species	Media used
E. coli	LB
S. Typhimurium	LB
C. perfringens	BHI-C
B. thetaoiotaomicron	BHI-H
B. longum	BHI
L. rhamnosus	MRS +glucose

4.4 Results

4.4.1 Evaluating the growth of independently cultured bacteria under conditions of iron chelation using chemical iron chelators

Small-scale experiments were performed to elucidate whether independently cultured bacteria would achieve optimal growth under chelated-iron conditions. For these experiments, the bacteria selected to investigate the effects of iron chelation were a range of bacteria that can be found in the colon.

4.4.1.1 Bathophenantrholine Disulphonic Acid (BPDS) and 2,2-dipyridyl (22D)

BPDS and 22D both act as metal chelators, with a very strong affinity for iron. They both form a complex with iron, rendering it unavailable to the surrounding environment. Taking this into consideration, a range of bacteria were cultured with and without BPDS or 22D. Figure 4.1 illustrates the impact of BPDS and 22D on potentially pathogenic bacteria, whilst Figure 4.2 demonstrates how the potentially beneficial bacteria are affected by the chelation of iron.

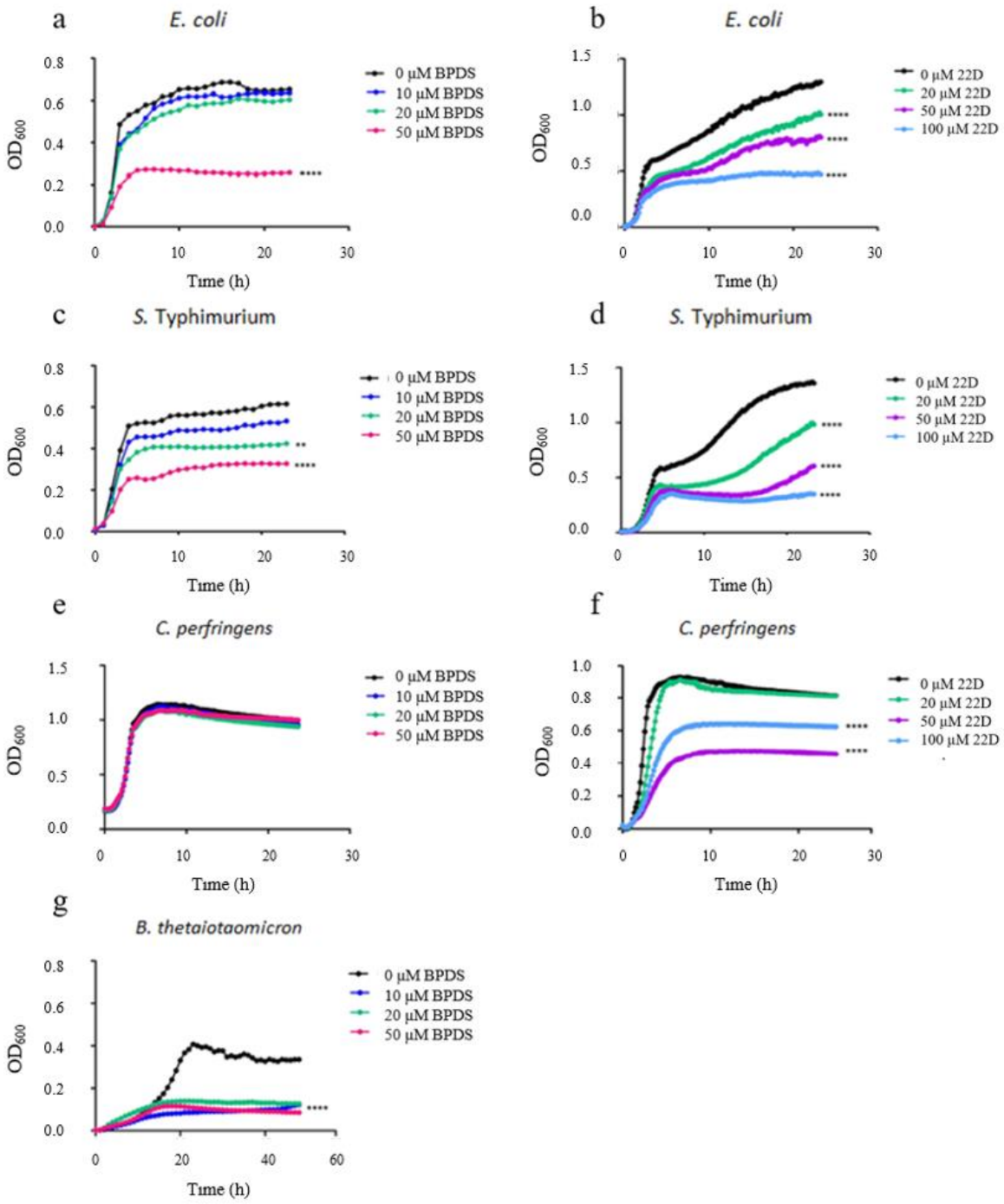


Figure 4.1 – The effects of BPDS and 22D on the growth of potentially pathogenic bacteria. *BPDS and 22D was added at various concentrations, ranging from 0 – 100 µM to* E. coli *(a and b, respectively),* S. *Typhimurium (c and d, respectively),* C. perfringens *(e and f, respectively) and* B. thetaiotaomicron *(g) grown anaerobically, at 1% inoculation, with a total working volume of 300 µL. One-way ANOVA was performed to analyse growth curves, comparing each curve to the control (0 µM chelator). **p<0.01 and ****p<0.0001.*

The presence of either BPDS or 22D in the medium had a marked influence on the growth of the majority of the bacteria cultured. Both *E. coli* (Figure 4.1a and 4.1b) and *S.*

Typhimurium (Figure 4.1c and 4.1d) displayed a dose-dependent decrease that was statistically significant when cultured in LB media containing either of the iron chelators. It was concluded that for *E. coli*, a concentration of 50 μM BPDS was deemed sufficient for significant growth impairment ($p<0.0001$), whilst for *S.* Typhimurium, a lower concentration of 20 μM BPDS was sufficient to exert significant growth-limiting effects ($p<0.01$). Water-soluble iron (Fe^{2+}) was also quantified in from the pure culture, and results showed that the presence of both chelators in the solution led to a decrease in iron in comparison to the control (no chelator) for both *E. coli* and *S.* Typhimurium. Fe^{2+} levels in the control media were 0.11 nmol and 0.531 nmol for *E. coli* and *S.* Typhimurium, respectively, whilst negligible amounts of Fe^{2+} were found in the presence of both BPDS and 22D for both bacteria.

Although not dose-dependent, similar results were observed for *C. perfringens* when cultured in the presence of 22D (Figure 4.1f), but this was not observed when grown with BPDS (Figure 4.1e). Interestingly, when Fe^{2+} was quantified, the presence of both chelators led to a decrease in iron in comparison to the control (0.645 nmol in control and negligible amounts in the presence of both chelators). For *C. perfringens*, a minimum concentration of 50 μM 22D elicited a statistically significant decrease in growth ($p<0.0001$). Finally, the growth of *B. thetaiotamicron* was negatively impacted by BPDS (Figure 4.1g), causing a statistically significant reduction in its growth at all concentrations of this chelator ($p<0.0001$).

Next, the effects of the same chelators were investigated on bacteria that are considered as beneficial for gut health. In general, beneficial bacteria, such as members of the *Lactobacillus* genera, are unaffected by a lack of iron and can grow optimally even when iron is scarce. Figure 4.2 illustrates the effects of both iron chelators on two beneficial species, *B. longum* and *L. rhamnosus*.

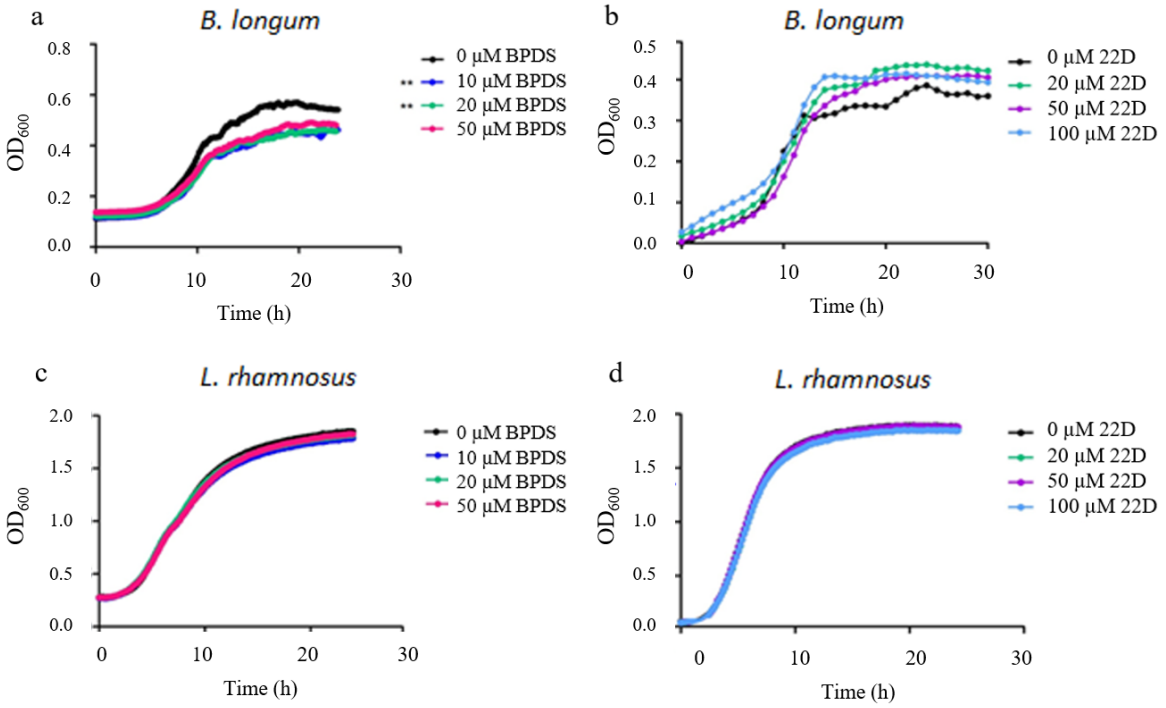


Figure 4.2 – The effects of BPDS and 22D on the growth of beneficial bacteria. *BPDS and 22D was added at various concentrations, ranging from 0 – 100 µM to* B. longum *(a and b, respectively) and* L. rhamnosus *(c and d, respectively) grown anaerobically, at 1% inoculation, with a total working volume of 300 µL. One-way ANOVA was performed to analyse growth curves, comparing each curve to the control (0 µM chelator). **p<0.01.*

There has been some research which suggests that some *Bifidobacterium* species do not require iron for its growth [253], and interestingly, when *B. longum* was cultured in the presence of BPDS, there was a significant decrease in its growth ($p<0.01$ for both 10 µM and 20 µM) in comparison to the control (0 µM) (Figure 4.2a). This decrease correlated with a reduction in Fe^{2+} in the culture in the presence of BPDS. 22D illustrated no significant impact on *B. longum* growth (Figure 4.2b). As expected, the growth of *L. rhamnosus* was unaffected when cultured with either chelator, despite Fe^{2+} levels reducing in the presence of both chelators (Figure 4.2c and 4.2d). *B. longum* presented with Fe^{2+} levels of 0.026 nmol in the control culture, whilst negligible amounts of Fe^{2+} were found in the presence of both chelators.

4.4.1.2 Effects of BPDS on the growth of different species of bifidobacteria

Given the interesting decrease in *B. longum* growth observed when cultured with BPDS, a range of different *Bifidobacterium* species and *B. longum* strains were cultured with BPDS to confirm whether similar effects were observed. Figure 4.3 shows the influence of BPDS

on the growth of the following bacteria – *B. longum subsp. longum; B. longum subsp. infantis* (x2 different strains)*; B pseudocatenulatum* and *B. adolescentis.*

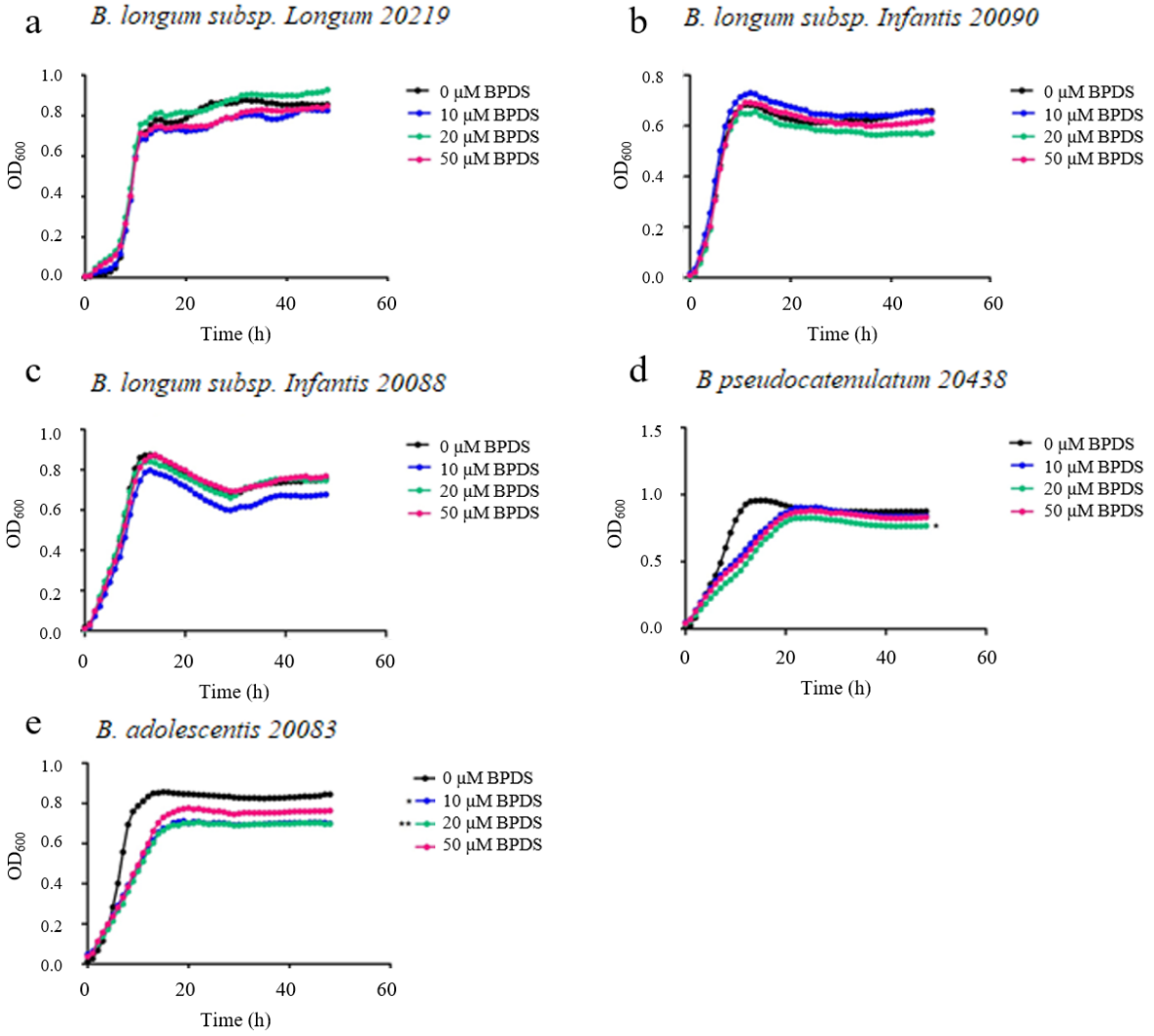


***Figure 4.3 – The effects of BPDS on different species and strains of* Bifidobacterium.** *BPDS was added at various concentrations, ranging from 0 – 50 µM to* B. longum subsp. Longum 20219 *(a);* B. longum subsp. Infantis 20090 *and* 20088 *(b and c, respectively);* B pseudocatenulatum 20438 *(d) and* B. adolescentis 20083 *(e) grown anaerobically, at 1% inoculation, with a total working volume of 300 µL. One-way ANOVA was performed to analyse growth curves, comparing each curve to the control (0 µM BPDS). *p<0.05 and **p<0.01.*

No decrease in growth was observed when any of the *B. longum* species were cultured in BHI media with BPDS (Figure 4.3a, 4.3b and 4.3c). Conversely, in the presence of BPDS at a concentration of 20 µM, both *B. pseudocatenulatum* (Figure 4.3d) and *B. adolescentis* (Figure 4.3e) exhibited a statistically significant reduction in growth ($p<0.01$ and $p<0.05$, respectively), with 10 µM BPDS being sufficient to also significantly impair the growth of *B. adolescentis* ($p<0.05$). These observations suggest that the iron-limiting effects of the

iron chelator is species/strain dependent, and reliant on the iron-regulatory genes, if any, that the different species/strains express.

4.4.2 Evaluating the growth of cultured bacteria under conditions of iron chelation achieved by naturally-derived iron chelators

There are many naturally-occurring compounds within our diet that have the ability to strongly bind iron and withhold it from bacteria present in the colonic environment. A range of naturally-derived iron chelators identical to those outlined in section 4.4.1, were added to media used to independently culture bacteria. The next series of figures displays the impact of the following chelators, sodium alginates, lactoferrin, tannic acid and phytic acid on the growth of different bacteria.

4.4.2.1 Sodium alginate

Alginates have been reported to inhibit the growth of a range of bacteria, and therefore Manucol LD (mwt 145 kDa; G:M ratio 38:62), a sodium alginate was added at a final concentration of 0.3% (w/v) to pure cultures of bacteria to investigate this.

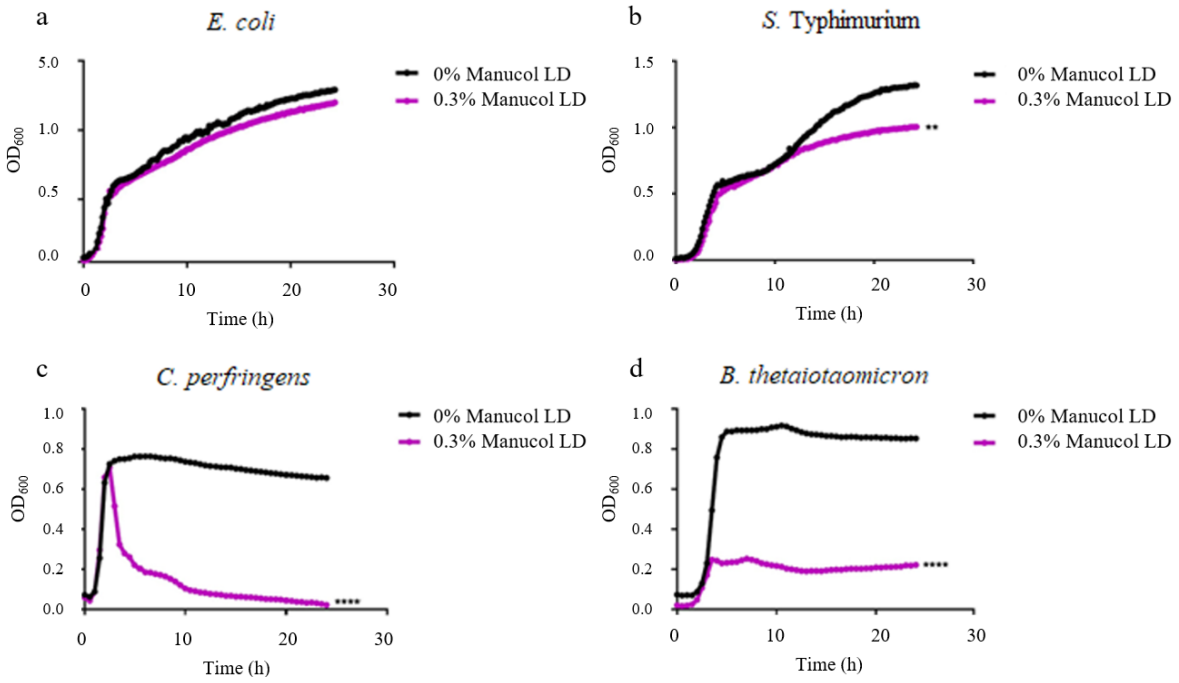


Figure 4.4 – The effects of Manucol LD on the growth of potentially pathogenic bacteria. *Manucol LD was added at a final concentration of 0.3% (w/v) to pure cultures of* E. coli *(a),* S. Typhimurium *(b),* C. perfringens *(c) and* B. thetaiotaomicron *(d) grown anaerobically, at 1% inoculation, with a total working volume of 300 µL. One-way ANOVA was performed to analyse growth curves, comparing each curve to the control (no alginate). **p<0.01 and ****p<0.0001.*

From these data, it was clear that the growth of most of the pure cultures tested (*S.* Typhimurium, *C. perfringens* and *B. thetaiotaomicron,* Figure 4.4b, 4.4c and 4.4d, respectively) were significantly impaired in the presence of Manucol LD ($p<0.01$, $p<0.0001$ and $p<0.0001$, respectively), correlating with lower Fe^{2+} levels in the media when compared to the control (*S.* Typhimurium - 0.531 nmol and 0.199 nmol from control and chelator cultures, respectively; *C. perfringens* – 0.645 nmol and 0.254 nmol from control and chelator cultures, respectively). *E. coli* growth was unaffected by Manucol LD (Figure 4.4a), as has been reported in other studies, where *E. coli* displayed the most resistance to the antibacterial effects of the alginates tested [254]. This is also further strengthened by the unaffected levels of Fe^{2+} concentrations in the presence of Manucol LD (0.105 nmol present in control culture compared to 0.274 nmol present in chelator culture), suggesting strong iron-scavenging properties of *E. coli.*

The next set of figures illustrate the effects of Manucol LD on the beneficial species, *B. longum* and *L. rhamnosus* (Figure 4.5a and 4.5b, respectively).

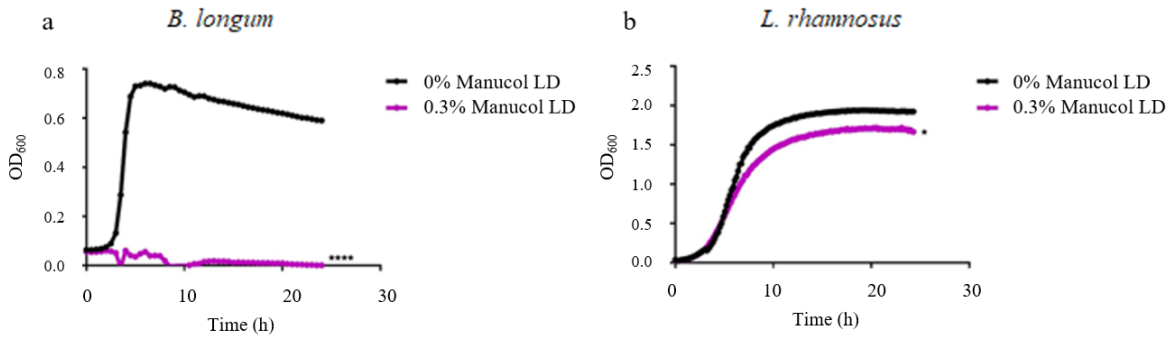


Figure 4.5 – The effects of Manucol LD on the growth of beneficial bacteria. *Manucol LD was added at a final concentration of 0.3% (w/v) to pure cultures of* B. longum *(a) and* L. rhamnosus *(b) grown anaerobically, at 1% inoculation, with a total working volume of 300 µL. One-way ANOVA was performed to analyse growth curves, comparing each curve to the control (0% Manucol LD). *p<0.05 and ****p<0.0001.*

As seen in Figures 4.5, the addition of Manucol LD had a detrimental effect on the beneficial species, *B. longum* (Figure 4.5a), with its growth being significantly impaired ($p<0.0001$). This correlated with a decrease observed in Fe^{2+} levels in the culture compared to the control (0.026 nmol from control culture compared to negligible amounts of Fe^{2+} in the presence of Manucol LD). *L. rhamnosus* growth was also significantly stunted ($p<0.05$) (Figure 4.5b). Fe^{2+} levels show no reduction in concentrations when cultured with Manucol LD.

These data suggest that although removal of iron could reduce the pathogenic profile of the gut microbiota, as illustrated by the pure cultures in Figure 4.5, it may be important to ensure that the beneficial bacterial population are not negatively affected by iron removal.

4.4.2.2 Human lactoferrin

Human lactoferrin (Lf) is a key protein in host defences, with its iron-binding capabilities contributing towards this [255-257]. The same bacterial species from the previous experiments were cultured independently with and without human Lf (apolactoferrin) (Figure 4.6).

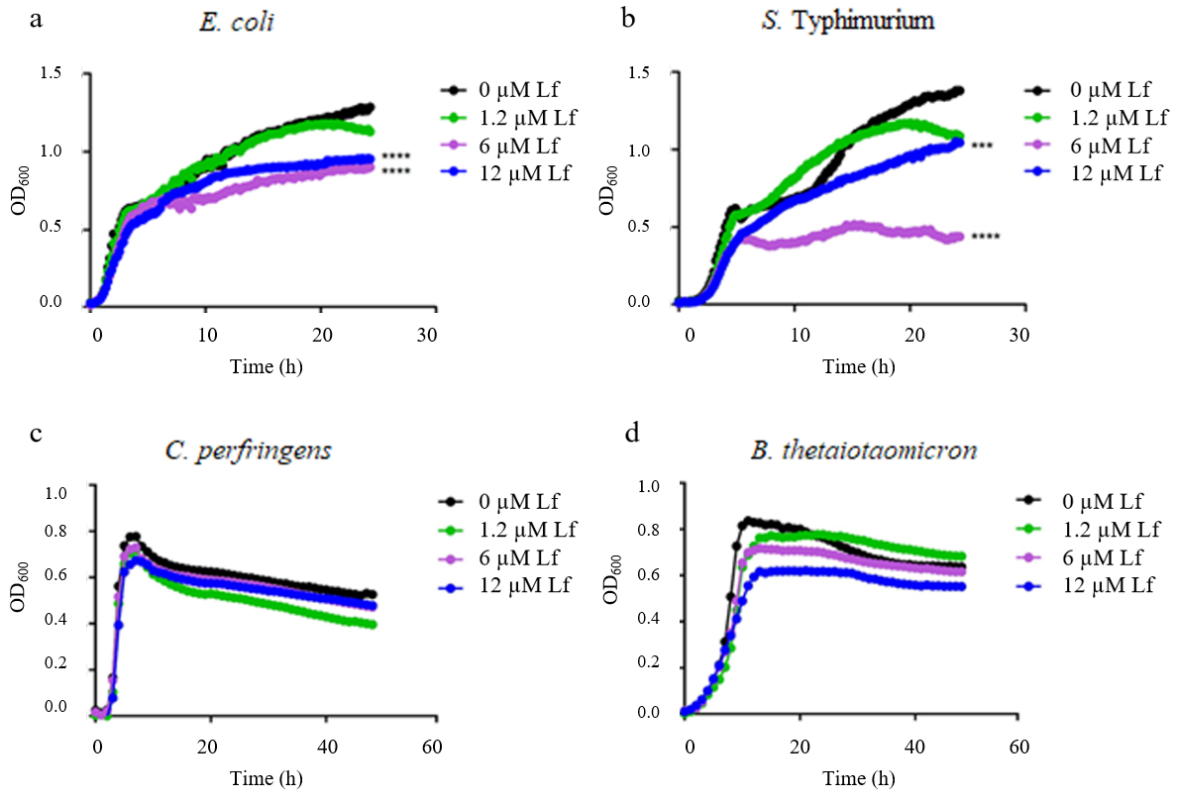


Figure 4.6 – The effects of lactoferrin on the growth of potentially pathogenic bacteria. *Lf was added at various concentrations (0 – 12 µM) to pure cultures of* E. coli *(a),* S. Typhimurium *(b),* C. perfringens *(c) and* B. thetaiotaomicron *(d) grown anaerobically, at 1% inoculation, with a total working volume of 300 µL. One-way ANOVA was performed to analyse growth curves, comparing each curve to the control (0 µM Lf). ***p<0.001 and ****p<0.0001.*

Statistically significant effects were only observed for *E. coli* ($p<0.0001$, Figure 4.6a) and *S.* Typhimurium ($p<0.001$ and $p<0.0001$ at 6 µM and 12 µM, respectively, Figure 4.6b), where concentrations of 6 µM and 12 µM Lf impaired the growth of these bacteria, though not in a dose-dependent manner. For *C. perfringens* and *B. thetaiotaomicron*, (Figure 4.6c and 4.6d, respectively) no effect on their growth was observed when Lf was present in the media, suggesting Lf may not be as potent an iron chelator as some of the previously investigated chelators in this Chapter.

The next set of figures illustrate the effects of human Lf on the beneficial species, *B. longum* and *L. rhamnosus* (Figure 4.7).

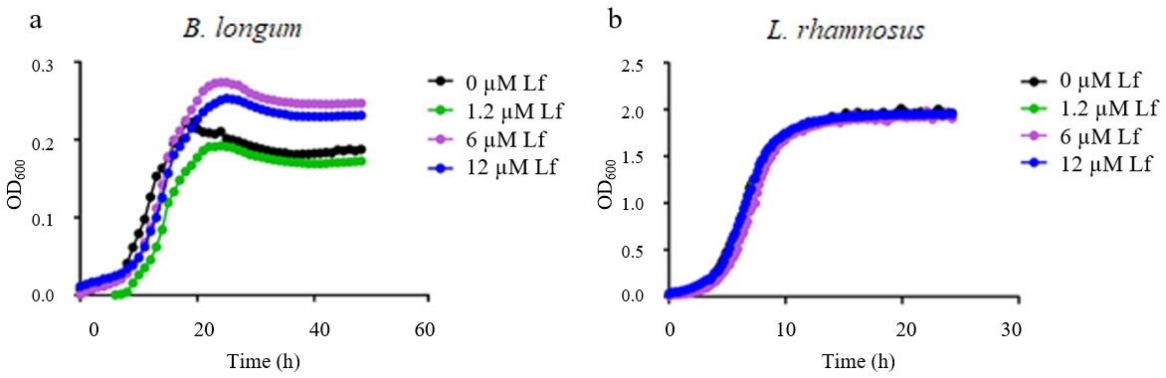


Figure 4.7 – The effects of lactoferrin on the growth of beneficial bacteria. *Lf was added at various concentrations (0 – 12 µM) to pure cultures of* B. longum *(a) and* L. rhamnosus *(b) grown anaerobically, at 1% inoculation, with a total working volume of 300 µL. One-way ANOVA was performed to analyse growth curves, comparing each curve to the control (0 µM Lf).*

Interestingly, no statistically relevant effect of Lf was observed on the growth of *B. longum* (Figure 4.7a), as was also the case with *L. rhamnosus* (Figure 4.7b), where its growth remained unaffected in the presence of Lf in comparison to the control.

4.4.2.3 Tannic acid

As briefly discussed in the introduction of this Chapter, tannins (a compound present in a wide variety of fruits and vegetables) also have iron-chelating properties. Therefore, tannic acid (TA) (final concentration of 60 µM), the salt form of tannins, was cultured with the same set of bacteria outlined in the previous sections, to investigate its effects on bacterial growth.

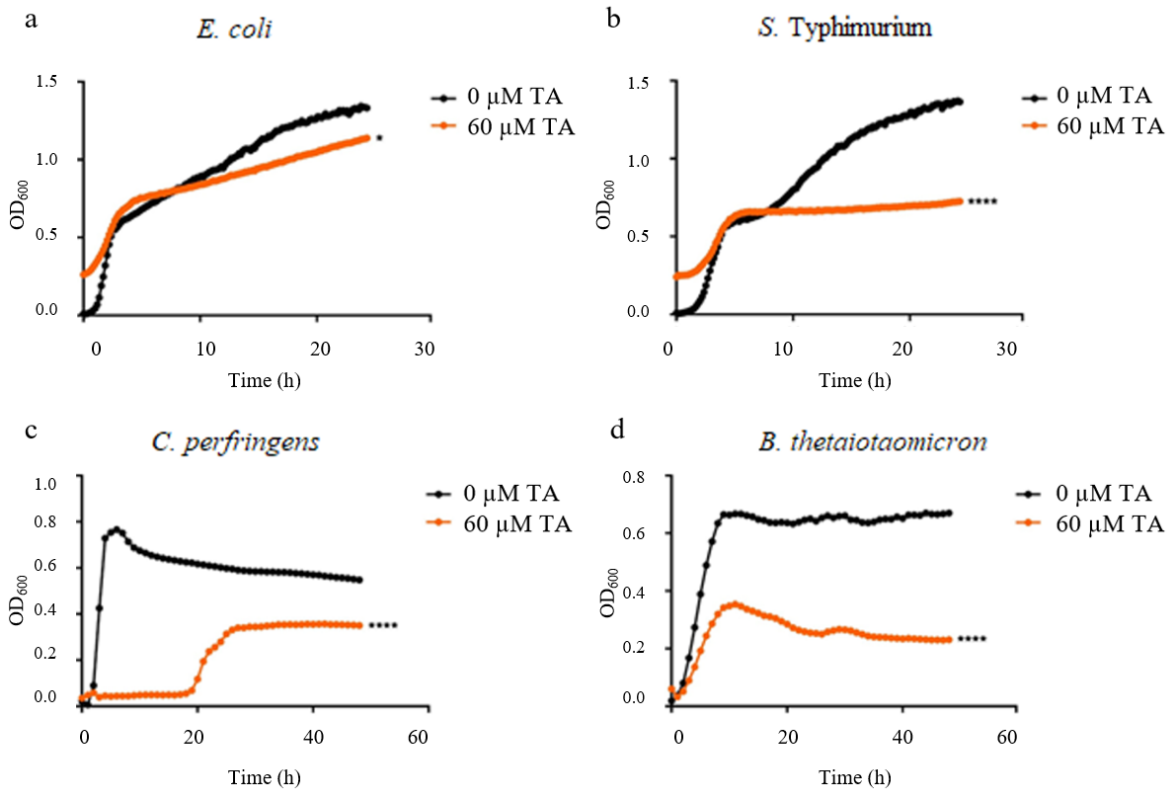


Figure 4.8 – The effects of tannic acid on the growth of potentially pathogenic bacteria. *TA was added at a final concentration of 60 µM to pure cultures of* E. coli *(a),* S. *Typhimurium (b),* C. perfringens *(c) and* B. thetaiotaomicron *(d) grown anaerobically, at 1% inoculation, with a total working volume of 300 µL. One-way ANOVA was performed to analyse growth curves, comparing each curve to the control (0 µM TA). *p<0.05 and ****p<0.0001.*

The growth of all bacterial cultures was impacted by the presence of TA (Figure 4.8). *E. coli* growth was significantly impaired when TA was present in the culture media ($p<0.05$, Figure 4.8a), however, this growth was not as significant as observed for the growth of *S.* Typhimurium ($p<0.0001$, Figure 4.8b), *C. perfringens* ($p<0.0001$, Figure 4.8c) and *B. thetaiotaomicron* ($p<0.0001$, Figure 4.8d). *E. coli* and *C. perfringens* displayed comparable levels of Fe^{2+} to the control when in the presence of TA whilst Fe^{2+} concentrations in the control culture of *S.* Typhimurium was 0.531 nmol compared to 0.194 nmol when in the presence of TA.

Following this, the effects of TA were investigated on the beneficial bacteria, *B. longum* and *L. rhamnosus*.

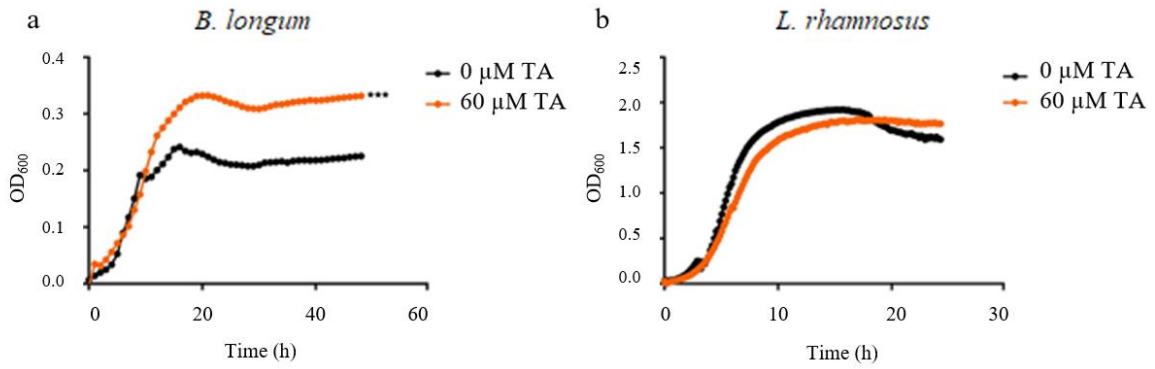


Figure 4.9 – The effects of tannic acid on the growth of beneficial bacteria. *TA was added at a final concentration of 60 µM to pure cultures of* B. longum *(a) and* L. rhamnosus *(b) grown anaerobically, at 1% inoculation, with a total working volume of 300 µL. One-way ANOVA was performed to analyse growth curves, comparing each curve to the control (0 µM TA). ***p<0.001.*

As expected, the growth of *L. rhamnosus* (Figure 4.9b) was unaffected when cultured in media containing TA. A positive effect on *B. longum* was observed (Figure 4.9a), where the addition of TA significantly increased its growth in comparison to the control ($p<0.001$). Iron quantification showed comparable levels of Fe^{2+} to the control for both bacterial species.

4.4.2.4 Phytic acid

Phytic acid (PA) is also a very potent iron chelator found in plant-based foods. For this reason, a range of bacteria were independently cultured with and without PA to observe its effects, if any, on bacterial growth.

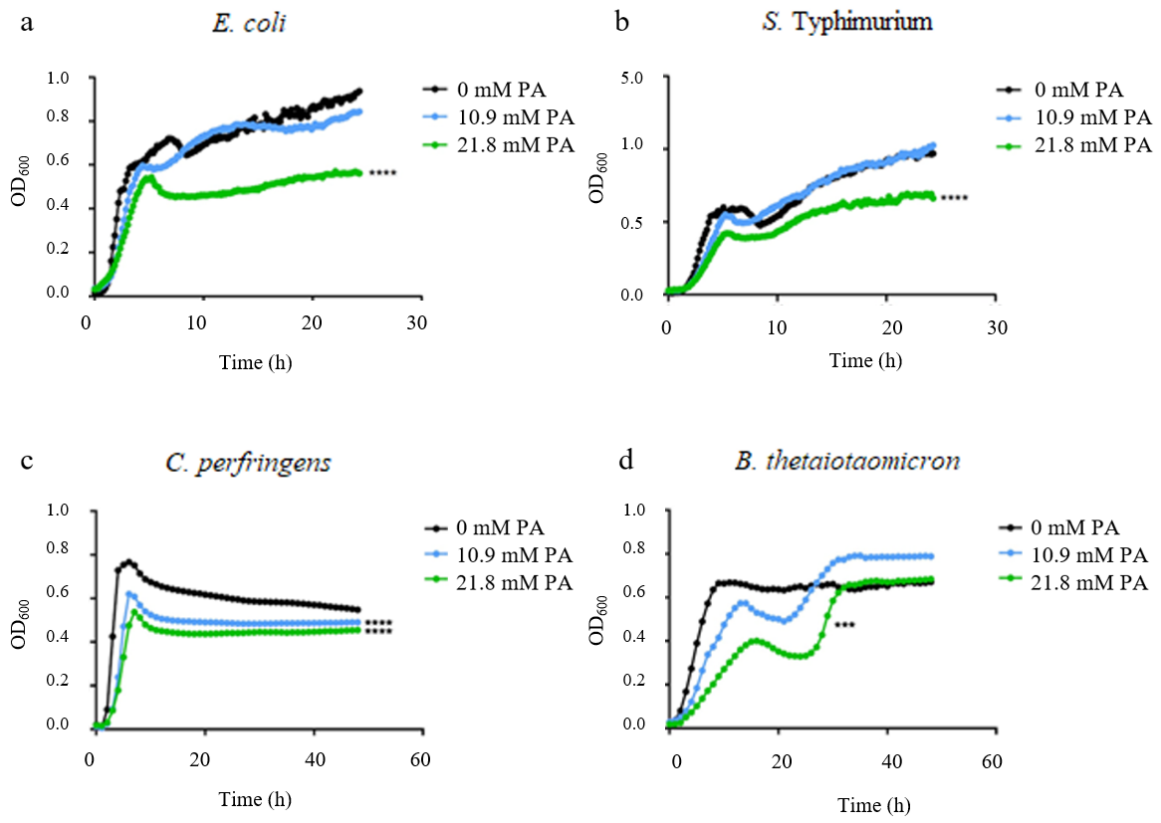


Figure 4.10 – The effects of phytic acid on the growth of potentially pathogenic bacteria. *PA was added at various concentrations (0 mM – 21.8 mM) to pure cultures of* E. coli *(a),* S. Typhimurium *(b),* C. perfringens *(c) and* B. thetaiotaomicron *(d) grown anaerobically, at 1% inoculation, with a total working volume of 300 µL. One-way ANOVA was performed to analyse growth curves, comparing each curve to the control (0 mM PA). ***p<0.001 and ****p<0.0001.*

The highest concentration of PA tested (21. 8 mM) elicited a statistically negative effect on all potentially pathogenic bacteria investigated (*E. coli*, *S.* Typhimurium and *C. perfringens* p<0.0001, Figure 4.10a – 4.10c, respectively) in comparison to the control, with *C. perfringens'* growth also being impaired at a lower PA concentration of 10.9 mM (Figure 4.10c). In the presence of PA, Fe^{2} concentrations were lower in comparison to when no chelator was cultured with these bacterial species. Fe^{2+} concentrations were present at 0.006 nmol in the presence of PA in comparison to 0.105 nmol obtained from the control culture of *E. coli.* For *S.* Typhimurium, PA reduced the levels of Fe^{2+} to 0.071 nmol from 0.531 nmol. Finally, Fe^{2+} levels were reduced to 0.318 nmol from 0.645 nmol in the presence of PA for *C. perfringens.*

Interestingly, for *B. thetaiotaomicron* after an initial significant decrease in growth observed in the presence of 21.8 mM PA (up to 24 h, p<0.001, Figure 4.10d), a resumption of growth occurred after approximately 24 h, after which the growth of *B.*

thetaiotaomicron is comparable to that of the control. Many bacteria have been found to have outer-membrane vesicles, which contain phytate-binding enzymes [258], so this effect may be due to phytate degradation releasing iron back into the media.

Finally, the growth of beneficial bacteria was also observed in the presence of PA, as seen in Figure 4.11.

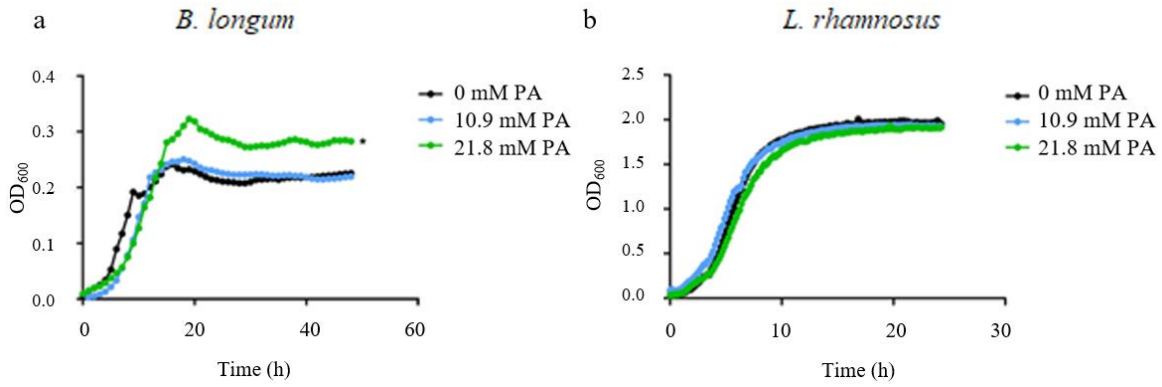


Figure 4.11 – The effects of phytic acid on the growth of beneficial bacteria. *PA was added at various concentrations (0 mM – 21.8 mM) to pure cultures B. longum (a) and* L. rhamnosus *(b) grown anaerobically, at 1% inoculation, with a total working volume of 300 µL. One-way ANOVA was performed to analyse growth curves, comparing each curve to the control (0 mM PA). *p<0.05.*

The addition of 21.8 mM PA to the media led to a statistically relevant increase in the growth of *B. longum* ($p<0.05$, Figure 4.11a), whilst *L. rhamnosus* growth was unaffected when PA was added to the culture (Figure 4.11b). Iron levels in the presence of PA when cultured with *L. rhamnosus* was comparable to the control (4.394 nmol control vs 4.127 nmol PA), whilst iron levels were considerably reduced when *B. longum* was cultured in the presence of PA (0.026 nmol control vs negligible concentrations in the presence of PA).

Table 4.2 summarises the effects of all the chelators tested on the different bacterial species presented in this chapter.

Table 4.2 - Effects of iron chelators on different bacterial species. *Significance shown for highest concentration of chelator tested (- $p<0.05$, -- $p<0.01$ and ---- $p<0.0001$ and + $p<0.05$, +++ $p<0.001$. '-' indicates a significant decrease and '+' indicates a significant increase*

Species	BPDS	22D	Manucol LD	Lf	TA	PA
E. coli	----	----	No effect	----	-	----
S. Typhimurium	----	----	--	---	----	----
C. perfringens	No effect	----	----	No effect	----	----
B. thetaiotaomicron	----	No growth	----	No effect	----	----
B. longum	--	No effect	----	No effect	+++	+
L. rhamnosus	No effect	No effect	-	No effect	No effect	No effect

4.5 General Discussion

The main objective of these experiments was to establish whether the growth of independently cultured bacteria would be affected by the addition of a range of iron chelators to the media. Before inoculation with bacteria, nutrient rich media was placed in an anaerobic cabinet for a minimum of 12 h to ensure deoxygenated media was used in the experiments. It was found that for all bacteria tested, the lag phase ended approximately between 6-8 h, after which the exponential phase commenced, and therefore, running the experiments for a period of 24 – 48 h was deemed sufficient for the observations.

A range of iron chelators was used for these experiments in order to assess the impact of iron withdrawal on various bacterial species. The chelators were divided into two groups, chemical and dietary, and both sets of chelators were tested on bacteria that have the potential to be pathogenic as well as those that are beneficial for gut health. The concentrations of chelators to be used in these pure culture experiments were based on previously published literature to ensure that concentrations were low enough to elicit inhibitory effects and at the same time not cause toxic effects on the bacteria [254, 259, 260]. All iron chelators elicited an iron-reduced environment when cultured with majority of the bacteria, with Fe^{2+} concentrations being considerably lower in the media when compared to the control. Firstly, when cultured with the chemical iron chelators, BPDS and 2,2-Dipyridyl, the growth of all potentially pathogenic bacteria was significantly impaired,

except for *C. perfringens* when cultured with BPDS. Similar results were seen in another study, which investigated the survival of bacteria derived from lake water when treated with bathophenanthroline, a chemical iron chelator [261]. This study showed that the administration of bathophenanthroline strongly inhibited *E. coli* growth by up to 88%. Our data also showed BPDS significantly reducing the growth of *B. longum*, whilst 2,2-Dipyridyl had no effect on its growth. As expected, the growth of L. rhamnosus was unaffected by either of the chelators. It can be seen from the control culture (i.e. cultures that were grown without the addition of any chelator) that the growth of all bacteria progressed without any restriction and it can therefore be inferred that the reduction in the growth of the bacterial species tested was mediated through lack of iron.

Likewise, when bacterial species were cultured with dietary iron chelators, a similar trend was observed. Phytic and tannic acid both decreased the growth of all potentially pathogenic bacteria to a significant extent, whilst simultaneously increasing the growth of *B. longum* and *L. rhamnosus.* Interestingly, the addition of PA led to a temporary reduction in the growth of *B. thetaiotaomicron* until 24 h, after which growth resumed. *B. thetaiotaomicron* have been shown to have outer membrane vesicles, which contain phytate degrading enzymes [258, 262]. These enzymes allow the phytate to enter the cell for nutrient processing whilst also preventing it from being destroyed by the hosts own protein degrading enzymes. In the case of *B. thetaiotaomicron*, it has been reported that it produces a histidine acid phosphatase, which is characterised by a subtle change in amino acid structure, therefore, providing this enzyme with catalytic properties. From Figure 4.10d, it could be speculated that this enzyme takes approximately 24 h to be produced, as before that time, the growth of *B. thetaiotaomicron* is significantly impaired, possibly due to undigested phytate. When comparing phytic and tannic acid together, PA appears to have an overall higher significant impact on the growth of potentially pathogenic bacteria, whereby PA reduced the growth of *E. coli* to a greater extent than TA (Figure 4.10a and 4.8a, respectively).

Lactoferrin only decreased the growth of *E. coli* and *S.* Typhimurium, whilst the growth of all other bacterial species tested remained unaffected in the presence of lactoferrin, suggesting it may not be as strong a chelator compared to the ones previously discussed. The decrease observed in *E. coli* and *S.* Typhimurium has been observed in other studies also. Iron binding related growth inhibition or cell death has been regarded as a major antibacterial activity of lactoferrin. For instance, human apo lactoferrin (iron free

lactoferrin) has a bactericidal effect on a variety of microorganism (gram-positive/negative bacteria, rods and cocci, facultative anaerobes and aerotolerant anaerobes) [147, 263]. Some studies have described lactoferrin as consistently exhibiting bactericidal activity against gram-negative bacteria [264, 265], whilst other studies have suggested the use of lactoferrin in the control of *Salmonella* food poisoning as an additive to dry foods [266]. Over the recent years, studies have shown that lactoferrin not only chelates and sequesters iron, it also binds to the lipid A portion of lipopolysaccharide (LPS), found on the bacterial cell wall and therefore disrupting the surface [267, 268]. The binding of lactoferrin to the lipid A section of LPS can therefore reduce the virulence of some of the major enteropathogens [269, 270].

Finally, Manucol LD significantly decreased the growth of all bacteria, apart from *E. coli*, including the beneficial species, to a statistically significant extent. Our results reflect what has been seen in another study, which had the aim of culturing a panel of bacteria, including *E. coli* and *S.* Typhimurium, with various concentrations of alginate, followed by assessment of bacterial growth [254]. As with our study, the growth of *Salmonella* was significantly reduced by the addition of alginate, whilst *E. coli* displayed the most resistance to the antibacterial properties of the alginates. Again, given the successful growth of bacteria cultured without any chelator present in the media, it can be inferred that these effects observed are mediated by the depletion of iron.

One can speculate the direct interaction of the sodium alginate with the bacterial species, in regard to *L. rhamnosus.* For all chelators tested, Fe^{2+} levels were reduced in comparison to the control, except for when cultured with Manucol LD. However, the presence of Manucol LD subsequently led to a significant decrease in the growth of this bacteria. This suggests that the reduction in growth observed may not be linked to the levels of iron, but a direct effect of sodium, which has been reported to be toxic to the *Lactobacillus* genus [271].

4.6 Conclusions

Using a variety of independently cultured bacteria, the effects on growth were observed with and without iron chelators. It was confirmed that the addition of a range of iron chelators, both dietary and chemical, restricted the growth of many potentially pathogenic bacterial species, such as *E. coli, S.* Typhimurium, *C. perfringens* and *B. longum*. However, this effect was not observed for all chelators tested. Furthermore, it was also

observed that the growth of beneficial bacterial species, such as *B. longum* and *L. rhamnosus* was largely unaffected in the presence of an iron chelator, and in some cases, the growth of these bacteria was positively impacted. Furthermore, the reduction in the potentially pathogenic bacteria was positively correlated with the amount of Fe^{2+} available in the media, suggesting that the decreases observed in various bacterial species was iron-dependent. When choosing which chelator to use with the objective to improve gut health, the impacts on all groups of bacteria have to be considered. Out of the naturally-derived chelators, phytic acid appeared to have the most positive impact overall. Therefore, to further complement these results, the next chapter will look at how the chelation of iron with phytin (calcium magnesium salt of phytic acid) and BPDS (a commonly used chemical iron chelator) impacts the function and composition of the human gut microbiota when iron chelators are cultured with human faecal microbiota in a colonic batch fermentation model.

CHAPTER FIVE

5 Iron chelation and the gut microbiota: in vitro colonic fermentations to investigate the impact of iron chelation on the function and composition of the human gut microbiota

5.0 Summary

The previous chapter described the effect of a range of iron chelators on bacteria that were cultured independently. It was observed that the growth of many pure cultures of bacteria were negatively impacted in the presence of an iron chelator. However, bacteria are largely present as a mixed community, therefore, the next step was to examine what effect iron chelation has on human faecal microbiota derived from apparently healthy donors. The relative abundance of different groups of bacteria which have the potential to display pathogenic phenotypes, such *Enterobacteriaceae*, were reduced in the presence of an iron chelator, whilst the presence of alternative chelators also led to an increase in the relative abundance of bacterial groups beneficial for the host, such as bifidobacteria. Part of the work described in this chapter has been published previously [272].

5.1 Introduction

Dietary components have a large influence on iron availability. Organic acids, such as citrate, have been demonstrated to form a weak, soluble chelate with iron, which potentially prevents the precipitation of iron, keeping it in its soluble form, once it has left the acidic conditions of the stomach and entered the duodenum at a higher pH [51]. A well-known enhancer of iron absorption is ascorbic acid (Vitamin C) that can chelate iron and also reduce Fe^{3+} to Fe^{2+}, the species that is absorbed in the duodenum [82, 84]. As well as increasing the absorption of iron, there are many dietary components that restrict this process. PA, also known as inositol hexakisphosphate (InsP_6), or phytate when present as the calcium-magnesium salt, is the principle storage form of phosphorus in many plants, such as seeds, nuts, cereals and legumes [250]. Beneficial properties of PA have been reported, including anti-cancer and anti-oxidant activities [273, 274]. However, *in vivo* and *in vitro* studies have shown that it forms insoluble complexes with several divalent minerals such as Ca^{2+}, Fe^{2+}, Zn^{2+} and Mg^{2+}, thereby preventing absorption [275, 276] and it has therefore been referred to as an anti-nutrient. Inorganic phosphate is released as a result of PA degradation, resulting in the production of PA's lower inositol forms (penta-, tetra-, tri-, di- and mono-*myo*-inositol phosphates; InsP_5, InsP_4, InsP_3, InsP_2 and InsP_1, respectively) [277]. Of these lower inositol phosphates, only InsP_5 and InsP_4 has anti-nutritional properties, thereby decreasing mineral availability [251, 278]. Other derivatives of PA display no anti-nutritional activity.

Degradation of PA can occur during the processing of foods [279], through the actions of enzymes called phytases, derived from plant-based foods. Any ingested PA will enter the gastrointestinal tract but no substantial PA degradation is achieved due to the absence of phytase activity in human intestinal cells [280]. Enzymatic degradation of PA, however, is achievable via intrinsic plant-based phytases or via the colonic microbiota [278].

Certain gut micro-organisms (*Bifidobacteriaceae* and coliforms) are able to break down phytates. One study showed that the highest phytate degrading activity belonged to *Lactobacillus reuteri*, *Lactobacillus salivarius* and *Bifidobacterium dentium* [201]. Notably, phytate-bound iron found in the colon is present in the insoluble form making it difficult to degrade [52, 202], suggesting a potential role for phytate in the withholding of iron from potentially pathogenic bacteria.

Few studies have examined the effects of iron chelation on the gut microbiota of healthy individuals, and therefore, the aim of the experiments detailed in this chapter was to investigate the effects of limiting the availability of iron to mixed cultures of bacteria obtained from the gut microbiota. For the experiments outlined in this chapter, BPDS and PA were used as examples of iron chelators to examine the hypothesis that iron-chelation in the colonic environment would alter the composition of the gut microbiota, with potentially pathogenic groups of bacteria reducing in abundance.

5.2 Objectives

The aim of the research presented in this chapter was to investigate the hypothesis that culturing human faecal microbiota with an iron chelator will decrease the relative abundance of those bacteria which are potentially pathogenic, whilst simultaneously increasing the relative abundance of beneficial bacteria. This chapter will be split into two sections: the effect on human faecal microbiota by i) the chemical iron chelator, BPDS, and ii) the natural iron chelator, phytin (calcium magnesium salt of phytic acid).

5.3 Materials and Methods

The data presented in this chapter was obtained from experiments using faecal material from 6 healthy donors, details for whom are given in Table 5.1. This table also outlines for which chelator the faecal microbiota were used.

Table 5.1 – Age, gender and BMI status of faecal donors

Donor ID	Age	Gender	BMI (kg/m^2)	Chelator
CM031	33	M	22.4	BPDS
CM036	31	F	23.2	BPDS
CM075	70	M	18	BPDS
CM011	51	M	25.7	Phytin
CM026	27	F	25.1	Phytin
CM052	25	F	25.4	Phytin

5.3.1 In vitro *colonic fermentations*

Faecal samples used in the colon model experiments were obtained from participants recruited to the QIB Colon Model study. Further details regarding the criteria for participants recruited, all of which were non-smokers, can be found in chapter 2, section 2.5.1. Aliquots of fresh faecal samples obtained from the healthy volunteers were diluted in deoxygenated phosphate buffered saline (pH 7.7), and homogenised using a Stomacher 400 (Seward, United Kingdom) at 230 rpm for 45 s. Further details regarding the processing of the homogenised faecal samples can be found in chapter 2, section 2.5.2.

Conditions tested were either nutritive media (composition can be found in chapter 2) with faecal inocula only (control), or with faecal inocula supplemented with either BPDS (70 μM) or phytin (50 μM). Chelator concentrations tested were feasible for human intake levels. For each donor, 1 vessel was used for each condition. Samples were taken at 0, 4, 8, and 24 h, serially diluted in PBS and enumerated on selective agar plates (described previously in chapter 2, section 2.5.2).

5.3.2 Extraction of microbial DNA from human faecal microbiota

From the fermentation samples collected during the *in vitro* colonic batch fermentation model experiments, microbial DNA was extracted using the commercially available kit, FastDNA SPIN Kit for Soil. An additional bead-beating step was incorporated into this method, as detailed in chapter 2, section 2.6.1. Following extraction, the QIIME 1.9.0 pipeline was implemented to perform bioinformatic analysis on the sequencing output files, with RDP as the reference sequence database (chapter 2, section 2.6.3).

5.4 Results – Bathophenanthroline disulphonic acid

5.4.1 Evaluating the impact of BPDS-mediated iron chelation on the viable counts of some bacterial groups

For the experiments in this section, the range of bacterial groups enumerated were a selection of beneficial bacteria and those that have the potential of displaying pathogenic phenotypes. Table 5.2 depict the effects of BPDS on the viable counts of a variety of bacterial groups derived from the human faecal microbiota of three individual healthy donors (CM031, CM036 and CM075). The viable counts for the 'control' vessel (i.e. this vessel contained nutritive media and faecal inocula only) were first normalised to 100% and thereafter, viable counts from the iron-chelated vessels ('+BPDS') were shown as percentages in relation to the control at the corresponding time point ('T8' and 'T24'). Significant changes of viable counts in comparison to the control are marked at the relevant timepoints for each bacterial group.

Table 5.2 – Viable counts (%) of different bacterial groups derived from the human faecal microbiota of donors after iron chelation with BPDS. ***p<0.01, ***p<0.001 and ****p<0.0001. Control normalised to 100%.*

Bacterial Group	T8 +BPDS			T24 +BPDS		
	CM031	CM036	CM075	CM031	CM036	CM075
Total Anaerobes	34 ****	10 ****	42 ****	38 ****	14 ****	36 ****
Bacteroides	103	6 ****	99	8 ***	15 ****	69
Bifidobacteria	7 ****	5 ****	4	4 ****	4 ****	324 **
Clostridia	75	15 ****	103	1012 ****	10 ****	278 ****
Lactobacilli	2 ****	18 ****	5 ****	39 ****	24 ****	44 ****
Enterobacteriaceae	2 ****	44 ***	16 ****	2 ****	48 ***	3 ****

Numerous trends can be seen that are consistent in the donors. For example, the faecal material from all three donors displayed a significant reduction ($p<0.0001$) in the viable counts of three bacterial groups at both 8 h and 24 h when cultured in the presence of BPDS, in comparison to the control at the respective timepoints. These three groups of bacteria are (i) total anaerobes, (ii) the beneficial group lactobacilli and (iii) *Enterobacteriaceae*, a family containing potentially pathogenic bacterial species. Decreases as high as 98% were observed for the viable counts of *Enterobacteriaceae* at 24 h of the fermentation period in the presence of BPDS.

The viable counts of *Bacteroides*, a genus of bacteria that are also known to contain bacterial species with pathogenic phenotypes, displayed a statistically significant decrease from the faecal material of two of the three donors at 24 h (CM031, $p<0.001$ and CM036, $p<0.001$). Both donors displayed decreases in viable counts ranging from 85% - 92%. Faecal material from donor CM075 displayed a non-significant decrease in the viable counts of *Bacteroides* when their human faecal microbiota was cultured with BPDS.

Bifidobacteria, a group of bacteria that is linked to a healthy gut profile, were observed to decrease in viable counts at both 8 h and 24 h when the human faecal microbiota of two of the three donors were cultured with BPDS. Faecal material from donor CM031 displayed decreases of 92% and 96% at 8 h and 24 h ($p<0.0001$ at both timepoints), respectively, whilst faecal material from donor CM036 illustrated reductions of 95% and 96% at 8 h and 24 h ($p<0.0001$ at both timepoints), respectively. Interestingly, although faecal material from donor CM075 showed no change in viable counts of bifidobacteria when in the presence of BPDS at 8 h, by the end of the fermentation period, viable counts had significantly increased in comparison to the control ($p<0.01$).

For clostridia, another group of bacteria that may contain species of a pathogenic nature, faecal material from two of the three donors exhibited no change in viable counts in the presence of BPDS after 8 h of the fermentation. However, at the end of the 24 h fermentation period, statistically significant increases were observed for both donors in comparison to the control. The viable counts from the faecal material of donor CM031 increased by 912% ($p<0.0001$) by 24 h, whilst viable counts in the faecal material from donor CM075 increased by 178% by the end of the fermentation cycle, compared to the control vessel. Such substantial increases could suggest that clostridia were presented with a competitive advantage due to other bacterial groups rapidly declining in the neighbouring environment. However, the opposite effect was observed in the faecal material from donor CM036 when cultured in the presence of BPDS. By 8 h, the viable counts of clostridia had significantly reduced by 85% ($p<0.0001$) and by the end of the fermentation cycle, clostridia counts had further reduced to 90% ($p<0.0001$) in comparison to the control vessel.

5.4.2 Extraction of microbial DNA from human faecal microbiota

From the fermentation samples collected during the *in vitro* colonic batch fermentation model experiments, microbial DNA was extracted. Extraction effectiveness was confirmed

using gel electrophoresis (Figure 5.1) and the yield and purity quantified using a NanoDrop Spectrophotometer.

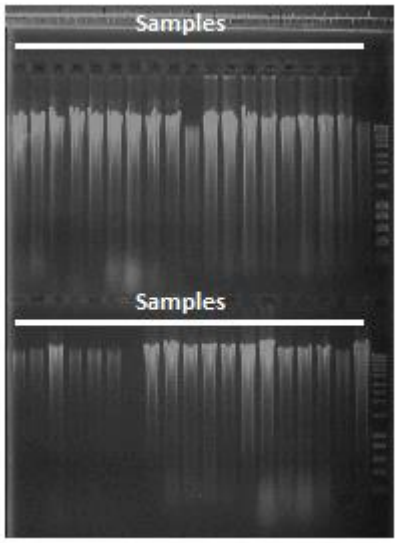


Figure 5.1 – Representative image of a 1% agarose gel containing extracted microbial DNA from fermented human faecal microbiota.

5.4.3 Compositional analysis of cultured human faecal microbiota

The V4 variable region of the 16S rDNA of the extracted microbial DNA obtained from the cultured faecal microbiota was investigated using high throughput 16S rRNA gene sequencing using the Illumina Miseq platform, followed by data analysis using the Quantitative Insights into Microbial Ecology (QIIME, V1.9) pipeline. Faecal samples from 3 healthy humans (CM031, CM036 and CM075; Table 5.1) aged between 31-70 y (mean age of 45 y; mean BMI of 21.2 kg/m^2) were collected, microbial DNA extracted and sequenced. Sequencing produced 103,954 high-quality reads.

5.4.3.1 Compositional analysis of cultured human faecal microbiota – phylum level

For each sample sequenced, the relative abundance of the bacterial taxa is illustrated as a proportion of each taxonomic unit within the human faecal microbiota. Both phylum and genus levels have been displayed to illustrate the differences observed between the taxonomic groups within the microbiota under both control and iron-chelated conditions.

Tables 5.3a-c display the relative abundances (%) for the 4 main phyla present in the human gut microbiota sequenced for each donor. These phyla are Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria.

Table 5.3 – Relative abundances (%) of phyla sequenced from human faecal microbiota of donor CM031(a), CM036 (b) and CM075 (c)

a.

CM031					
Phyla	**T0**	**T8 control**	**T8 BPDS**	**T24 control**	**T24 BPDS**
Actinobacteria	4	37	13	53	12
Bacteroidetes	31	28	49	3	12
Firmicutes	62	20	32	36	72
Proteobacteria	2	15	6	8	3

b.

CM036					
Phyla	**T0**	**T8 control**	**T8 BPDS**	**T24 control**	**T24 BPDS**
Actinobacteria	13	25	4	46	27
Bacteroidetes	3	9	11	5	1
Firmicutes	34	44	82	38	65
Proteobacteria	50	22	2	11	6

c.

CM075					
Phyla	**T0**	**T8 control**	**T8 BPDS**	**T24 control**	**T24 BPDS**
Actinobacteria	3	2	2	2	6
Bacteroidetes	17	7	7	2	2
Firmicutes	60	2	9	14	15
Proteobacteria	1	89	80	81	76

Tables 5.3a-c illustrate the large differences observed in the human faecal microbiota at the phylum level when the microbiota were cultured in the presence of BPDS. However, consistent trends were observed for 4 of the major phyla found in the human gut.

Actinobacteria are a phylum of Gram-positive bacteria that are generally associated with species contributing towards a healthy gut profile. For two of the three donors, when cultured with BPDS, the human faecal microbiota displayed a reduction in the relative abundance of Actinobacteria at both 8 h and 24 h.

The phylum Bacteroidetes is composed of Gram-negative bacteria and comprises bacterial species that exhibit pathogenic behaviour but also contain species that contribute positively towards gut health. All three donors illustrated an increase in the relative abundance of Bacteroidetes at 8 h when iron was chelated.

Firmicutes are a phylum of mostly Gram-positive bacteria. It includes many well-known genera, which can either be beneficial or harmful to gut health. When BPDS was cultured with the human faecal microbiota, all three donors showed an increase in the relative abundance of Firmicutes in comparison to the control at the respective time-points at 8 h and 24 h.

Finally, Proteobacteria were adversely also affected by the presence of BPDS in the cultured human faecal microbiota. Proteobacteria are a major phylum of Gram-negative bacteria and include a wide variety of pathogens that can lead to a negative microbial profile. When iron was chelated from the fermentation, all three donors exhibited a decrease in the relative abundance of Proteobacteria at 8 h and 24 h in relation to the respective control.

5.4.3.2 Compositional analysis of cultured human faecal microbiota – genus level

Figure 5.2 illustrates the differences observed in the same human faecal microbiota at the genus level when the microbiota were cultured in the presence of BPDS. As with the phyla, consistent trends can be observed throughout all donors for many of the genera when iron chelation occurs via BPDS.

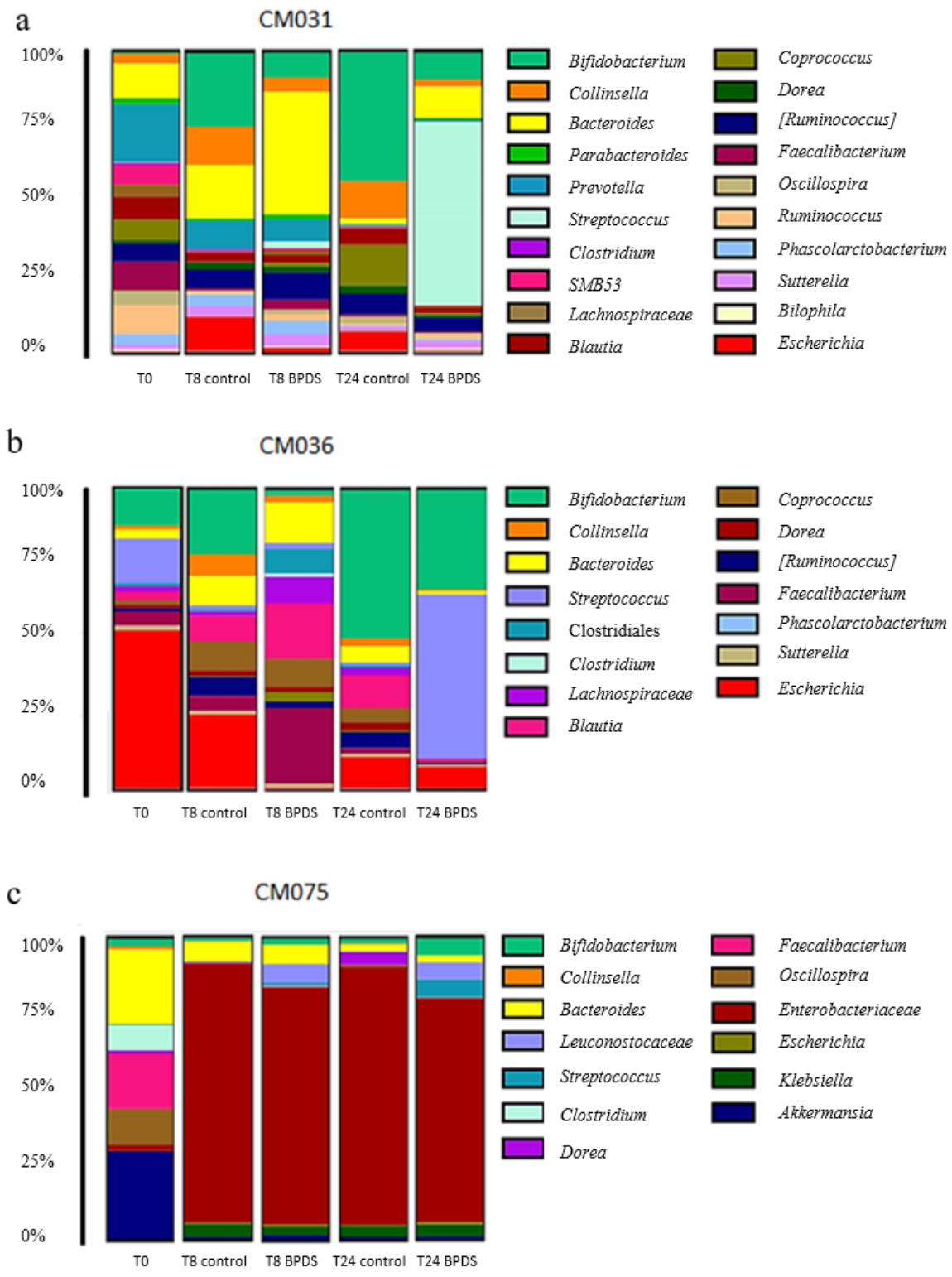


Figure 5.2 – Bar chart representing relative abundances (%) of different genera sequenced through 16S rDNA. *Human faecal microbiota derived from donors CM031 (a), CM036 (b) and CM075 (c).*

Similar to that seen at the phyla level, genera identified from the human faecal microbiota of the three donors highlighted similar trends when cultured in the presence of BPDS.

Streptococcus is a genus of Gram-positive bacteria belonging to the phylum Firmicutes. *Streptococcus* species can contribute towards both negative and positive host health with *S. thermophilus* being exploited industrially as a probiotic [281-284]. For all three donors, upon chelation of iron from the fermentation through BPDS, subtle increases in the relative abundance of *Streptococcus* were seen in the faecal material at 8 h of the fermentation. However, by the end of the 24 h fermentation, a notable bloom in *Streptococcus* was observed in comparison to the 24 h control vessel for donors CM031 (59% T24 BPDS vs 0.2% T24 control) and CM036 (43% T24 BPDS vs 1% T24 control).

Bifidobacterium, a beneficial genus of bacteria belonging to the phylum Actinobacteria, decreased in relative abundance for two of the three donors when the faecal microbiota were cultured in the presence of BPDS. Faecal material from donor CM031 had relative abundances of 8% at T8 BPDS in comparison to 24% at T8 control. Similarly, the relative abundance of *Bifidobacterium* also decreased at 24 h (9% T24 BPDS vs 41% T8 control). Likewise, the faecal material from donor CM036 also displayed lower levels of *Bifidobacterium* in the presence of BPDS. At 8 h, the BPDS vessel contained 2% *Bifidobacterium* compared to 19% in the control. By the end of the fermentation, the relative abundance of *Bifidobacterium* was 27% (T24 BPDS) compared to 43% (T24 control).

Another genus belonging to Actinobacteria is *Collinsella* whose levels decreased in all donors when BPDS was added. Faecal material from donor CM031 had relative abundances of 4% at T8 BPDS compared to the T8 control, which displayed a relative abundance level of 24%. Similarly, at 24 h, *Collinsella* relative abundance was 9% compared to 41% (T24 control). Faecal material from donor CM036 also showed a similar trend though not to the same extent as CM031; 2% T8 BPDS vs 6% T8 control and 0.4% T24 BPDS vs 3% T24 control. However, negligible decreases were observed in the faecal material from donor CM075 (0.04% T8 BPDS vs 0.3% T8 control and 0.02% T24 BPDS vs 0.3% T24 control).

The genus *Bacteroides* generally increased in the presence of BPDS for two of the three donors. For donor CM031, the relative abundance of *Bacteroides* at T8 BPDS and T24 BPDS was 38% and 10%, respectively, in comparison to 17% and 2% at T8 control and T24 control, respectively.

Escherichia, a genus associated with negative health belonging to the Proteobacteria phyla, decreased in relative abundance in two of the three microbiota in which it was identified (CM031 and CM036) when cultured with the iron chelator. For donor CM031, when the faecal material was cultured with BPDS, the relative abundance of *Escherichia* was 1% and 0.4% at 8 h and 24 h, respectively, in comparison to 11% and 6% in the control vessels at 8 h and 24 h, respectively. Likewise, when the faecal microbiota of donor CM036 was cultured with BPDS, the relative abundance of *Escherichia* was lower in comparison to the control vessel at both timepoints (0.4% vs 21% T8 and 6% vs 9% T24). For the microbiota of donor CM075, the community displayed relatively high levels of *Enterobacteriaceae*, the family to which *Escherichia* and other pathogenic species belong. Relative abundances of *Enterobacteriaceae* decreased when the faecal microbiota was cultured with BPDS at both T8 and T24 in comparison to the respective control (77% vs 84% T8 and 72% vs 77% T24). The sample obtained from donor CM075 was the only of the three faecal samples that was found to contain measurable levels of *Enterobacteriaceae* at T0.

The compositional data from all three donors combined to illustrate the overall trends observed when iron was chelated from the culture media through BPDS is described in Table 5.4.

Table 5.4 – Average viable counts (%) of different bacterial groups before and after BPDS-mediated iron chelation derived from the human faecal microbiota of donors (+SEM). *Control normalised to 100%.*

	Viable Counts % ('control' normalised to 100%, ±SEM)	
Bacterial Group	**T8**	**T24**
	(+)BPDS	**(+)BPDS**
Total Anaerobes	28 ±10 *	29 ±8 *
Bacteroides	69 ±32	30 ±19
Bifidobacteria	5 ±1***	110 ±106
Clostridia	64 ±26	433 ±299
Lactobacilli	8 ±5 **	36 ±6 **
Enterobacteriaceae	19 ±11 *	18 ±15 *

Compared to when the human faecal microbiota were analysed individually (Table 5.2), only four bacterial groups displayed statistically different viable counts data at the same timepoint, when analysed as an average of the human faecal microbiota (Table 5.4). The viable counts for total anaerobes ($p<0.05$), lactobacilli ($p<0.01$) and *Enterobacteriaceae*

($p<0.05$) showed a statistically significant reduction at both 8 h and 24 h when cultured under iron-chelated conditions. Similar observations were seen for the viable counts for bifidobacteria at 8 h, where a reduction was observed in viable counts under iron-chelated conditions ($p<0.001$). The increase in viable counts observed at 24 h for bifidobacteria is attributed entirely to one donor, CM075 (Table 5.2).

After combining and averaging the abundance values from the three individual experiments, the data revealed that most abundant genera at 0 h were *Escherichia* (16%), followed by *Bacteroides* (9%) (Figure 5.3).

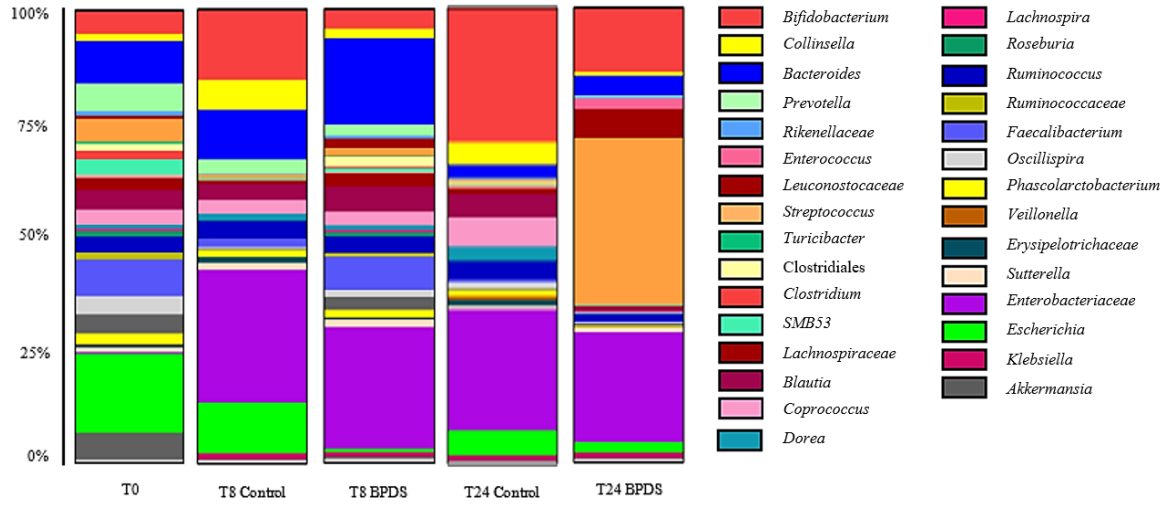


Figure 5.3 – Microbial community profiles assessed by 16S rRNA gene analysis illustrating relative abundances of different genera from the three donors averaged

High-throughput paired-end sequencing of the 16S rRNA gene (V4 region) was performed on fermentation samples using the Illumina Miseq platform, and microbial communities present in relative abundances ≥0.5% are illustrated in Figure 5.3. After combining and averaging the abundance values from the three individual experiments, the data revealed that the most abundant genera at 0 h were *Escherichia* (16.1%), followed by *Bacteroides* (8.7%). It is worth noting that the relative abundance of *Escherichia* was entirely attributed to one donor only, as the remaining two donors had no more than 0.1% *Escherichia* present at T0.

Briefly, the relative abundance of *Escherichia* was reduced substantially in the iron chelated fermentation vessel in comparison to the control vessel at 8 h (0.8% vs 10.7%) and 24 h (2.3% vs 5.3%). This correlates well with the reduction in the viable counts for *Enterobacteriaceae* (Table 5.4). Again, it is worth noting that the high relative abundance of *Enterobacteriaceae* at T8 and T24 for both control and chelator conditions, is entirely

attributed to the levels observed in one donor only, since in the other two donors, the levels were below the detectable limit. A similar trend was observed for *Bifidobacterium*, where the relative abundance was much lower at T8 and T24 under iron-chelated conditions (4.1% vs 15% and 14% vs 29%, respectively), which is also reflected in the viable counts results for bifidobacteria at 8 h. Interestingly, 16S rDNA analysis indicated that at 24 h, the relative abundance of *Streptococcus* increased to 36% in the iron chelated condition compared to the starting proportion of 4.7%. Iron-depleted conditions have been shown to proportionally decrease many bacterial groups, and this may provide other bacteria, such as *Streptococcus*, with a competitive advantage resulting in their increased growth. This could also be true for *Bacteroides*, as the relative abundances of this genus increased at both 8 h (10.6% to 18.1%) and 24 h (2.7% to 4.4%) under iron chelated conditions. Finally, 16S rDNA analysis indicated that *Clostridium* abundance was largely unaffected by iron removal, and this was reflected in the viable counts for clostridia (Table 5.4).

5.4.3.3 Microbial diversity within human faecal microbiota

α-and β- diversity was measured for all *in vitro* colonic fermentation samples tested.

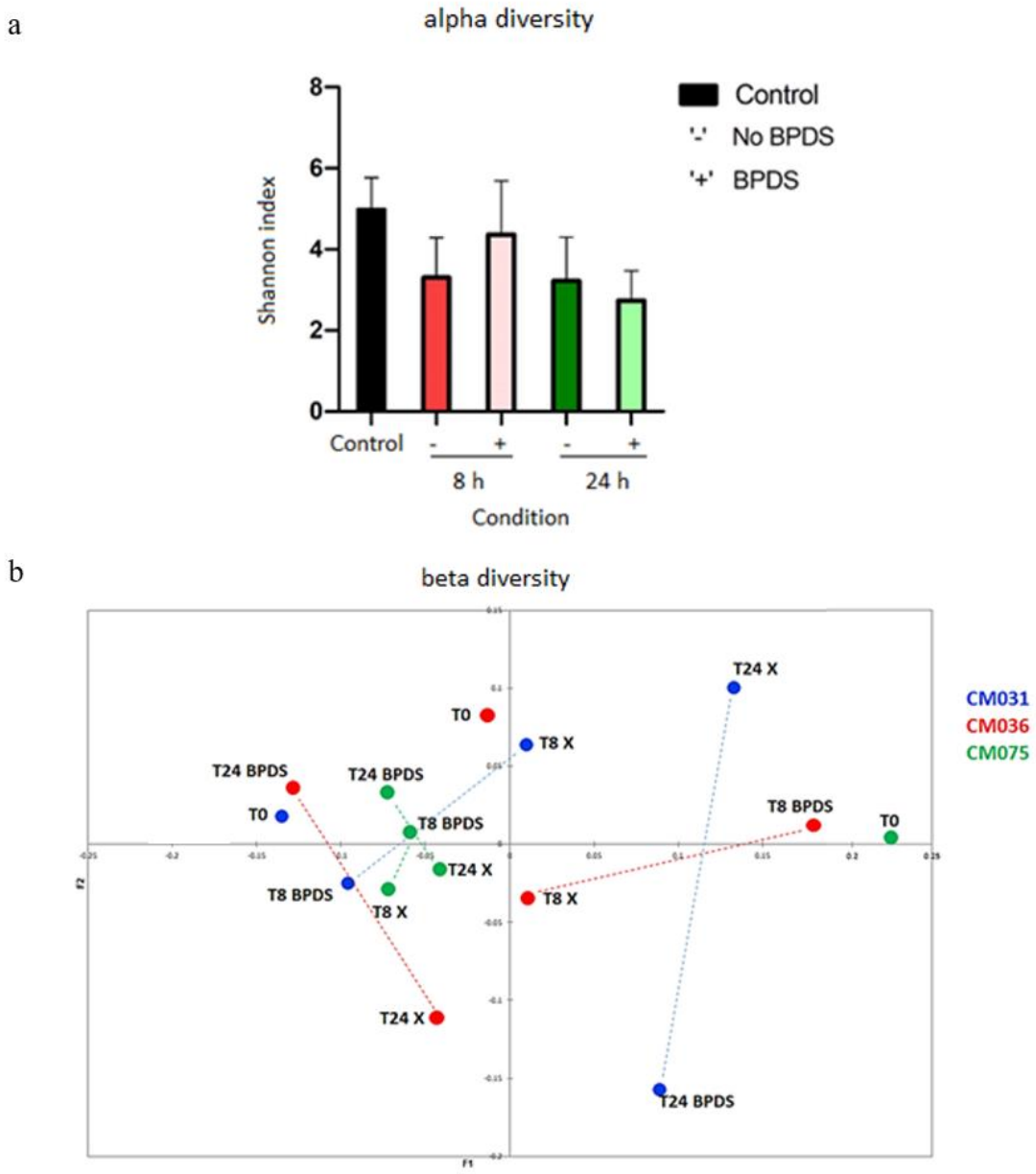


Figure 5.4 – Bacterial diversity profiles of colonic fermentation samples cultured with BPDS. *(a) α- diversity analysis of batch fermentation samples with and without iron chelator (BPDS) using the Shannon index and (b) β- diversity analysis of batch fermentation samples portraying weighted analysis of samples with (BPDS) and without (X) iron chelator using the UniFrac metric and presented as a PCoA plot. Data shown is the average Eigenvalues from the 3 individual experiments using 3 independent donors. Each colour represents a different donor. Analysis was performed using QIIME (V1.9) and visualised using the XLSTAT add-on package in Microsoft Excel.*

The Shannon index, which is a measure of α- diversity, did not indicate a difference in diversity within the population in the absence of the chelator, between 8 h and 24 h (Figure 5.4a). However, an increase in population diversity was observed at 8 h in the presence of

BPDS, compared to the control vessel at the same timepoint. The PCoA plot, using the weighted UniFrac metric, which depicts β- diversity, indicates that the microbiota of the subjects before and after iron chelation did not have a large range of taxa in common, (Figure 5.4b). Interestingly, at both 8 h and 24 h, a shift in β-diversity was observed upon iron chelation when compared to the control at the respective time points, depicted by the dashed line.

5.4.3.4 Metabolite profiling in human faecal microbiota cultured with BPDS

^{1}H NMR spectroscopy was used to determine the levels of over 70 metabolites from samples taken from the fermenters at 0, 8, and 24 h. The three metabolites which showed the most substantial changes between treatments, SCFAs, acetate, propionate and butyrate (Figure 5.5).

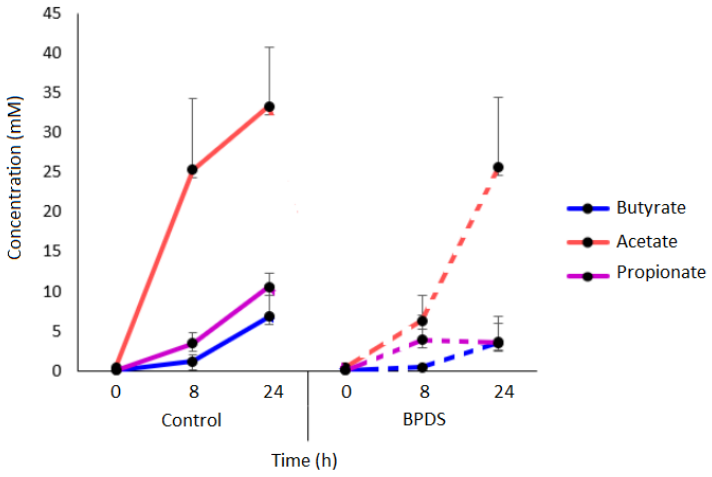


Figure 5.5 - Metabolite concentrations of batch fermentation samples cultured with BPDS. *Short chain fatty acid concentrations in both vessels (Control 'X' and BPDS 'BPDS') were measured using ^{1}H NMR spectroscopy, with samples being screened for multiple metabolites against a spiked standard (TSP) and a validated reference library. Data shown is the average ±SEM metabolite concentrations from the individual experiments from 3 independent donors (microbiota of donors CM031, CM036 and CM075).*

Acetate concentrations was ~25.3 mM under control conditions (i.e. 0 µM BPDS) at 8 h with lower levels (~6.3 mM) found when iron was chelated (with 70 µM BPDS) at the same time-point. The same trend was observed at 24 h where acetate concentrations were ~33.3 mM in the control vessel whereas under iron-limitation, its levels were reduced to ~25.6 mM. Levels of propionate and butyrate exhibited the same pattern under control and iron-chelated conditions. In the control condition, ~1.1 mM (8 h) and ~6.8 mM (24 h) of butyrate was measured whereas under iron-chelation, butyrate levels were reduced (~0.4 mM at 8h and

~3.6 mM 24 h) representing 47% reduction at 24 h. This is reflected in the reduction observed for the relative abundances of the members of the *Ruminococcus* (3.9% to 1.5%) genera, which are common butyrate producers.

Although similar propionate levels were observed between the different conditions at 8 h, propionate concentrations were lower in the iron-chelated vessel compared to the control at 24 h (~10.5 mM vs ~3.5 mM). This represents a 67% decrease in production at 24 h under iron-limiting conditions. This is in line with the 70% decrease observed in the viable counts of *Bacteroides* (a genus containing propionate producers) under low iron conditions (Table 5.4).

5.5 Results - Phytin

5.5.1 Evaluating the impact of phytin-mediated iron chelation on the viable counts of a variety of bacterial groups

For the experiments in this section, the range of bacterial groups enumerated were a selection of beneficial bacteria and those that have the potential of displaying pathogenic phenotypes.

Table 5.5 depicts the effect of phytin on the viable counts of a variety of bacterial groups derived from the human faecal microbiota of three individual healthy donors (CM011, CM026 and CM052). The viable counts for the 'control' vessel (i.e. this vessel contained nutritive media and faecal inocula only) were first normalised to 100% and thereafter, viable counts from the iron-chelated vessels ('+Phy') were shown as percentages in relation to the control at the corresponding time point (8 and 24 h, 'T8' and 'T24', respectively). Significant changes of viable counts in comparison to the control are marked at the relevant timepoints for each bacterial group.

Table 5.5 – Viable counts (%) of different bacterial groups derived from the human faecal microbiota of after iron chelation with phytin. **p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Control normalised to 100%.*

Bacterial Group	T8 +Phy			T24 +Phy		
	CM011	CM026	CM052	CM011	CM026	CM052
Total Anaerobes	268 *	97	101	184.2	13 ****	137
Bacteroides	1588 *	337 ***	65	185	39	233
Bifidobacteria	147	553 *	91	144	623 *	55.4
Clostridia	840 ***	769 ****	51 ****	170	22	133 ***
Lactobacilli	139	47 ****	189 ***	136	37 ****	22.2 ***
Enterobacteriaceae	83	17 ****	61 *	295 ***	4 ****	38 **

As with BPDS, trends can be observed for two bacterial groups in the viable counts of various bacterial groups when cultured in the presence of phytin.

In general, *Enterobacteriaceae*, to which many potentially pathogenic species belong to, exhibited decreases in its viable counts when the human faecal microbiota were cultured with phytin. The faecal materials from donors CM026 and CM052 displayed significant decreases in viable counts at both 8 h and 24 h when compared to the control at the respective timepoint (CM026 $p<0.0001$ at T8 and T24; CM052 $p<0.05$ and $p<0.01$ at T8 and T24, respectively). The microbiota of donor CM011 showed a decrease, though not significant, at 8 h, however this was reversed at 24 h, where a significant increase in counts was observed ($p<0.001$).

The faecal materials from two of the three donors displayed increases in the viable counts of bifidobacteria when cultured with phytin. At 8 h and 24 h, the viable counts of bifidobacteria for the microbiota of donors CM011, increased to 147% and 144%, respectively, compared to the control vessel at these timepoints. A similar trend was observed for the microbiota of donor CM026 where viable counts at 8 h and 24 h were 553% ($p<0.05$) and 623% ($p<0.05$) in comparison to the control.

5.5.2 Compositional analysis of cultured human faecal microbiota

The sequencing and bioinformatic analyses of the extracted microbial DNA obtained from the cultured faecal microbiota was performed as described in section 5.4.3. Faecal samples from 3 apparently healthy humans (CM011, CM026 and CM052; Table 5.1) aged between 25-51 y (mean age of 34 y; mean BMI of 25.4 kg/m^2) were collected, microbial DNA

extracted and sequenced using the Illumina Miseq platform. Sequencing produced 103,954 high-quality reads.

5.5.2.1 Compositional analysis of cultured human faecal microbiota – phylum level

For each sample sequenced, the relative abundance of the bacterial taxa is illustrated as a proportion of each taxonomic unit within the human faecal microbiota. Both phylum and genus levels have been displayed to illustrate the differences observed between the taxonomic groups within the microbiota under control and iron-chelated conditions.

Tables 5.6a-c display the relative abundances (%) for each of the phyla sequenced for each donor.

Table 5.6 – Relative abundances (%) of phyla sequenced from the human faecal microbiota of donor CM011 (a), CM026 (b) and CM052 (c).

a.

	CM011				
Phyla	**T0**	**T8 control**	**T8 Phy**	**T24 control**	**T24 Phy**
Actinobacteria	11	44	30	61	51
Bacteroidetes	22	14	12	1	1
Firmicutes	58	36	52	36	45
Proteobacteria	9	6	6	3	3

b.

	CM026				
Phyla	**T0**	**T8 control**	**T8 Phy**	**T24 control**	**T24 Phy**
Actinobacteria	21	11	31	16	32
Bacteroidetes	10	4	11	6	2
Firmicutes	60	18	4	2	35
Proteobacteria	6	68	13	57	30

c.

	CM052				
Phyla	**T0**	**T8 control**	**T8 Phy**	**T24 control**	**T24 Phy**
Actinobacteria	5	9	35	19	65
Bacteroidetes	7	11	4	4	2
Firmicutes	82	36	36	22	16
Proteobacteria	3	43	24	51	17

As with the BPDS experiments, consistent trends can be observed throughout all donors for 4 of the major phyla found in the human gut: Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria.

Unlike BPDS, the presence of phytin increased the relative abundance of Actinobacteria, a phylum associated with a healthy gut profile. When the faecal microbiota of donors CM026 and CM052 were cultured with phytin, an increase in Actinobacteria was observed at both 8 h and 24 h (11% to 31% and 16% to 32% T8 and T24, respectively, CM026; 9% to 35% and 19% to 65% T8 and T24, respectively, CM052). The microbiota of donor CM011 exhibited decreases in the relative abundance of Actinobacteria at both timepoints in the presence of phytin (44% to 30% and 61% to 51% T8 and T24, respectively).

Another difference between the iron chelators tested was that phytin addition led to a decrease in the relative abundance of Bacteroidetes, a phylum which has the potential to contain pathogenic species. The microbiota of donors CM011 and CM052 illustrated decreases in Bacteroidetes abundance, although CM011 displayed decreases to a reduced extent than CM052 (14% vs 12% and negligible decrease T8 and T24, respectively, CM011; 11% vs 4% and 4% vs 2% T8 and T24, respectively, CM052).

Similar to BPDS, an increase in the relative abundance of Firmicutes was observed in the presence of phytin for two of the three donors. At 8 h and 24 h, there was an increase observed in the relative abundance of Firmicutes in the faecal materials of donors CM011(36% vs 52% and 36% vs 45% T8 and T24, respectively) and CM026 (18% vs 45% and 22% vs 35% T8 and T24, respectively).

Finally, as with BPDS, when cultured with phytin, a decrease in the relative abundance of the Proteobacteria phyla was observed for two of the three donors. The microbiota of donors CM026 and CM052 displayed decreases in Proteobacteria at both 8 h (CM026 68% vs 13%; CM052 43% vs 24%) and 24 h (CM026 57% vs 30%; CM052 51% vs 17%).

5.5.2.2 Compositional analysis of cultured human faecal microbiota – genus level

Figures 5.6a-c illustrate the differences observed in the same human faecal microbiota at the genus level when the microbiota were cultured in the presence of phytin.

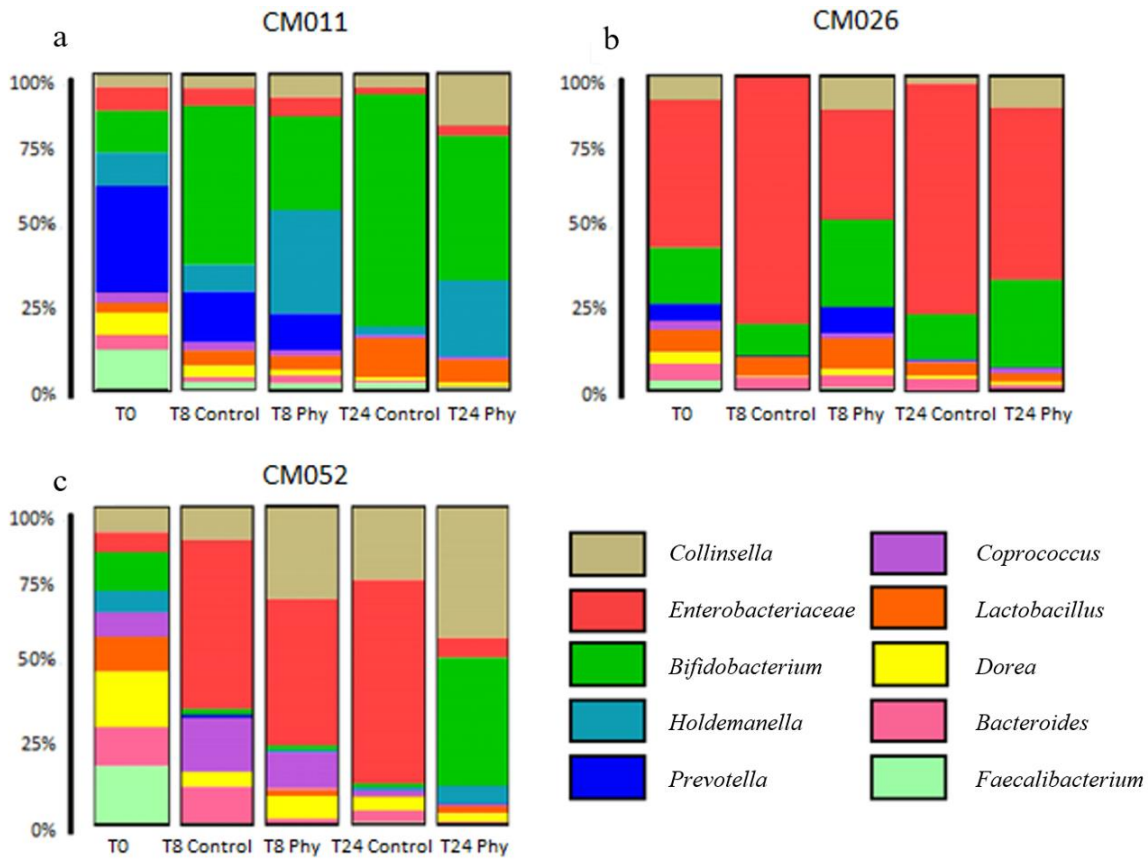


Figure 5.6 – Bar chart representing relative abundances (%) of different genera sequenced through 16S rDNA. *Human faecal microbiota derived from donors CM011 (a), CM026 (b) and CM052 (c).*

As with the phyla, consistent trends can be observed throughout all donors tested for many of the genera when iron chelation occurs through the addition of phytin.

A consistent pattern observed is that of *Collinsella*, a genus associated with positive gut profiles. The relative abundance of *Collinsella* increased in all donors when the human faecal microbiota were cultured with phytin, in comparison to the control. These increases were seen at both 8 h and 24 h (CM011 4% vs 6% and 4% vs 14%; CM026 0.5% vs 9% and 2% vs 9%, respectively; CM052 8% vs 24% and 18% vs 35%, respectively).

Another genus belonging to Actinobacteria is *Bifidobacterium*. For donors CM026 and CM052, increases in the relative abundance of *Bifidobacterium* was observed throughout the fermentation process (CM026 10% to 22% and 13% to 23% T8 and T24, respectively, CM052 no change at T8 and 1% to 35% at T24). Donor CM011 displayed opposite results, where at 8 h and 24 h, decreases were observed in the abundance of *Bifidobacterium*, which could be a reflection of the decrease observed in Actinobacteria relative abundance for donor CM011.

16S rDNA sequencing was unable to resolve some members of the *Enterobacteriaceae* family down to the genus level. However, from the microbiota of donors CM026 and CM052, a noticeable decrease in *Enterobacteriaceae* abundance was observed in the presence of phytin throughout the fermentation. For the faecal material from donor CM026, the relative abundance changed from 74% to 28% (T8) and 68% to 45% (T24), whilst for donor CM052, the relative abundance of *Enterobacteriaceae* in the faecal material remained the same at T8 but changed from 49% to 6% at T24.

The next table (Table 5.7) shows the viable counts data from all three donors combined to illustrate the overall trends observed when iron was chelated from the culture through phytin.

Table 5.7 – Average viable counts (%) of different bacterial groups before and after phytin-mediated iron chelation derived from the human faecal microbiota of donors (+SEM). *Control normalised to 100%.*

	T8	**T24**
Bacterial Group	**(+) Phytin**	**(+) Phytin**
Total Anaerobes	143 ±45	105 ±48
Bacteroides	546 ±370	147 ±61
Bifidobacteria	259 ±147	212 ±119
Clostridia	538 ±244	104 ±42
Lactobacilli	123 ±41	65 ±36
Enterobacteriaceae	50 ±19	108 ±88

Unlike the data for BPDS, when the viable counts for the three faecal microbiota are combined and the control data normalised to 100%, although overall trends are defined, no significant differences in the counts are seen when comparing the control microbiota to those cultured with phytin. This highlights the variability of the human gut microbiota in each of the donors and despite significant changes being present when analysed individually, these may be masked when combined with human faecal microbiota of other donors.

In general, similar trends are observed in the averaged data as with the individuals. Most importantly, the viable counts for bifidobacteria noticeably increase in the presence of phytin throughout the fermentation period when compared to the control microbiota.

Secondly, the growth of *Enterobacteriaceae* is reduced, at least at the 8 h time point, when cultured in the presence of phytin. When analysed individually, this is also true for 24 h, however, due to such high variability amongst the donors, this is not reflected in the data when combined.

Next, high-throughput paired-end sequencing of the 16S rRNA gene (V4 region) was performed on fermentation samples using the Illumina Miseq platform, and microbial communities present in relative abundances ≥0.5% from the three donors are illustrated in Figure 5.7.

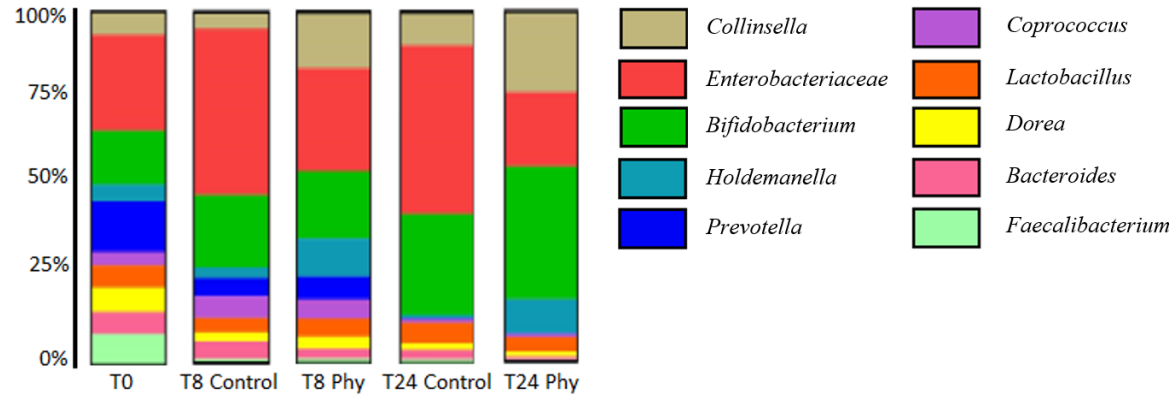


Figure 5.7 – Microbial community profiles assessed by 16S rRNA gene analysis illustrating relative abundances of different genera from the three donors combined

After combining and averaging the abundance values from the three individual experiments, the data revealed that the most abundant taxa at 0 h were *Enterobacteriaceae* (14%), followed by *Bifidobacterium* (8%) and *Prevotella* (8%), (Figure 5.7).

As with the individual analysis of the human faecal microbiota, the most noticeable changes were observed in the following three taxa: *Collinsella*, *Bifidobacterium* and *Enterobacteriaceae*.

On average, the relative abundance of *Collinsella* increased to 13% compared to 4% at 8 h. This was again observed at 24 h, where the relative abundance of *Collinsella* in the microbiota cultured with phytin was 19% compared to the control microbiota, which displayed an abundance of 8%.

Similarly, the relative abundance of *Bifidobacterium* also increased in the presence of phytin at 24 h (32% compared to 24%). This increase is well correlated with the increase in average viable counts observed for bifidobacteria (Table 5.7). The relative abundance of

Bifidobacterium was unchanged at 8 h when comparing the control microbiota to those cultured with phytin.

Lastly, *Enterobacteriaceae* relative abundance displayed a general decrease when the microbiota were cultured with phytin. At 8 h, the relative abundance of *Enterobacteriaceae* decreased to 24% from 39%, and at 24 h, the relative abundance decreased to 18% from 40%. This decrease is reflected in the average viable counts data (Table 5.7), for at least the 8 h timepoint.

5.5.2.3 Microbial diversity within human faecal microbiota

Alpha (α) and beta (β) diversity was analysed for *in vitro* colonic fermentation samples at 0, 8 and 24 h. Unlike BPDS, results for this section are presented for individual donors as opposed to combined data, due to the high variability observed throughout the donors' microbiota.

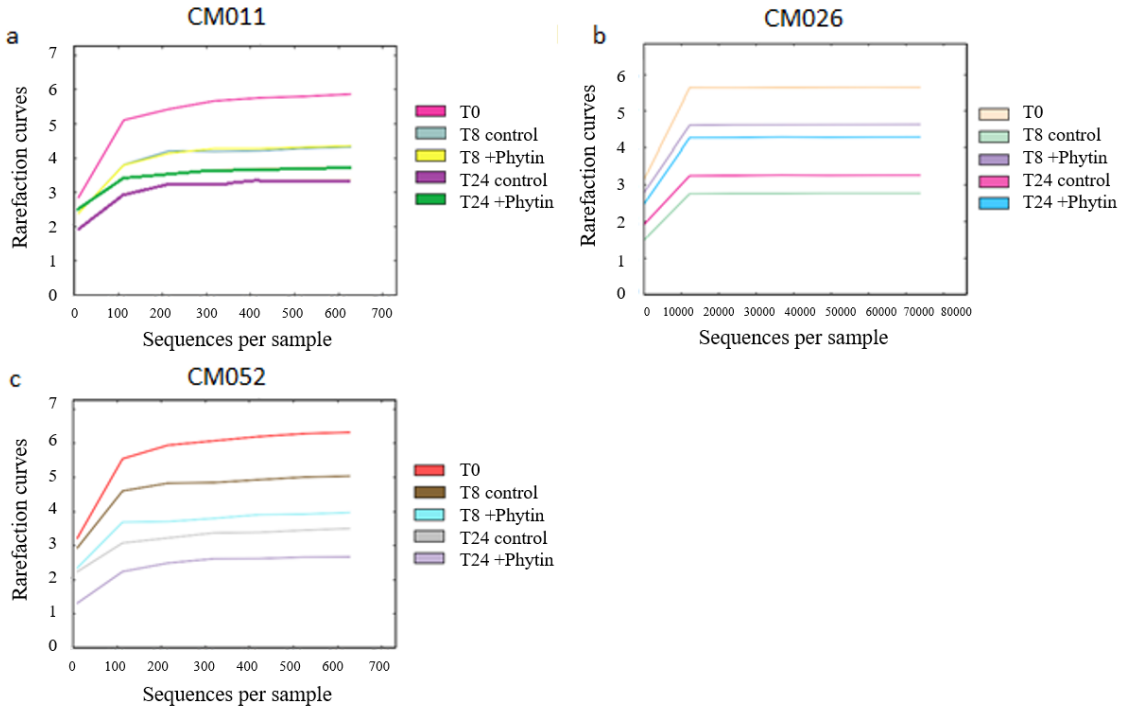


Figure 5.8 – α- diversity analysis of microbiota cultured with phytin. *α- diversity analysis of batch fermentation samples with (Phytin) and without iron chelator (control) using the Shannon index. Donor CM011 (a), CM026 (b) and CM052 (c).*

The Shannon index, a form of α-diversity, showed a shift in diversity within the population in the presence of phytin for two of the three donors in comparison to the control fermenter. The microbiota of donor CM026 illustrated a noticeable increase in population

diversity in the presence of phytin at both 8 and 24 h (Figure 5.8b), whilst for donor CM011, when phytin was added to the fermentation (Figure 5.8a).

PCoA plots, which illustrates β- diversity, was also performed on all three faecal microbiota.

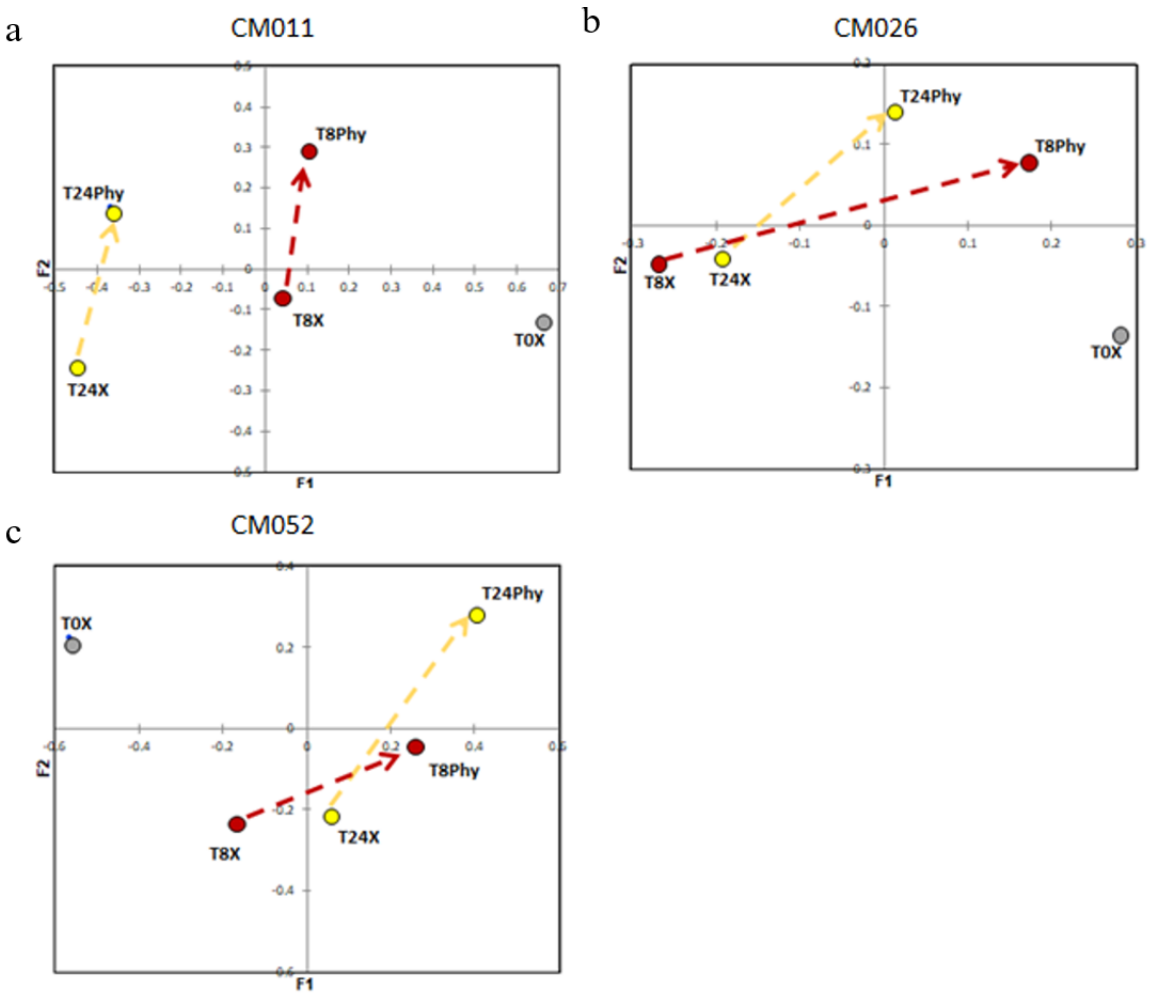


Figure 5.9 – β- diversity analysis of microbiota cultured with phytin. *β- diversity analysis of batch fermentation samples portraying weighted analysis of samples with (Phy) and without (X) iron chelator using the UniFrac metric and presented as a PCoA plot. Donor CM011 (a), CM026 (b) and CM052 (c).*

The PCoAs indicates that for all three donors, microbiota of the subjects after iron chelation through the addition of phytin, did not have a large range of taxa in common. Moreover, at both 8 and 24 h, for all three donors, a shift in β- diversity was observed upon phytin addition when compared to the control at the respective time points, depicted by the dashed.

5.5.2.4 Metabolite profiling within human faecal microbiota cultured with phytin

^{1}H NMR spectroscopy was used to determine the levels of over 70 metabolites from samples taken from the fermenters at 0, 8, and 24 h.

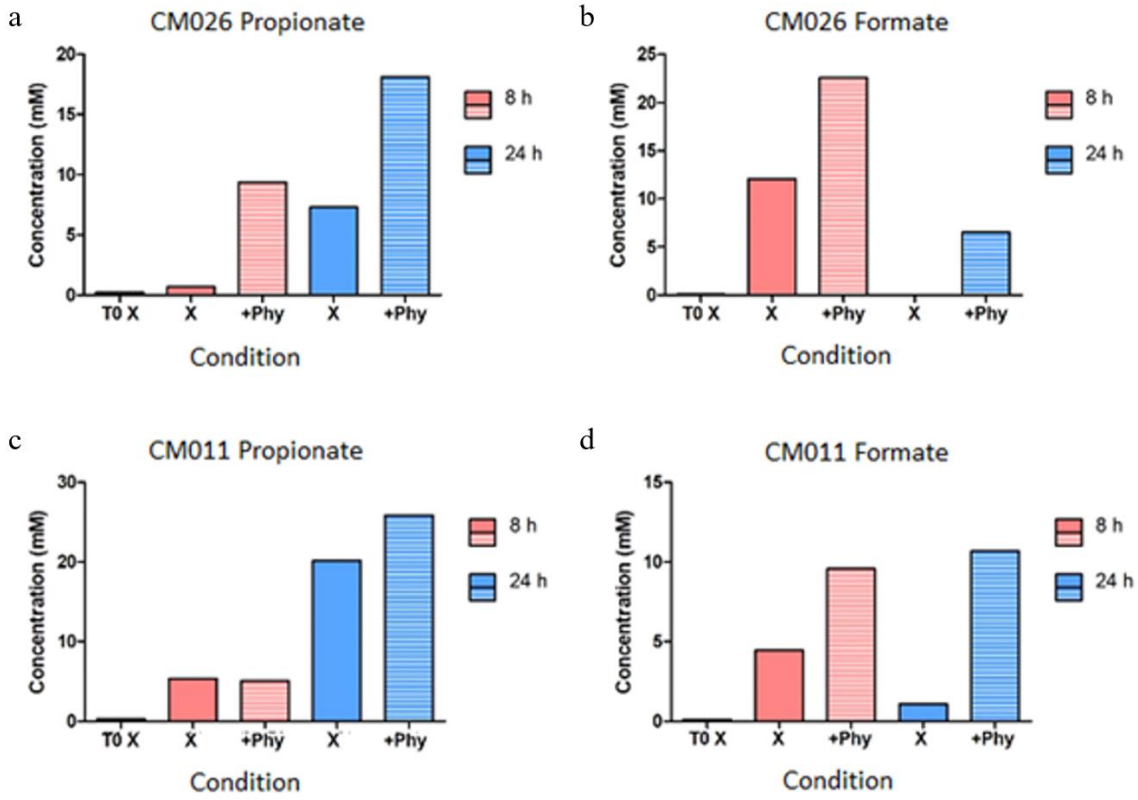


Figure 5.10 - Metabolite concentrations of batch fermentation samples cultured with phytin. *Short chain fatty acid concentrations in both vessels (Control 'X' and Phytin 'Phy') were measured using ^{1}H NMR spectroscopy, with samples being screened for multiple metabolites against a spiked standard (TSP) and a validated reference library. Figure a and b represent the microbiota of donor CM026 and Figure c and d represent the microbiota of donor CM011.*

In two of the three donors, formate and propionate showed the most substantial changes between treatments, (Figure 5.10). For donor CM026, concentrations of propionate increased from 0.6 mM to 9.4 mM and 7.3 mM to 18 mM at 8 and 24 h, respectively, in the presence of phytin in comparison to the control (Figure 5.10a). Similarly, levels of propionate increased from 20 mM to 26 mM at 24 h for donor CM011 in the presence of phytin when compared to the control fermenter (Figure 5.10c).

Similar trends were observed for formate. Donor CM026 displayed a 150% and 100% increase in formate concentrations at 8 and 24 h, respectively when phytin was present (Figure 5.10b), whilst donor CM011 illustrated increases of 92% and 721% at 8 and 24 h,

respectively (Figure 5.10d). This is reflected in the increase observed for the relative abundance of *Collinsella* (Figures 5.6a-b), a genus well-recognised to ferment glycogen to produce formate [285].

5.6 Discussion

5.6.1 BPDS

This study was designed to assess the effects of colonic iron chelation on the human gut microbiota in an *in vitro* colon model system.

The net effects of iron on the gut microbial composition are unclear and need further research as the results reported are inconsistent [6, 39, 44, 45]. The experiments detailed in this chapter showed a marked effect on the global microbiota composition when iron was chelated in the fermentation vessels with BPDS. BPDS acts as a chelator of various metals and, in particular, it has been shown to bind iron with very high affinity and has therefore been used as an iron chelator in many studies [286, 287]. Comparison of the relative abundance of bacterial taxa between the two conditions (control and BPDS) illustrated that the most apparent differences were the decreased relative abundance of the potentially pathogenic *Escherichia*. The observed decrease of *Bifidobacterium* is interesting as other studies have reported different outcomes, which could be due to strain specificity, inter-individual variability of the host, as well as the effects of neighbouring taxa and metabolites. However, the relative abundance of *Clostridium* remained relatively stable, which has been observed in other studies. Notably, *Streptococcus*, a member of the lactic acid bacteria group to which *Lactobacillus* also belongs, was seen to increase upon iron removal, and this could potentially lead to restricted growth of other bacteria in the gut environment.

Studies investigating the effects of iron supplementation report comparable results to those found in this study in relation to the relative abundance of various bacterial taxa, including *Escherichia*. Jaeggi *et al.,* (2015) examined the effects of low and high doses of in-home iron supplementation on the gut microbiota of Kenyan children [157]. In this setting, provision of iron-fortified porridge led to an increase in the abundance of known/potential pathogens, with proportions of enterobacteria, *Clostridium* and *E. coli* increasing, whilst bifidobacteria decreased. In addition, children given iron-fortified porridge had elevated levels of faecal calprotectin, a marker of gut inflammation, which is likely to reflect an increased pathogenic profile.

Much of the published literature focuses on the effects of iron on the infant microbiota which differs in composition to that of adults. In addition, geographical location is likely an important factor, as studies have shown that individuals from developing countries tend to have a greater pathogenic microbial profile due to the lack of clean food and water, and, in turn, a compromised gut function. This is reflected in two studies, where one investigated the effects of iron addition on the gut microbiota of children from Cote d'Ivoire [229], the second study focused on children from South Africa [155]. No detrimental effects of iron supplementation were observed in those that had access to clean water and food, yet the children that did not have access to clean water had adverse side effects.

α- diversity analysis using the Shannon index, which accounts for the distribution and richness of OTUs within a population, showed an increase in population diversity at 8 h in the presence of the iron chelator. It can be speculated that the chelation of iron may lead to certain taxa exploiting other metals as a means of replacing iron, and therefore temporarily facilitating growth in a micronutrient-restricted environment. However, by 24 h, a substantial decrease in diversity was observed under iron-chelated conditions, suggesting an exhaustion of these metals and nutrients. β-diversity analysis demonstrated that there was not a large range of taxa in common when comparing the control samples to those that were cultured in iron-limiting conditions. Interestingly, at both 8 h and 24 h, there was a shift in β-diversity between the two groups for all three of the cultured donor samples. This is in line with a study performed by Dostal *et al*., (2015) [288], which investigated the effect of iron on butyrate production in the child gut microbiota, where altering the iron concentration in the medium affected microbial community structure as well as causing a shift in β-diversity.

Microbial metabolic activity contributes to human health. When iron was chelated, there was a decrease in the three main SCFAs that are produced in the gut, which presumably reflects poor growth of fermentative microbes. Based on the 16S rRNA gene sequencing data from this study and those published elsewhere, we can infer the potential mechanisms behind certain metabolic changes. Firstly, iron-dependent enzymes are critical operators of many metabolic pathways, and therefore these processes can be affected by differing iron concentrations. Moreover, any microbial fermentation that takes place requires the redox balance to be sustained. Due to the dual role of iron as an electron donor and acceptor, we speculate that changes in iron levels could have a large effect on redox balance. During the

batch fermentation, the most prominent effect observed on metabolite production was that of acetate levels. Many gut bacteria produce acetate by either the reductive acetyl-CoA pathway, which uses H_2 and CO_2 [289], or via the regular glycolytic pathway through pyruvate metabolism [290]. The former pathway consists of numerous iron-dependent enzymes and can account for >25% of acetate produced in the gut [291]. It is therefore plausible to speculate a lack of conversion of H_2 and CO_2 to acetate under iron-limiting conditions, resulting in an overall decrease in acetate levels. This observation also correlated with the viable counts and 16S rRNA gene analysis, where a decrease in the members of *Bifidobacterium*, a prominent bacterial group which produces acetate, was observed under iron-limiting conditions. Although correlations of metabolite levels with the relative abundance of bacterial taxa do not provide a causal relationship, it may still provide some indications as to which taxa are responsible for any observed differences.

5.6.2 Phytin

Experiments were designed to assess the effects of the dietary compound, phytin, and its iron-chelating abilities in the human colonic environment. It was found that the addition of phytin, the salt form of phytic acid, significantly reduced the viable counts of *Enterobacteriaceae* in two of the three donors that were tested. This is in line with our previously published study, which illustrated a significant reduction in this group of potentially pathogenic bacteria in the presence of the chemical iron chelator BPDS [272]. Interestingly, contrary to our previous study, the viable counts for bifidobacteria significantly increased in the presence of phytin for two of the three donors, suggesting that the effects of iron chelation on other bacterial groups could have a subsequent effect on neighbouring taxa.

Next, a closer look at the effect of phytin on the composition and function of the human gut microbiota revealed a marked reduction in the relative abundance of *Enterobacteriaceae* for two of the three donors. Similar observations have been reported in other studies looking at the effects of iron supplementation on the gut microbiota, where an increase in iron led to a rise in the potentially pathogenic genus, *Escherichia* [157, 229]. Although other studies have not been performed with the aim of observing the effects of phytic acid's iron-chelating capabilities on the human gut microbial composition, several studies have looked at the effects of whole grain foods, a major source of phytic acid, on the human gut microbiota. Three studies reported the decrease in *Enterobacteriaceae* levels when volunteers were provided with foods rich in whole grain content [292-294].

Other studies have looked at the impact of phytic acid on pure bacterial cultures. One study carried out in Korea examining the protective role of sodium phytate against *E. coli* in meats revealed similar observations, whereby sodium phytate exhibited bactericidal effects on *E. coli* in a dose-dependent manner [295]. In another study, phytic acid from rice bran was reported to inhibit the growth of both *S.* Typhimurium and *E. coli*, species belonging to the *Enterobacteriaceae* family [296].

Interestingly, 16S rDNA analysis revealed an increase in the relative abundance of the beneficial genus, *Bifidobacterium*, in the presence of phytin in two of the three donors, which was also reflected in the viable counts data for bifidobacteria. One study investigated the effects of dietary sodium phytate on the colonic luminal environment of rats fed a high-fat diet and found that bifidobacterial profiles increased in the presence of sodium phytate [297], which is in line with the results illustrated in our study. Furthermore, Bifidobacteria have recently been recognised as a group of bacteria with phytate-degrading abilities. Numerous studies have reported that various strains of bifidobacteria to possess phytase-producing properties, whereby phytate is degraded to its lower inositol forms [201, 298-300]. Many of these studies indicated the conservation of InsP6 as well as the production of both InsP5 and InsP4 [201, 298, 299]. This suggests that though phytate is being degraded into forms lower than InsP5, therefore releasing micronutrients from phytate-bound complexes, with InsP6 present and InsP5 still being generated, phytic acid's iron-chelating capabilities are still active. Another study looked at how diet can influence the ability of human intestinal microbiota to degrade phytate [277], which showed that unlike previous studies, gram-positive anaerobes had in fact the least effective hydrolysing properties, with no more than 20% of the phytic acid being degraded. However, it is important to note two crucial differences in this study: (i) the faecal material was separately inoculated in media selective for specific bacterial groups, thereby negating any community effects and (ii) faecal material was derived from volunteers on different diets. In light of this, results of studies on the impact of phytic acid on the gut microbiota demonstrate that regardless of the dietary group of the volunteers, lactobacilli and gram-positive anaerobes had the least phytate-degrading ability, whereas proteobacteria-bacteroides cultures and coliforms displayed the highest phytate-degrading potential. The authors concluded that a community environment with both aerobic and anaerobic bacteria is vital for the degradation of phytate to take place.

A study carried out by Steer *et al*., (2004) [260] investigated the biodiversity of human faecal bacteria that had been cultured in continuous *in vitro* fermentations in the presence

of phytic acid. This study found that, unlike the data presented in this chapter, bifidobacteria were less able to maintain viable counts when derived from phytic acid enriched chemostat fermenters. This was further reflected in the decrease observed for *Bifidobacterium* relative abundance. However, this study also showed that lactobacilli viable counts and *Lactobacillus* relative abundance decreased when cultured with phytic acid, and this is in line with the data presented in this chapter.

Another interesting observation was that of the genus, *Collinsella*, which increased in relative abundance in all three donors when the faecal inocula was supplemented with phytin. *Collinsella* is the most dominant of the group Coriobacteriales and is frequently detected in the human colonic microbiota [301-303]. Previous studies have reported similar effects on *Collinsella*, whereby the provision of phytate-rich whole grain foods to volunteers increased the levels of *Collinsella* [304, 305]. Moreover, *Collinsella* have been observed to ferment a vast range of different carbohydrates, such as glycogen, resulting in the production of metabolites such as formate. This is reflected in the metabolite analysis performed in this study, where formate concentrations, along with propionate, are increased in the presence of phytin. From these data, it can be speculated that the increase in formate could be as a result of the rise in *Collinsella* relative abundance under phytin-supplemented conditions.

The diversity of the microbial population was also affected by the presence of phytin (Figures 5.11 – 5.16). It was found that when iron was chelated through phytin, α- diversity was altered. α- diversity measures the richness and distribution of OTUs within a population, and the results illustrated that the presence of phytin led to an increase in population diversity in two of the donors. A similar observation was reported in our previous study looking at the effects of BPDS on the gut microbiota [272]. β-diversity analysis, which looks at the similarity of taxa between different populations, displayed a shift in diversity at both 8 and 24 h upon the addition of phytin. This agrees with a study performed by another group using a continuous colonic fermentation system, where a shift in β-diversity was observed as well as an alteration in the microbial community structure in response to a change in iron concentrations [288].

5.7 Conclusions

These results highlight the potential role that iron chelators can play in relation to decreasing the growth of potentially pathogenic bacteria in the human gut. Data presented

in this chapter have shown that iron chelation through BPDS or phytin resulted in a decrease of *Enterobacteriaceae*, a group to which pathogenic bacteria belong. Simultaneously, the addition of phytin, but not BPDS, resulted in a rise of *Bifidobacterium* relative abundance¸ a genus with properties beneficial to intestinal health. The results from these *in vitro* colonic fermentations suggest the importance of iron to bacterial growth, as well as the potential use of a dietary component that has iron chelating properties, in having a positive impact on gut health and homeostasis. Based on the results detailed in this chapter, the next step was to see whether these observations could be replicated *in vivo*. The next chapter details the first step towards reproducing these results *in vivo*.

CHAPTER SIX

6 Optimisation of Phloral® - a specialised coating formulation for targeted colonic drug delivery

6.0 Summary

The results detailed in chapter 5 illustrate the iron-chelating properties of phytin and the subsequent alteration in gut microbial composition. The next step was to see whether the same effects could be observed *in vivo*. However, to achieve this, it was vital to ensure that phytin was only active once it reached the colon. The concept of targeted drug delivery has been well established and has been used in the treatment of cancers and inflammatory bowel disorders. It was hypothesised that the same approach could be used to deliver encapsulated compounds with iron-chelating properties into the colon. This chapter examines the use of a specialised coating formulation, known as Phloral®, to deliver encapsulated compounds to the colon. Through dynamic dissolution assays, it was found that Phloral® successfully maintained its dissolution properties until it had reached the colon.

6.1 Introduction

The benefits of delivering a drug directly to the desired organ or compartment in a human has long been recognised. During the last decades, research and technology in controlled drug delivery has advanced and has the potential to contribute significantly towards the clinical treatment of patients [306].

Phloral® is a technology invented by Intract Pharma, a research-based company, which specialises in oral drug delivery and hold a range of licensable technologies for targeted delivery and drug release into the gastrointestinal tract. Phloral® specifically provides precise and consistent delivery to the colon by exploiting both the alterations in gastrointestinal pH and the enzymatic activity of the colonic microbiota: this allows a complementary-based release mechanism to provide site-specific release.

One of the properties of Phloral® is the pH-dependent release of the desired drug achieved through a polymer coating. However, spatial pH differences are found throughout the colon. The pH of the proximal colon is 6.4 ± 0.8, this gradually increases to 6.6 ± 0.8 in the traverse colon, and finally reaches a pH of 7 ± 0.7 in the distal colon [307]. pH sensitive systems aim to exploit the changes that are observed in the different regions of the human gut to enable a successful colonic delivery. One way in which this system is developed is through the use of enteric polymers, which are designed to resist low pH levels found in the stomach and exhibit properties allowing for dissolution at higher pH values [308]. These enteric polymers can be used to coat the active pharmaceutical ingredient (API),

Once the coated capsule enters an environment with an appropriately high pH, the coating is broken down and dissolved, releasing the API in the desired section of the GIT. Eudragit® is one of the most widely used polymers, which contains derivatives of acrylic acid [309] and cellulose, such as cellulose acetate phthalate and hypromellose cellulose (HPMC) acetate phthalate. Eudragit® polymers vary in the properties they exhibit. For example, some Eudragit® polymers that are utilised in colonic drug delivery are water soluble whilst others are water insoluble. Eudragit® S 100 and FS30D are examples of polymers which dissolve at a pH of 7 [310, 311], and utilisation of this acrylic based polymer was proposed by Dew *et al*., (1982) [312]. Capsules were filled with sulfapyridine (5 aminosalicylic acid) before being coated with Eudragit® S 100, which is a copolymer of methacrylic acid and methyl methacrylate with a high dissolution threshold (pH>7). X-ray imaging confirmed that the capsules ruptured in either the distal small intestine or the colon [312]. This led to the availability of mesalazine drugs, such as Asacol MR, Ipocol and Mesren, which have been indicated for treatment of ulcerative colitis (UC) [313]. In addition, mesalazine were also formulated with Eudragit L which dissolves at a pH>6. Furthermore, diffusion mediated drug release has also been developed. Pentasa, a product of mesalazine, is released from the ethylcellulose coated film via diffusion along the GI tract. Two marketed products of Budesonide (Entocort EC and Budenofalk) are also available. Entocort is formulated by the combination of ethylcellulose granules and Eudragit L 100-55. On the other hand, Budenofalk has been developed from budesonide pellets coated with a mixture of Eudragit L and S. This fusion of two polymers allows for drug release at pH> 6.4 and delivers the drug to the colon. Both Budesonide products are indicated for the treatment of acute Crohn's disease [313]. However, Eudragit S 100 showed poor site specificity in many experimental assays. The colonic pH in patients of UC can be low, and the pH drop increases with the severity of the disease [314]. If a pH responsive delivery system does not meet the desired intestinal pH, it will fail to dissolve in the required site of action. Consequently, due to the varying nature of the gut environment, not only within individuals but also between individuals, a coating consisting of only a single mechanism polymer system is unreliable due to the uncertainty of where the API will be released [315].

Another consideration when developing coating suspensions is the exploitation of the natural microbial system that resides within the gut. The large intestine is home to trillions of bacteria and are responsible for the fermentation of many proteins and polysaccharides,

which evade degradation in the upper gastrointestinal tract, subsequently forming SCFAs. For the purpose of colonic targeted delivery, naturally occurring polymers which also have properties that allow them to avoid any degradation prior to reaching the colon are preferred. There are many naturally occurring polymers, such as chondroitin sulphate, pectin, chitosan, guar gum, locust bean gum, alginate, dextran, inulin and amylose [316]. Bacteria dependant systems are of two types: delivery of prodrugs and a universal system. A prodrug is an inactive form of therapeutics which undergoes bio-transformation to become a pharmacologically active ingredient.

In vitro dissolution assays are a critical tool to control the quality of pharmaceutical products and guide formulation development. However, traditional approaches are often crude, with inaccurate measures of actual intestinal release behaviour in humans. The Dynamic Dissolution Model builds upon conventional apparatus but incorporates a unique bicarbonate-based media controlled by a patented Auto pH System™. These innovative features reflect the dynamic environment of the GI tract and modulate parameters crucial to dissolution and drug release. This provides a greater ability to predict which coating formulations are most likely to release the API at the desired site of action within the human GI tract.

A universal delivery system was required to be developed that can carry any drug to the colon without being specific to a particular group of enzymes. Pectin was used as a direct compression coat on tablets and an *in-vivo* experiment on human subjects confirmed drug release in the colon [317]. Further investigations on this polysaccharide demonstrated pectin as a potential element for colonic drug delivery [318]. CODES is a bacteria-triggered system that includes three layers of polymers coated on the core tablet. First, the core tablet is coated with an acid soluble polymer which is further coated with a hydrophilic polymer and enteric layer, respectively. The two outer layers dissolve in the small intestine and though the innermost acid soluble layer remains intact, it allows slow diffusion of water into the core tablet. When the tablet reaches the colon, the polysaccharide of the core tablet is fermented by the microflora and organic acids are formed. This lowers the pH of the surrounding gut milieu and dissolves the acid soluble layer, which initiates drug release [319]. Amylose is a widely used polysaccharide for colon targeted delivery and is the starch component of Phloral®. It is known as resistant starch because it escapes degradation in the upper gut but is fermented by the colonic

microflora. However, when used individually as a coating material, it sometimes swells in the stomach. Therefore, it is necessary to add a water insoluble or pH sensitive polymer. Starch is the most abundant storage polysaccharide in plants and is made of two fractions, amylopectin and amylose. Phloral® consists of amylose (Figure 6.1), which is made of glucose and is a linear α-1,4 linked D-glucan.

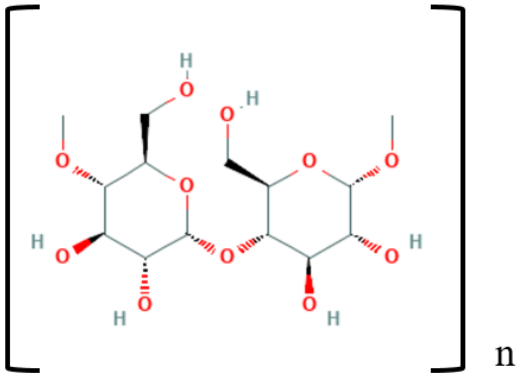


Figure 6.1 - Chemical structure of amylose. *Source: https://www.ncbi.nlm.nih.gov/pccompound*

Starch occurs as granules in the chloroplast of green leaves and the amyloplast of seeds, pulses and tubers [320]. Crystallinity of the starch granule is an extremely vital property that contributes towards the formation of resistant starch. When in crystallised form, starch granules have been shown to confer resistance to hydrolysis by enzymes, therefore increasing the formation of resistant starch. This is reversed when crystallised starch is treated to remove crystallinity and a subsequent decrease in resistant starch is observed [321]. It has been reported that when the content of amylose is high, the digestibility of the starch is reduced [322]. Moreover, through a hydration process, starch crystallinity can be increased [323].

Retrogradation is a process by which resistant starch can be prepared and involves a two-step process [324] (Figure 6.2). The first step, gelatinisation, which causes the release of the starch into solution. Once suspended in solution, the starch granules are then heated to a high temperature (usually between 60^{o}C – 70^{o}C). Once heated, irreversible swelling of the granules causes the starch to be released into the solution, in the form of a random coil [325]. The second stage of retrogradation is the recrystallisation of the starch granules. This occurs once the granules have cooled down from the first step. During this process,

the polymer chains of the starch granules start to reform as double helices, which are stabilised by hydrogen bonds.

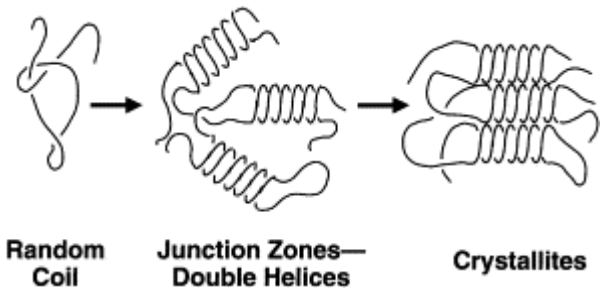


Figure 6.2 - Retrogradation process of amylose starch granules*. Amylose is leached from the granules into solution as a random coil polymer. Upon cooling, the polymer chains begin to re-associate as double helices, stabilised by hydrogen bonds. The individual strands in the helix contain six glucose units per turn. Upon further retrogradation the double helices pack in a hexagonal unit cell (taken from Haralampu (2000) [326]).*

Resistant starches are classified further into four fractions, I-IV. Resistant starch Type I is enclosed in a non-digestible matrix and therefore evades digestion. Type II is ungelatinised starch, whilst Type III is retrograded amylose starch. Finally, resistant starch Type IV is starch that has been chemically modified. Type III is completely resistant to the enzymatic activity exhibited by pancreatic amylases due to its retrograded properties [321].

In previous chapters, various types of iron chelators were investigated with the aim of finding ones which have the most positive impact on gut microbial function and composition. From the panel of chelators previously tested, phytin was chosen for further *in vivo* analysis. The next step was to create a delivery system whereby phytin could be delivered to the colon in order to exert its iron-chelating properties directly in the environment where high levels of iron are present.

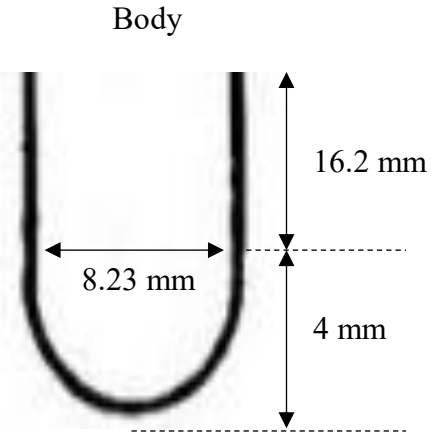
6.2 Objectives

The research presented in this chapter examines the optimisation of an already existing formulation, Phloral®. Phloral® allows colon-specific delivery of the desired agent, in either tablet or capsule form. The aim of this chapter was to explore how changing parameters of the ingredients used in Phloral® affects the delivery of the desired agent, including pharmacokinetics and to establish the best procedure for encapsulating phytin for use in a human clinical trial.

6.3 Materials and Methods

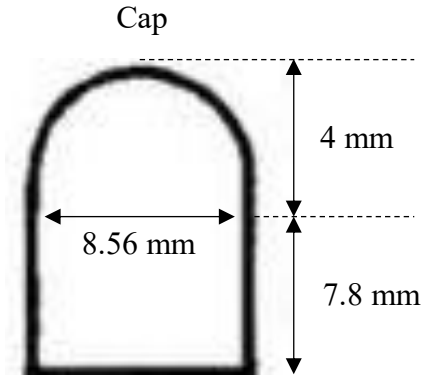
6.3.1 Calculating coating thickness of capsules

The calculation of the amount of coating required for the capsules was based on the measurements of an average size 00 HPMC capsule. Surface areas of the body and caps were measured, and these values were then used to calculate final weight gain per body/cap to achieve required coating thickness. Figure 6.3 depicts the surface area of size 00 HPMC capsules, whilst Table 6.1 outlines the range of final weights required for different coating thicknesses per capsule.



$[16.2 \text{ mm} \times 8.23 \text{ mm} \times \pi] + [(4 \times \pi \times 4^2) \div 2]$

Surface area = **519.12 mm^2 (5.1912 cm^2)**



$[7.8 \text{ mm} \times 8.56 \text{ mm} \times \pi] + [(4 \times \pi \times 4^2) \div 2]$

Surface area = **310.13 mm^2 (3.1013 cm^2)**

Figure 6.3 – Surface area of size 00 HPMC capsule*. The body and cap of size 00 HPMC capsules were measured to obtain a surface area value for both parts. These were then combined to achieve a total surface area, which was then used to calculate coating thickness.*

Table 6.1 – Final weight gain required for different coating thicknesses

Weight gain required (mg/cm^2)	Weight gain per body/cap (mg)	
	Body	**Cap**
3	15.6	9.3
4	20.8	12.4
5	26.0	15.5
6	31.1	18.6
7	36.3	21.7
8	41.5	24.8

6.3.2 Phloral® coating suspension

Phloral® is a coating suspension made of two main components, starch and polymer. Two suspensions of Phloral® were investigated – semi-organic and fully organic.

6.3.2.1 Semi-organic Phloral® coating suspension

A semi-organic coating suspension of Phloral® was made (this suspension is identical to the fully-organic suspension, outlined in the next section, with the addition of butanol). For the starch dispersion, amylo-maize starch N-400 (Roquette, France) was added gradually to butanol. Next, deionised water was added to the starch/butanol suspension under agitation. This mixture was boiled for exactly 3 mins at 300^{o}C. The suspension was then transferred to a cold plate and left to stir overnight. For the polymer dispersion, absolute ethanol and deionised water were mixed to achieve a final ethanol concentration of 95%, before the polymer Eudragit® S 100 (Evonik, Germany) was very slowly added to the ethanol solution. Using the starch moisture factor (SMF), the amount of starch dispersion prepared the night before required was calculated (difference in weight before and after starch dispersion preparation, ΔW, multiplied by SMF, 0.879). Once calculated, the required amount of starch dispersion was slowly added to the polymer dispersion and left to stir for 30 mins. Next, triethyl citrate was added to the above mixture whilst still stirring, and finally, PlasACRYL™ T20 was added. This was left to stir for a minimum of 60 m, after which it was immediately used to coat the HPMC capsules.

6.3.2.2 Fully organic Phloral® coating suspension

A fully-organic coating suspension of Phloral® was made (6 g amylo-maize starch N-400, 14 g Eudgragit® S100, 20 g PlasACRYL™ T20, 3g triethyl citrate, 210.6 g absolute ethanol and 8.8 g deionised water). Absolute ethanol and deionised water were mixed to achieve a final ethanol concentration of 95%. Next, the polymer Eudragit® S 100 was very

slowly added to the ethanol solution. Amylo-maize starch N-400 was then slowly added to the polymer/ethanol solution. This was left to stir at a brisk pace for a minimum of 30 mins to ensure no aggregates developed. Next, triethyl citrate was added to the above mixture whilst still stirring and finally, PlasACRYL™ T20 was added. This was left to stir for a minimum of 60 m, after which it was immediately used to coat the capsules.

6.3.3 Capsule coating

HPMC capsules were filled with a known dose of prednisolone either before (Group 1) or after (Group 2) using a coating machine Aeromatic AG Strea-1 bottom spray fluidised bed coater (Aeromatic AG, Bubendorf, Switzerland). Prednisolone was chosen due to its known absorbance from previously performed experiments with this drug in the laboratory. Caps and bodies were coated separately to ensure capsules were coated with Phloral® evenly. Phloral® was left to stir throughout the coating process, at a consistent pace to ensure homogeneity of the suspension whilst simultaneously ensuring no bubbles were formed. The coating suspension was delivered into the coating machine via a peristaltic pump. Once the coating machine parameters were set up (Table 6.2), a known number of either bodies or caps (also known as cores) were added to the coating chamber. This chamber was then sealed to ensure it was void of atmospheric air and pressure. The temperature within the chamber was set to a minimum of 35^{o}C and the compressed air was set to 0.3 bar. To begin the coating process, the peristaltic pump was activated to start the flow of Phloral® into the coating chamber, and the cores were exposed to Phloral® until the required coating thickness was achieved (section 6.3.1). Once coated, the cores were placed in a sieve and dried overnight at room temperature to be subsequently used in the dissolution systems.

Table 6.2 – Parameters for Aeromatic AG coating machine

Parameter	Condition
Atomising air pressure	0.3 bar
Inlet temperature	30-40°C
Fan speed	Dependent on batch size
Pump speed	Pump speed set to 1 rpm for 15 mins to form initial coat and then increased to 1.5 rpm for the rest of the coating process
Curing conditions	Fluidised in coater for 5 mins and left to dry overnight at room temp

6.3.4 Static and dynamic dissolution assays

USP-II apparatus (Model PTWS, Pharmatest, Hainburg, Germany) was used to perform static and dynamic dissolution assays to record the release of prednisolone within the coated capsule and, by doing so, testing the efficiency of the coating prepared by exposing it to similar conditions to that of the human GI tract. The dynamic dissolution system was pH controlled by the Auto pH system™. In brief, two buffers were prepared for the dissolution assays. First, a 0.1M HCl buffer was prepared to simulate conditions in the stomach. Capsules coated with Phloral® were then submerged into the acidic buffer for a period of 2 h, known as the 'acidic phase'. Capsules were then transferred into a second buffer (Hank's buffer; 0.441 mM KH_2PO_4, 0.337 mM $Na_2HPO_4.2H_2O$, 136.9 mM NaCl, 5.37 mM KCl, 0.812 mM $MgSO_4.7H_2O$, 1.26 mM $CaCl_2.2H_2O$ and 4.17 mM $NaHCO_3$, pH 6.8) to simulate small intestinal conditions. The pH of the buffer was set to gradually increase from pH 5.6 to 6.8 for the first 35 mins. Following this, Pre-Krebs bicarbonate buffer (6.9 mM KH_2PO_4 and 400.7 mM $NaHCO_3$, pH 7.4) was added to Hank's buffer to increase the pH (Table 6.3) The release of prednisolone was measured at Abs_{247} for a period of 210 mins.

Table 6.3 – pH settings for dynamic dissolution system

Time (mins)	pH
0	5.6
5	6.0
10	6.5
20	6.8
36	7.0 (pre-krebs buffer added at 35 mins)
50	7.2
80	7.4
210	6.5 CO_2 flow increased to rapidly drop pH)

6.4 Results

6.4.1 Testing Phloral® efficiency on Group 1 capsules

Size 00 HPMC capsules were filled with 10 mg prednisolone prior to coating with semi-organic Phloral®. The remainder of the capsule was filled with a chemically inactive filler to provide weight to the capsules. Pre-filled capsules were placed in the coating chamber of the coater machine, and batches of capsules were coated with a semi-organic Phloral® suspension until a range of coating thicknesses were attained. These capsules were then subjected to static and dynamic dissolution assays.

Figure 6.4 – Disintegration of semi-organic Phloral® pre-filled capsules. *This figure illustrates the opened capsule after exposure to acidic phase during static dissolution*

When capsules that were pre-filled with prednisolone were subjected to the conditions of the human gastrointestinal tract, it was found that the capsules disintegrated within 1 h of the acidic (static) phase of the dissolution assay. This was indicated by an increase in absorbance values after 50 mins. Figure 6.4 depicts a disintegrated capsule that was removed from the static dissolution system, with no content left within. The dissolution assay was stopped at this stage. These results suggested that coating capsules in its entirety (i.e. coating closed capsules as opposed to coating the cores separately) most likely led to inefficient coating and therefore caused early release of the drug. Therefore, the next set of results show the drug release profile of prednisolone when placed within capsules that had their cores coated separately first and then filled with the drug and inert filler.

6.4.2 Testing Phloral® efficiency on Group 2 capsules

Size 00 HPMC capsules were coated with either a semi-organic (Figure 6.5a) or fully organic (Figure 6.5b) Phloral® coating suspension. Capsules were then filled with 10 mg prednisolone and inert filler. The capsules were then subjected to static and dynamic dissolution assays.

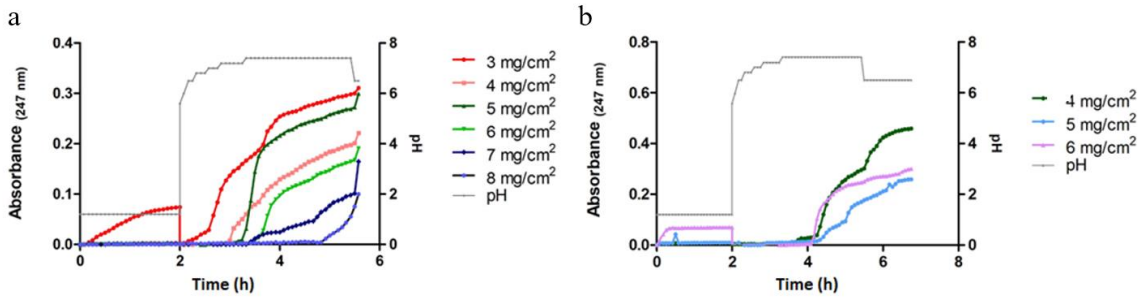


Figure 6.5 – Dissolution assay illustrating semi-organic and fully organic Phloral® efficiency of pre-coated capsules. *Figure a illustrates the prednisolone drug release profile for capsules coated with a semi-organic Phloral® suspension, with coating thickness ranging between 3 – 8 mg/cm^2. Figure b illustrates the prednisolone drug release profile for capsules coated with a fully organic Phloral® suspension, with coating thickness ranging between 4 – 6 mg/cm^2*

Figure 6.5 shows the results of dynamic dissolution assays on capsules that were coated with either a semi or fully organic Phloral® suspension. As illustrated in figure 6.5a, coating thicknesses of 3 and 4 mg/cm^2 were insufficient at retaining the capsule integrity for the required amount of time to reach the colon. Both coating thicknesses led to the release of the drug between approximately 2 and 3 h. This is too rapid as ideally drug release should occur roughly 4 to 5 h after exposure to the acidic conditions of the stomach to ensure drug release occurs in the colon. This ideal drug release time is reflected in the profile for capsules coated to a thickness of 5 mg/cm^2. Although coating thicknesses of 6 – 8 mg/cm^2 retained the drug for an extended period of time, a reduced release of prednisolone was observed compared to those coated to 5 mg/cm^2.

As illustrated in Figure 6.5b, all three coating thicknesses tested (4 – 6 mg/cm^2) exhibited similar release profiles of prednisolone. All three coating thicknesses indicate that drug release began at approximately 4 h. However, coating thicknesses of 5 and 6 mg/cm^2 show that prednisolone release is inhibited to an extent compared to capsules coated to a thickness of 4 mg/cm^2 and starts to plateau at approximately 6 h. Conversely, capsules coated to a thickness of 4 mg/cm^2 had an overall higher release of prednisolone over the course of the dissolution assay, as well as releasing the drug gradually at a consistent rate, illustrating the required delay in release.

6.5 Discussion

Due to the varying nature of the physiological gastrointestinal conditions in individuals, it is quite difficult to attain the ideal level of therapeutic efficiency by conventional methods. For example, mesalazine tablets (used in the treatment of inflammatory bowel disorders,

such as Crohn's disease) are coated with Eudragit® S 100, a coating that is subject to dissolution through pH [327]. In some cases, this pH triggered formulation has been reported to allow mesalazine tablets to reach the colon intact [328]. However, when consumed by healthy subjects, mesalazine tablets have also evaded dissolution [329-331], suggesting that besides pH, there must be other factors which impact the mechanism of delivery systems. Such factors could include feeding status, the duration of the delivery system at the ileo-caecal junction and gastrointestinal fluid composition [330].

To avoid relying on a single mechanism system, the formulation Phloral® was invented, which encompasses a dual coating system, thereby relying on both the pH conditions and enzymatic activity of the colon. Phloral® has been shown to successfully release the API at the ileo-caecal junction or the colon in eight healthy human volunteers [330]. This study investigated the *in vivo* targeting performance of Phloral®, which is made of a mixture of a pH-responsive enteric polymer (Eudragit® S 100) and biodegradable polysaccharide, amylo-maize (resistant starch), in a single layer matrix film. Briefly, tablets were film-coated with Phloral® and administered to eight healthy volunteers, with the site of intestinal disintegration assessed using gamma scintigraphy. It was found that the coated tablets were able to resist breakdown in the stomach and small intestine. Consistent disintegration of the dosage form was seen at the ileo-caecal junction/large intestine.

The aim of Phloral® is to protect the API from degradation in its transit through the gut and efficiently release the drug in the colon by using two triggering mechanisms which are independent, complementary, and act as a failsafe for each other: as a polysaccharide, starch can be hydrolysed by amylolytic enzymes which can cut one or both types of glycosidic bonds. The main enzyme involved in the hydrolysis of α-1,4 bonds is α-amylase [332, 333]. The rate and extent of hydrolysis depends on the physio-chemical form of the starch. It has been proven that retrograded amylose escapes degradation by pancreatic α-amylase which will avoid premature release of the API, but is susceptible to hydrolysis by colonic microflora [334]. For this reason, the amylose component in Phloral® adds an extra element of site-specificity to the system, since bacterial amylases can specifically digest resistant starch, as they are more efficient than mammalian pancreatic enzymes, and over fifty percent of the bacteria in the colon produce this enzyme [335]. When the amylose is degraded by bacterial amylases, it leaves small pores in the coating through which the drug can be released. This will ensure drug release even if the pH-sensitive polymer fails to dissolve. The polymer is also needed to control the swelling of the starch

[331], as this polysaccharide has the ability to form films through gelation which are very fragile and have a tendency to swell in water [336]. Due to this, Eudragit® S 100 is also used as a structuring agent in the pharmaceutical industry.

Formulating Phloral® coating is a very delicate and time-consuming process, since for the system to function the amylose should be randomly distributed throughout the structure of the mixed films, and without any degree of miscibility with the Eudragit® S 100 as this could alter the characteristics of the amylose, in particular its susceptibility to digestion by colonic bacterial enzymes [337]. This random distribution takes time, and the preparation of the starch suspension comprises many steps which are not well understood. For this reason, it is important to have a good knowledge of the transitions that occur regarding the physical form of starch during processing in order to try to achieve a form that will be resistant to pancreatic α-amylase but not to bacterial amylolytic enzymes, that is, type III resistant retrograded starch.

6.6 Conclusions

Two different types of Phloral® suspensions (semi and fully organic) were tested in order to attain the optimal coating suspension which provided the test drug with the most resistance to gastrointestinal conditions prior to reaching the colon. A fully organic Phloral® suspension displayed high resistant properties when the test drug was exposed to gastrointestinal conditions via dissolution systems. The dissolution systems also showed that a coating thickness of 4 mg/cm^2 was deemed sufficient in terms of drug release. To translate these *in vitro* assays, the coating system developed here was subsequently used in a human trial, detailed in the next chapter.

CHAPTER SEVEN

7 The **E**ffect of **P**hytin **o**n the Human Gut **M**icrobiome (EPoM) – a human trial

7.0 Summary

Previous chapters have measured the effect of different chelators on gut bacteria. Chapter 6 illustrated a successful targeted delivery system whereby, according to *in vitro* tests, compounds should be protected from gastric acid and GI digestive enzymes and only released when they reach the colon. This chapter describes a clinical trial in which volunteers were recruited to participate in a double-blind, randomised crossover dietary intervention trial. Participants were asked to consume encapsulated phytin or placebo for 2 weeks, followed by a 2-week wash-out, and then the alternative treatment was given. However, due to the lack of phytin dispersion in the colon, as indicated by white clumps of powder in the stool samples, it cannot be concluded whether the iron chelating properties of phytin had any effect on the composition of the gut microbiota.

7.1 Introduction

Phytic acid (Figure. 7.1), also known as inositol hexakisphosphate (IP6) or phytate (when in salt form), is the principal storage form of phosphorus in many plants, such as legumes, seeds, nuts and cereals [250]. Phytic acid content varies greatly among plants and is due to different factors, such as the type of seed, climate, and environmental conditions.

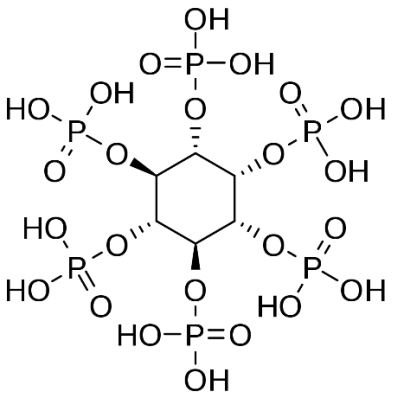


Figure 7.1 - Structure of phytic acid

Studies in humans report that between 37-66% of dietary phytate is degraded during digestion in the stomach and small intestine when the diet is rich in plant food phytases [278, 338, 339], a type of phosphatase enzyme that catalyses the hydrolysis of phytic acid.

Beneficial properties of phytic acid have been proposed, including antioxidant [252] and anticancer [273, 274] activities, but phytate is generally regarded as an antinutrient. *In vitro* and *in vivo* studies have demonstrated that phytic acid forms insoluble complexes with several divalent minerals, thereby preventing absorption, and can potentially result in zinc

and iron deficiencies [275, 276, 340, 341]. Once these insoluble complexes are formed, the mineral cannot be absorbed in the small intestine and therefore pass into the colon. Although phytic acid also binds the metals that the beneficial bacteria use, at pH 6-7 (representative of the colon), phytic acid preferentially binds iron, suggesting a protective role of phytic acid in preventing iron acquisition by potentially pathogenic bacteria.

Several published studies have found that the degradation of phytate in the gut varies between individuals and is largely based on the type of diet consumed. As most plant foods such as legumes, cereals and whole grain products, are processed or heat-treated during food production and the preparation of meals, many of the phytases present in these foods are likely to be inactivated. In individuals whose diets consist of high amounts of wheat or rye bran i.e. foods that contain native phytases, strong phytate hydrolysis occurs in the stomach, with the remaining small portion of non-degraded phytate being hydrolysed in the colon [203]. Notably, phytate-bound iron found in the colon is present in the insoluble form making it difficult to degrade [52, 202] suggesting a potential role for phytate in the withholding of iron from gut bacteria.

Despite numerous studies on the effects of iron supplementation on the gut microbiota, only a few studies have investigated the effects of reducing the level of iron in the gut lumen on the gut microbiota of healthy individuals. The earlier *in vitro* colonic fermentation experiments (chapter 5) investigated reducing the availability of iron to gut bacteria by means of iron-chelating compounds found in foods. The relative abundance of potentially pathogenic bacterial taxa, such as *Escherichia* and *Bacteroides*, decreased, and simultaneously increased the abundance of beneficial bacterial taxa, such as *Bifidobacterium*.

7.2 Objectives

The hypothesis tested in this trial was that the consumption of encapsulated phytin will cause a change in the composition of the colonic gut microbiota, and specifically decrease the proportions of potentially harmful *Enterobacteriaceae*, when compared to the faecal microbiota after consuming placebo capsules, as well as compared to the baseline faecal samples of individuals. It was considered that the consumption of phytin would alter the microbial metabolite profile and that the presence of phytin in the colon would reduce the concentration of faecal free iron in the individuals.

7.3 Materials and methods

Fourteen participants were recruited onto the 'Effect of Phytin on the Human Gut Microbiome' (EPoM) study, which investigated the differential effects of consuming encapsulated phytin on the composition of the gut microbiota. A detailed protocol on participant recruitment and the EPoM study design can be found in the Appendix, in the form of a study protocol and annexes.

Figure 7.2 summarises the EPoM study design and Table 7.1 presents participant information. The bacterial composition of faecal samples was determined using 16S rDNA paired-end sequencing (2x 250 bp) on the Illumina MiSeq platform, performed by Dave Baker (QIB), and bioinformatic analyses were performed by Dr Andrea Telatin (QIB) using the QIIME pipeline (section 2.8.3.1). Dr Gwenaelle Le Gall (UEA) performed ^{1}H NMR spectroscopy analysis (section 2.8.3.2) on faecal waters to investigate changes to microbial metabolic profiles associated with consumption of encapsulated phytin. MCC, which behaved as the placebo, was purchased from DFE Pharma and encapsulated in equal amounts as phytin.

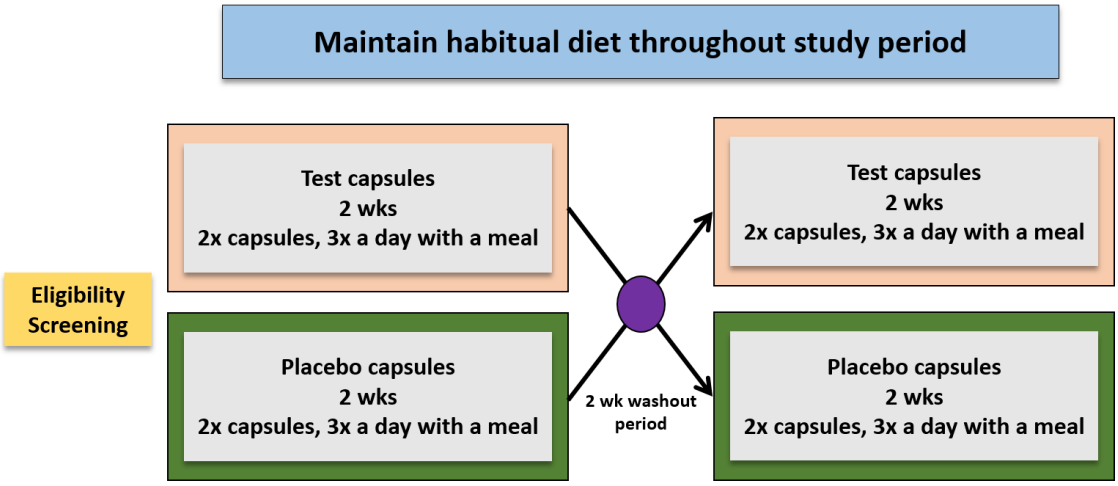


Figure 7.2 - Summary of EPoM study design. *The diagram represents a two-phase crossover, 6-week dietary intervention trial, where participants consumed a randomly allocated set of capsules during each phase. Phases were separated by a washout period, during which no capsule consumption took place. Habitual diet was maintained throughout the study period. Faecal samples were collected three times during each phase, at the start, middle and end. During each phase, stool charts, food frequency questionnaires and capsule checklists were completed by each participant.*

Table 7.1 – Age, gender, Body Mass Index (BMI) of EPoM study participants. *All participants were non-smokers.*

Participant Code	Age (y)	Gender	BMI (kg/cm^2)
EPoM114	27	Male	19.7
EPoM120	32	Female	27.0
EPoM125	33	Female	19.5
EPoM129	27	Female	26.5
EPoM134	26	Male	25.9
EPoM139	25	Female	25.0
EPoM148	28	Female	28.3
EPoM150	23	Female	21.4
EPoM151	29	Male	28.5
EPoM155	24	Male	20.9
EPoM156	23	Male	21.4
EPoM163	18	Male	25.8
EPoM169	25	Male	24.9
EPoM191	30	Female	23.8

7.4 Results

7.4.1 Serum ferritin and C-reactive protein measurements of EPoM samples

To confirm that the release of encapsulated phytin did not take place before it reached the colon, serum ferritin levels of EPoM participants were measured at the beginning and end of each phase. Blood results showed that serum ferritin levels remained stable for all participants throughout the duration of the trial when consuming either capsule.

C-reactive protein (CRP) was also measured, and as with serum ferritin, CRP levels also remained stable throughout the duration of the study in all participants, confirming the lack of systemic inflammation.

7.4.2 Community analysis of human faecal microbiota

Faecal samples were collected during the study from 14 healthy participants, aged 18 – 33 y with a mean age of 26 y, and an average BMI of 24.2 kg/m^2. The DNA was extracted (section 2.6.1) and normalised to 5 ng/µl. The normalised DNA samples were sequenced in-house at QIB, where the variable region, V4, of the 16S rRNA gene was amplified using PCR, and sequenced using the paired-end Illumina MiSeq platform, for downstream analysis using the QIIME pipeline (section 2.6.3). This produced 4,751,049 high-quality reads, with an average of 59,948 ± 10,024 reads per sample.

Performing the unweighted (Figure 7.3) and weighted (Figure 7.4) Unifrac β-diversity analysis with samples colour-coded for each participant, indicated that the samples obtained from each individual clustered together.

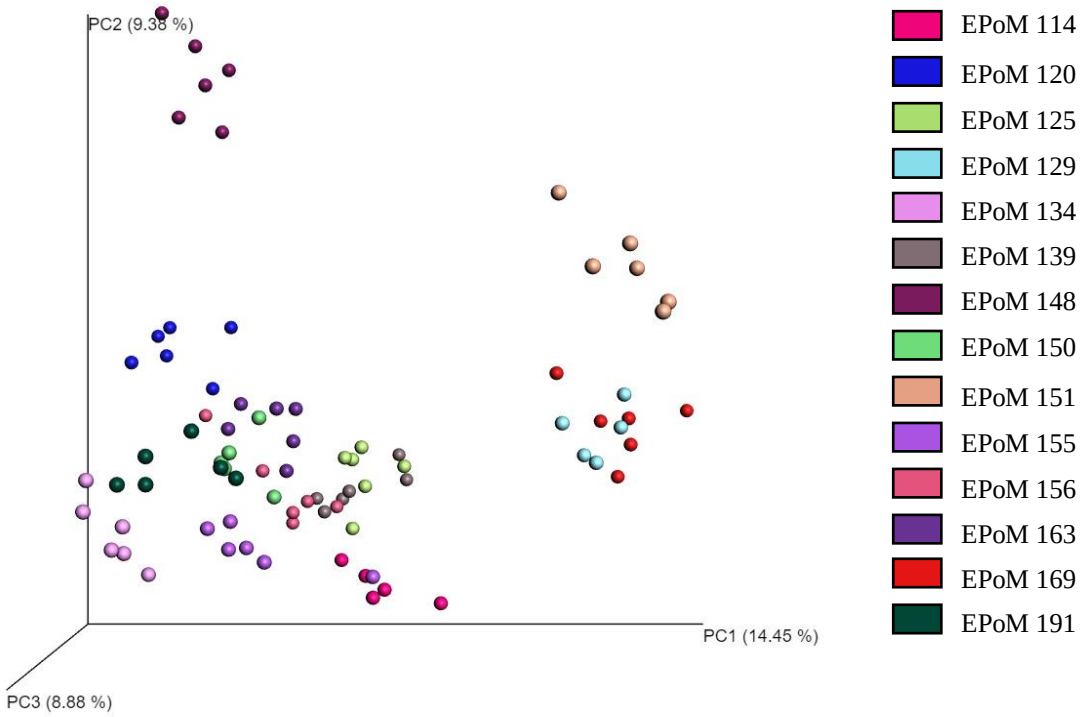


Figure 7.3 - Unweighted β-diversity analysis shows clustering of faecal microbiota based on the individual*. Unweighted β-diversity analysis of faecal microbiota from fourteen study participants; each participant collected a total of six faecal samples, three during each treatment phase. Analysis was performed using the UniFrac metric and visualised as a 3D PCoA plot. β-diversity analysis was performed using QIIME 1.9.1 and plotted using Emperor. The EPoM code in the key refers to different participants.*

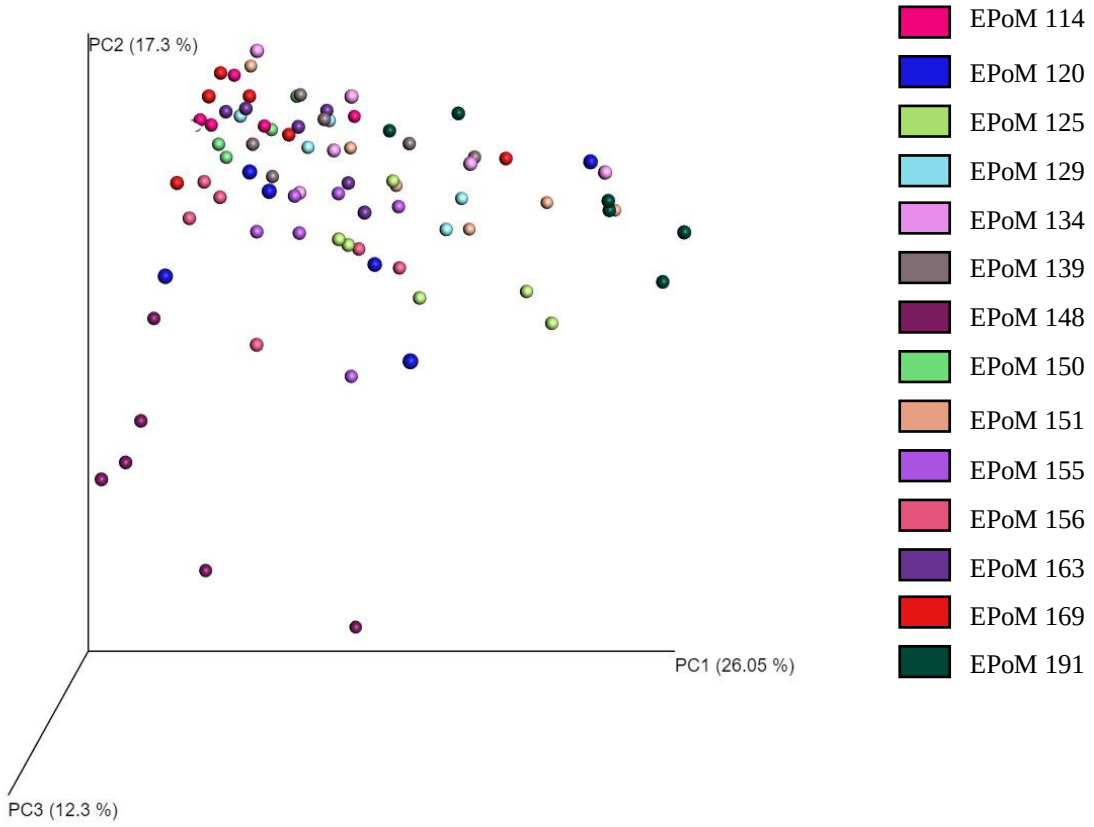


Figure 7.4 - Increased variation within individuals is observed when relative abundance of bacteria within the microbiota is considered*. Weighted β-diversity analysis of faecal microbiota from fourteen study participants; each participant collected a total of six faecal samples, three during each treatment phase. Analysis was performed using the UniFrac metric and visualised as a 3D PCoA plot. β-diversity analysis was performed using QIIME 1.9.1 and plotted using Emperor. The EPoM code in the key refers to different participants.*

Figure 7.3 suggests that the greatest degree of similarity between faecal microbiota can be found amongst the samples that originated from each individual. The faecal microbiota of EPoM148 appears to differ and are less similar to the microbiota of the other 13 participants, due to a lower number of shared bacterial taxa. Furthermore, EPoM129, EPoM151 and EPoM169 appear to have similar microbiota to one another but are less similar to the microbiota of the other 11 participants, due to a lower number of shared bacterial taxa.

Weighted Unifrac β-diversity analysis considers the similarity between the relative abundance of the bacterial taxa, alongside which taxa are shared between microbiota. Figure 7.4 indicates an increased variability amongst the faecal microbiota of individuals due to small fluctuations in the relative abundance of bacterial taxa.

7.4.3 Lack of phytin dispersion in the colon

Treatment A was encapsulated phytin and treatment B was encapsulated MCC (placebo). Faecal samples were collected from all participants and aliquoted for further analysis.

Figure 7.5 – Image of white, powdered clump in the faeces of EPoM participants when consuming encapsulated phytin. *The images above are faecal samples collected from two different participants consuming encapsulated phytin. These images are representative of all faecal samples collected when participants were consuming encapsulated phytin.*

When faecal samples were collected from participants consuming treatment A (i.e. encapsulated phytin), clumps of white powder were found in all faecal samples (Figure 7.5), indicating lack of dispersion in the colon. Furthermore, when these participants switched over to treatment B (i.e. placebo), no clumps of white powder were observed. Further examination indicated that the capsule outer layer had broken down and therefore only the content remained in the faeces.

As phytin did not mix in with the faecal material, any further conclusions could not be made in regard to whether phytin had any effect on water-soluble iron concentrations, faecal metabolites and gut microbial composition. This is reflected in the analysis performed on the faecal materials obtained from the trial participants, as shown in the following sections.

7.4.4 Water-soluble iron concentrations in faecal samples

Water-soluble iron concentrations were quantified from the stool samples of participants, collected at the start (pre), middle (mid) and end of each phase.

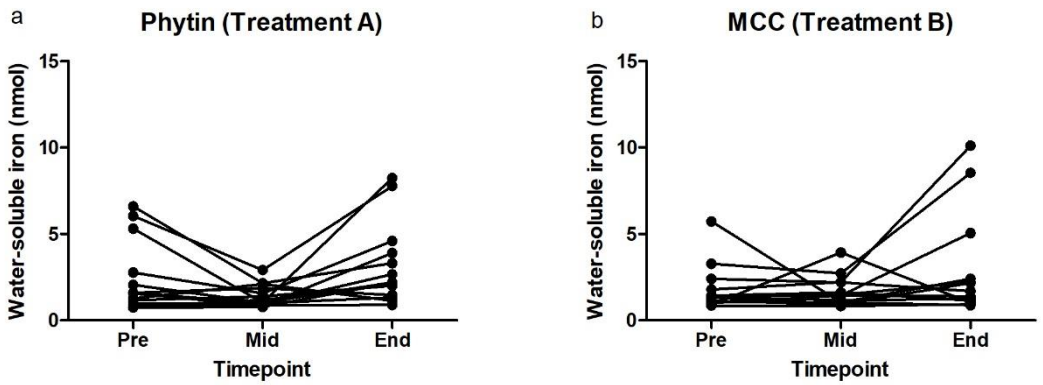


Figure 7.6 – Quantification of faecal water-soluble iron from both treatment arms of EPoM study. *Figure (a) illustrates water-soluble iron concentrations quantified from stool samples of participants when consuming encapsulated phytin, and figure (b) shows water-soluble iron concentrations quantified from stool samples of participants when consuming encapsulated MCC (placebo).*

As expected, due to lack of phytin dispersion, no difference was observed in water-soluble iron concentrations when quantified from the faecal waters of both treatment arms (Figure 7.6).

7.4.5 Short-chain fatty acid quantification in faecal samples

Acetate, butyrate and propionate concentrations were quantified from the stool samples of EPoM participants during both treatments.

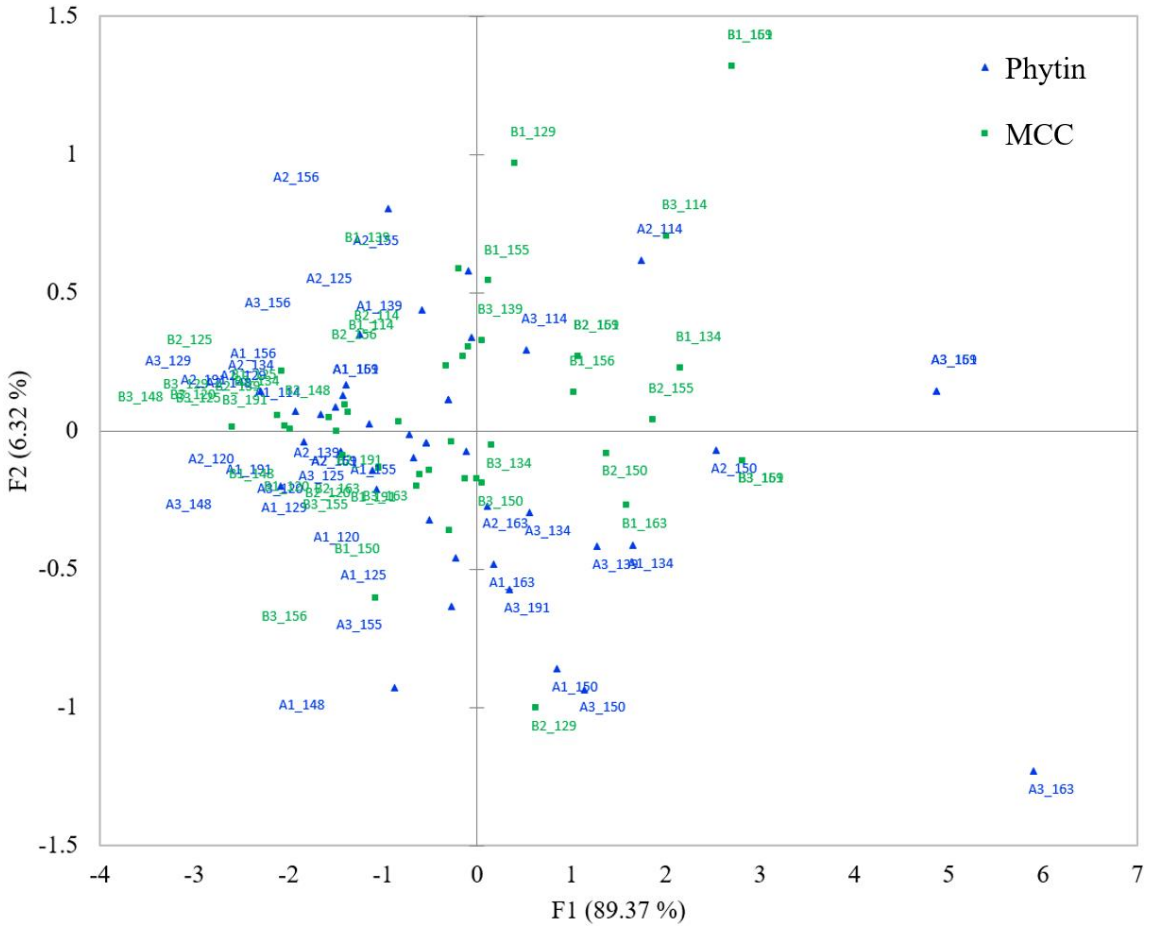


Figure 7.7 – Between-individual variation of the metabolite profile is generally stronger than the within-individual variation over time*. An aliquot of 84 faecal samples were each analysed using ^{1}H NMR spectroscopy. Representation of metabolite profiles from stool samples of participants consuming encapsulated phytin are shown in blue, whilst encapsulated MCC (placebo) is shown in green. The plot was generated using XLSTAT in Excel.*

The metabolite profiles of the 84 faecal waters showed a tendency to cluster based on the participant, indicating a low level of intra-individual variability over time (Figure 7.7). Furthermore, no association between the treatment arms and the faecal metabolite profile were detected due to the unsuccessful mixing of phytin with the faecal material.

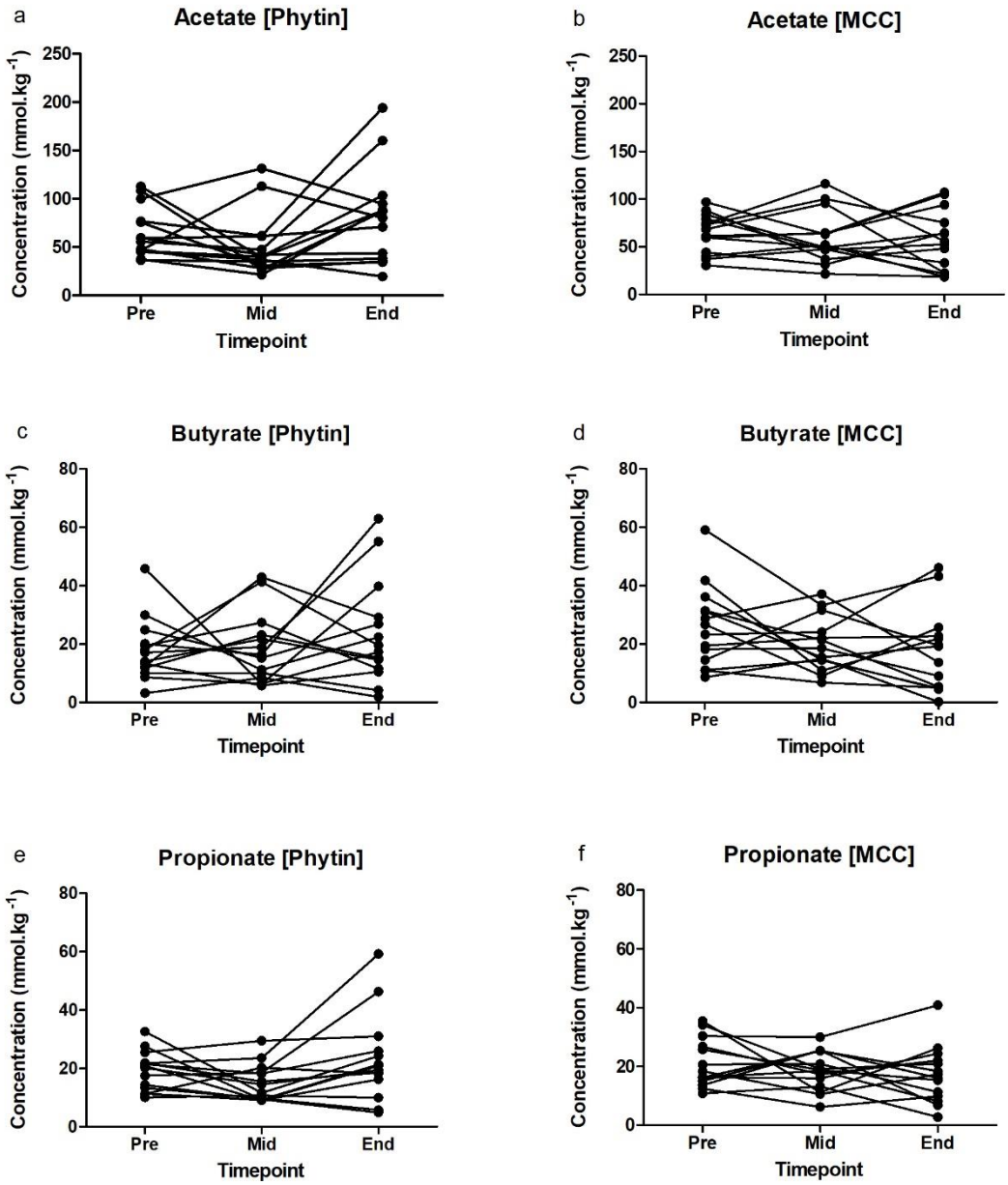


Figure 7.8 – Quantification of short-chain fatty acids from both treatment arms of EPoM study. *Acetate (a and b), butyrate (c and d) and propionate (e and f) levels were quantified from faecal samples of participants when consuming encapsulated phytin and MCC.*

Quantification of short-chain fatty acids confirmed no observable difference in the levels of acetate (Figures 7.8a and 7.8b), butyrate (Figures 7.8c and 7.8d) and propionate (Figures 7.8e and 7.8f), when comparing both treatment arms due to the lack of phytin dispersion in the faeces.

7.4.6 β-diversity analysis of faecal samples

The unweighted Unifrac β-diversity analysis calculates the similarities between samples, based on which bacterial taxa are shared amongst the microbiota. As phytin was not dispersed in the colon, no associations were observed between the consumption of either encapsulated phytin or encapsulated MCC (placebo).

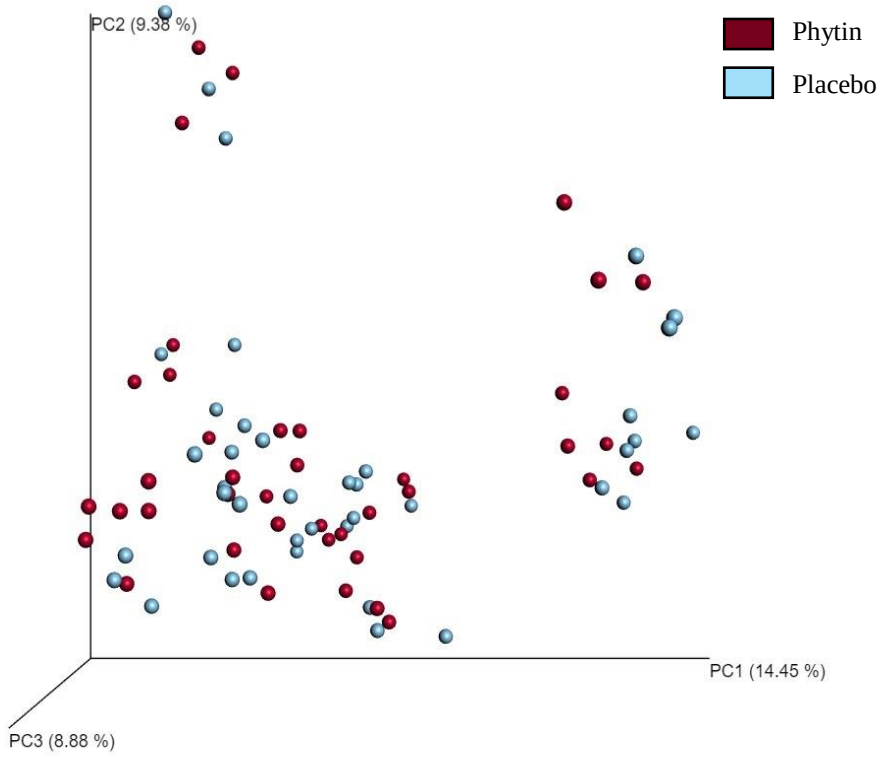


Figure 7.9 - Consumption of either phytin or placebo did not correlate with a common gut microbiota composition. *Unweighted β-diversity analysis of faecal microbiota from fourteen study participants; each participant collected a total of six faecal samples, three during each treatment phase. Analysis was performed using the UniFrac metric and visualised as a 3D PCoA plot. β-diversity analysis was performed using QIIME 1.9.1 and plotted using Emperor.*

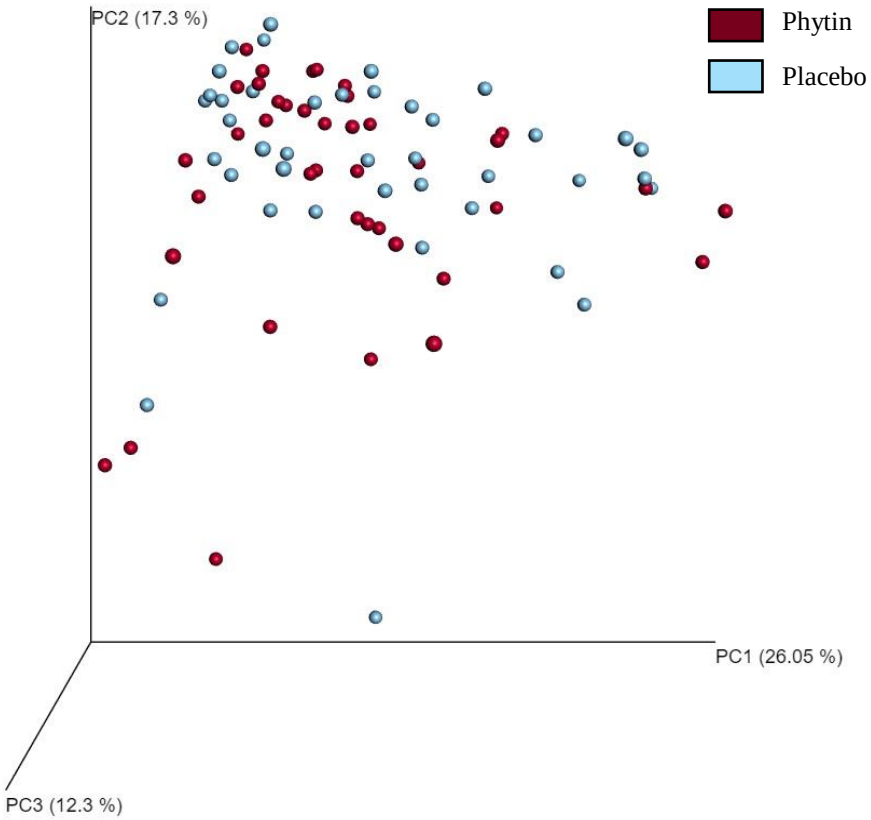


Figure 7.10 - No association between gut microbiota composition and phytin consumption was observed when accounting for bacterial relative abundance*. Weighted β-diversity analysis of faecal microbiota from fourteen study participants; each participant collected a total of six faecal samples, three during each treatment phase. Analysis was performed using the UniFrac metric and visualised as a 3D PCoA plot. β-diversity analysis was performed using QIIME 1.9.1 and plotted using Emperor.*

In line with previous findings, the unsuccessful mixing of phytin with the faecal material lead to no alteration of the faecal microbiota of the participants towards a common composition, as illustrated by the unweighted (Figure 7.9) and weighted (Figure 7.10) Unifrac β-diversity analysis.

α-diversity was performed to see if there was a difference in diversity within the population between the treatment arms (Figure 7.11).

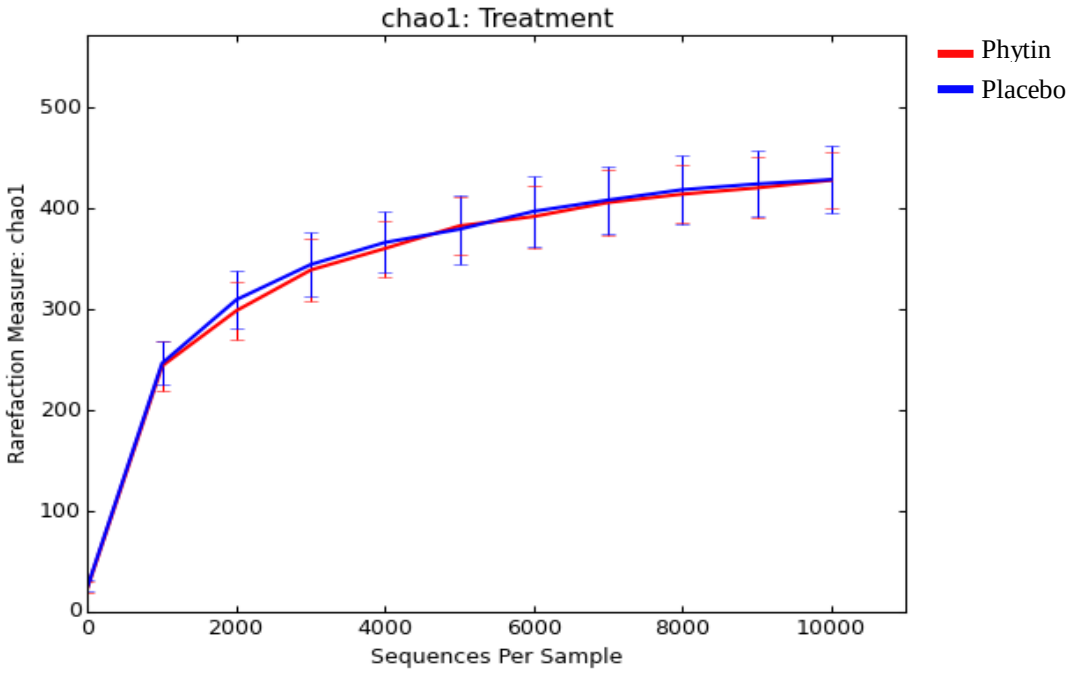


Figure 7.11 - α-diversity analysis indicates no difference in population diversity between treatment arms. *α-diversity rarefaction plot of species richness within the human faecal microbiota samples grouped for each of the fourteen individuals. The y-axis is a measure of diversity within each community, whilst the x-axis represents the number of sequences used per sample in the diversity calculation. Rarefaction plots were generated using QIIME 1.9.1.*

α-diversity analysis of samples derived from both treatment arms further confirms no difference in diversity within the population when consuming either encapsulated phytin or placebo (Figure 7.11).

7.4.7 Compositional analysis of the human gut microbiota

As phytin did not mix in with the faecal material, no changes were observed in the bacterial composition from the faecal samples derived from both treatment arms.

7.5 Discussion

Serum ferritin was measured to test whether the phytin affected iron status through reduction of iron absorption. In order to do this, it is important to rule out inflammation as this will increase ferritin levels and make it difficult to interpret whether phytin has affected body iron status. Without this measurement, the effect of phytin on iron absorption cannot be tested. CRP was therefore measured at the same time as serum ferritin throughout the duration of the study (before and after the start of each phase).

Blood tests revealed stable levels of serum ferritin and CRP in all participants during each phase of the trial.

When the microbiota profiles of all 84 faecal samples collected throughout the study were analysed, samples tended to cluster based on the participant (Figure 7.3), highlighting the unique nature and inherent stability of an individual's gut microbiota. Inter-individual differences in the composition of gut bacterial communities have been observed in other studies [342]. Weighted β-diversity analysis suggested that the microbiota clustered, but that there was variation in the abundances of bacteria within the communities over time (Figure 7.4). This potentially reflects the dynamic nature of the gut microbiota, which is able to adapt to environmental alterations, such as a change of dietary habits, while retaining its core structure.

Aliquoting of the stool samples collected by the EPoM participants revealed clumps of white powder in the samples derived from those consuming encapsulated phytin (Figure 7.5). No clumps were observed in the faeces of those consuming MCC. Further examination revealed that the capsule outer layer had broken down, suggesting the Phloral® coating (properties of which were tested *in vitro*) on the capsules worked successfully and allowed the encapsulated material to bypass and withstand the gastrointestinal conditions before being released in the colon. However, the content of the capsule remained in the faeces, and therefore it is not possible to conclude whether phytin had an effect on the parameters tested. The lack of phytin dispersion in the faecal material is reflected in the lack of effect of phytin on water-soluble iron concentrations (Figure 7.6), faecal metabolite profiles (Figures 7.7-7.8) and gut microbial composition (Figures 7.9-7.11).

The lack of phytin dispersion highlights the importance of the form of phytin to be administered in any future studies. For example, a liquid form of phytic acid could be encapsulated, which would allow for better dispersion in the gut.

7.6 Conclusions

A human dietary intervention study was performed to investigate the effects of encapsulated phytin on the human gut microbiota. It was shown that each participant had a unique bacterial community with small fluctuations in the relative abundances of bacterial members. β-diversity analysis suggested samples appeared to cluster when derived from the same participant over time. Due to the lack of successful dispersion of phytin in the

colon once released from the capsule, phytin appeared to clump in the stool and therefore did not mix with the faecal material, and subsequently was unable to bind iron. Therefore, it is not possible to make any conclusions on whether the iron chelating properties of phytin had any effect on the gut microbial composition.

CHAPTER EIGHT

8 Gut microbiota changes following systemic iron reduction in haemochromatosis patients

8.0 Summary

Hereditary haemochromatosis (HH) is an autosomal recessive genetic disease in which intestinal absorption of iron is increased resulting in accumulation of iron in tissues, primarily the liver, which can sometimes lead to liver damage. Currently, the line of treatment for HH is therapeutic iron reduction by phlebotomy (venesection therapy). However, during the course of repeated phlebotomy treatments, it is likely that intestinal iron absorption is enhanced to compensate for the iron loss during phlebotomy, and therefore may lead to alteration of the gut microbial composition due to changes in colonic luminal iron bioavailability. The aim of the work outlined in this chapter was to see whether iron removal through venesection reduced systemic and faecal iron levels in HH patients and whether iron removal had a subsequent effect on the composition of the gut microbiota. Results showed that a majority of the HH patients enrolled on this study displayed higher levels of iron removal after phlebotomy, as indicated by lower serum ferritin levels, along with changes in gut microbial composition towards a healthier profile.

8.1 Introduction

HH is an autosomal recessive disorder in which the regulation of iron is disrupted, leading to the toxic accumulation of iron in important organs, such as the liver, and the development of cirrhosis, bone and joint disease, diabetes mellitus, and heart disease [111]. HH occurs in approximately 1 in 250 individuals, with approximately 0.4% of people of northern European descent having the genetic mutation and thereby increasing the risk of developing haemochromatosis [112-114]. Most patients with HH are homozygous for the C282Y mutation in the HFE gene (HFE-HH), comprising up to 90% of phenotypically affected persons [111]. Disorders of iron excess, such as HH, have been suggested to effect gut microbial profiles [159]. HH patients have a higher risk of infection due to the increased availability of iron to potentially pathogenic bacterial species in the colon.

Therapeutic venesection has been the standard of care for patients with haemochromatosis for at least 60 y [343]. Venesection typically involves the removal of 500 mL of blood (equivalent to 250 mg of iron) weekly from patients until normal iron levels are achieved. Iron depletion is associated with an improvement in liver function tests, insulin resistance, liver fibrosis, enhanced quality of life, increased energy levels and a reduction in mortality and risk of several malignancies [344-348]. The mechanisms underlying these benefits are unknown. Paradoxically, venesection promotes iron absorption from the gut, and reduced

faecal iron levels have been reported during treatment. As iron is critical to the growth and proliferation of numerous gut microbes, and excess colonic iron has been implicated in inflammation of gut epithelium and carcinogenesis, changes in faecal iron levels during venesection could favourably alter the gut microbiota.

Oral iron supplementation has been shown to adversely affect the composition and function of the human gut microbiota [157], while differences in gut bacteria have been demonstrated when comparing iron deficient with iron replete individuals [349].

8.2 Objectives

In collaboration with Dr John Ryan at Oxford University Hospitals NHS Foundation Trust (OUHT)/Beaumont Hospital, we aimed to determine the relationship between gut bacteria and faecal iron levels before and during phlebotomy. Stool samples were collected from 20 patients before and after initiating venesection (characteristics outlined in Table 8.1), with paired samples obtained from 11 of these patients during follow up. Faecal iron levels were measured, and their relationship with the gut microbiota was assessed by faecal metataxanomic and metabolomic analyses.

Table 8.1 – Patient characteristics

Baseline Characteristics	**Venesection Cohort (n=20)**
Female (%)	8 (40%)
Age (y)	56 (11)
Weight (kg)	80.3 (16.9)
BMI (kg/m^2)	27.2 (4.5)
Serum ferritin (µg/L) *	717 (4500)
Transferrin Saturation (%)	71.9 (27.6)
Serum iron (µg/dL)	31 (9)
HFE Genotype C282Y/C282Y C282Y/H63D Carrier/Negative	 10 (50%) 4 (20%) 6 (30%)
Haemoglobin (g/L)	144 (14)
ALT (IU/mL)	40 (19)
AST (IU/mL)	38 (19)
CRP (mg/L)	3.2 (5.6)
HbA1c (mmol/mol)	32 (6)
^Mean+/- standard deviation in brackets unless indicated otherwise. *median (range). Alanine aminotransferase (ALT); aspartate aminotransferase (AST); C-reactive protein (CRP); haemoglobin A1c (HbA1c)	

8.3 Materials and Methods

8.3.1 Study design and cohort information

This study has been ethically approved by Yorkshire & The Humber - Sheffield Research Ethics Committee, reference number 16/YH/0247. This study was funded by Oxford comprehensive Biomedical Research Centre (OxBRC).

The Gastroenterological cohort was assembled from patients attending Oxford University Hospitals service, including those being referred for an endoscopic, radiological, or surgical procedure, including percutaneous biopsy or aspiration, as part of the normal care. Patients were recruited from the OUHT including the John Radcliffe hospital, the Children's Hospital, the Churchill Hospital and the Horton Hospital. Control and non-GI patient relatives were also invited to donate blood samples or a mucosal swab.

Patient recruitment took place from various locations and backgrounds. These included:

- Patients under the care of the gastroenterology unit;
- Patient relatives to allow for investigations of genetic influences;
- Patients undergoing tonsillectomy, adenotonsillectomy, laparoscopy, laparotomy, heart surgery, thymectomy, skin biopsy or appendectomy;
- Healthy blood donors to be used as controls.

Patients were presented with verbal and written information about this project along with a consent form. Family members of patients who were recruited as a 'healthy control' received information leaflets and consent forms as per patients (Adult, Child and Young Person information leaflets and consent forms). Healthy controls who were colleagues or students were recruited by advert and provided with the university guidelines.

Intended study duration is indefinite and planned to last for the duration of the disease or until patients withdraw consent. Continuing informed consent is necessary for participation and can be withdrawn by patients at any time, without giving a reason and without affecting the quality of future medical care. Samples and data collected and shared with other researchers up to the point of withdrawal of consent may still be used.

The inclusion criteria for this study is outlined below:

- patients attending the Gastroenterology service at Oxford University Hospitals, and referred for endoscopy, or radiology, percutaneous liver biopsy, genetic disorders and, or surgery;

- patients undergoing surgery such as tonsillectomy, adenectomy, or heart surgery/thymectomy or appendectomy, or skin biopsy;
- healthy controls and, in exceptional circumstances patient relatives, were invited to participate and donate a blood sample or oral mucosal swab and/or urine and stool samples;
- patients under the age of 16 y were offered participation using specially amended documentation, and consent was obtained from parents or guardians for those who were younger than 10 y old;
- for patients aged older than 10, and younger than 16 y old, their assent was sought using age-appropriate materials, in addition to consent from parents or guardians.

8.3.2 Microbial DNA extraction

Faecal samples collected from patients were frozen and sent to QIB for analysis. DNA was extracted from all samples using a commercially available kit (FastDNA spin kit for soil; MP Biomedicals, USA, Cat No. 6560200). Samples were thawed on ice, homogenised, and approximately 200 mg of each were used to extract DNA following the manufacturer's instruction, with an additional bead beating step using FastPrep (MP Biomedicals, USA), as detailed previously described (chapter 2, section 2.6.1).

8.3.3 16S rRNA gene amplification and sequencing

The impact of therapeutic venesection on the composition of the human gut microbiota was investigated using high throughput 16S rRNA gene (V4 region) sequencing using the Illumina Miseq platform, followed by data analysis using the Quantitative Insights into Microbial Ecology (QIIME, V1.9) pipeline. Details for this method can be found in chapter 2, section 2.6.3.

8.3.4 Short chain fatty acid quantification in stool samples

Faecal water was prepared to quantify short chain fatty acids in stool. Briefly, 0.2 g of faecal sample was mixed with 12x volume of NMR buffer (0.26 g NaH_2PO_4 and 1.41 g K_2HPO_4 made up in 100 mL D_2O, containing 0.1% NaN_3 (100 mg), and 1 mM sodium 3-(Trimethylsilyl)-propionate-d4, (TSP) (17 mg) as a chemical shift reference). The samples were then centrifuged at 3220 xg for 15 mins at 4^{o}C and the ^{1}H NMR spectra were recorded as detailed in chapter 2, section 2.7.

8.3.5 Measurement of total iron concentrations in stool samples

FAAS was used to determine the concentration of total iron in faecal samples. Faecal samples were weighed and then dried at 110ºC in an oven and then further processed for total iron analysis as outlined in chapter 2, section 2.3.1.

8.3.6 Measurement of water-soluble iron iron in stool samples

A 0.2 g faecal sample was homogenised with 0.2 g of Milli-Q water, mixed on a rotator stirrer for 30 mins at room temperature and centrifuged at 3,000 xg for 15 mins at 4ºC. Supernatants were then analysed using the Ferrozine assay, as outlined in chapter 2, section 2.3.1.

8.3.7 Bacterial species profiling using qPCR

Profiling of gut bacterial species was performed at the laboratories at the John Radcliffe Hospital, Oxford using the Metabolic Disorders qPCR array for microbial DNA testing (Qiagen) and Microbial DNA qPCR Assay for Hs.GAPDH. Data was analysed using the 2-ΔΔCt method with (glyceraldehyde 3-phosphate dehydrogenase) GAPDH as an internal control gene and using the mean of the control duplicates as control.

8.3.8 Serum ferritin and liver enzyme quantification

Routine haematological and biochemical tests were performed in the clinical laboratories of the John Radcliffe Hospital, Oxford.

8.4 Results

8.4.1 Effects of venesection on serum ferritin, faecal free iron and liver enzymes

Free iron levels in stool samples were significantly higher in HH patients at baseline when compared with those of healthy controls (Figure 8.1).

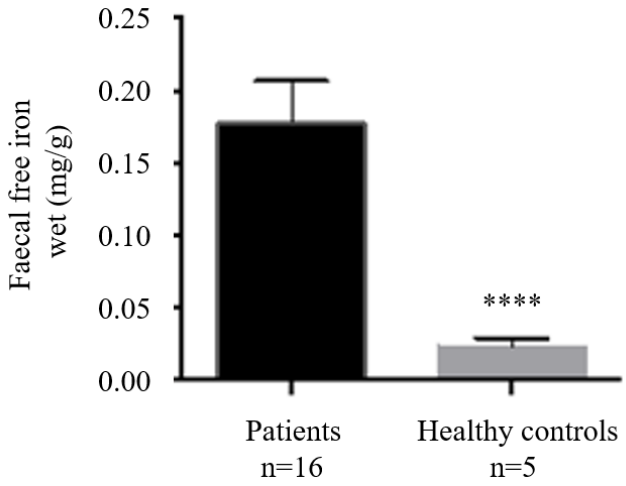


Figure 8.1 – Faecal free iron levels in healthy controls and HH patients. *Patients with iron overload had significantly higher faecal free iron levels compared to healthy controls. Data available on 16 HH patients. ****p<0.0001, data represented as mean ±SEM.*

As expected, treatment with venesection was associated with a significant reduction in serum ferritin levels, and an improvement in the levels of the liver enzyme, alanine aminotransferase (ALT) (Figure 8.2a and 8.2b).

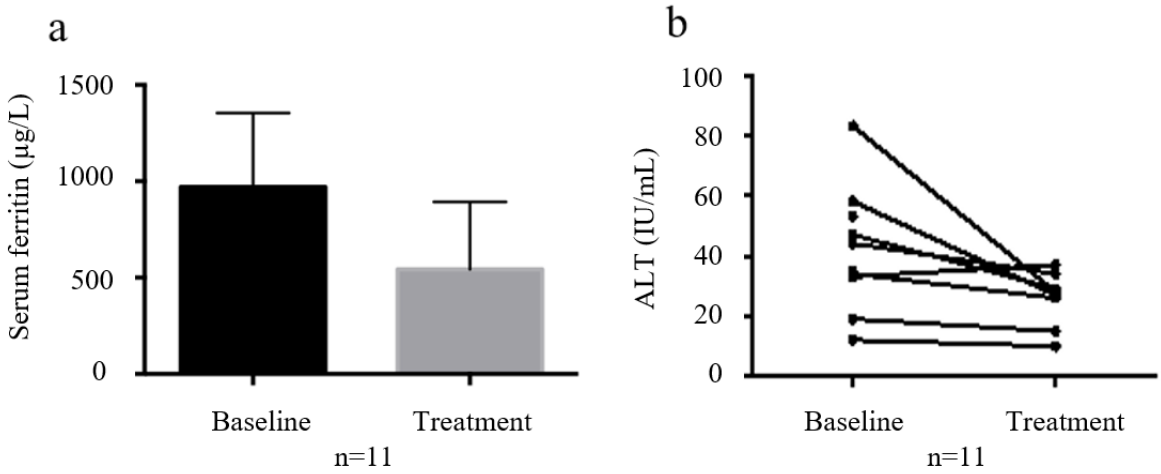


Figure 8.2 – Serum ferritin and liver ALT of HH patients before and after venesection. *Significant reductions in (a) serum ferritin (a; p<0.0001) and (b) liver ALT (b; p<0.05) were observed in paired samples after treatment with venesection.*

Faecal free iron levels were measured in 11 HH patients (Figure 8.3).

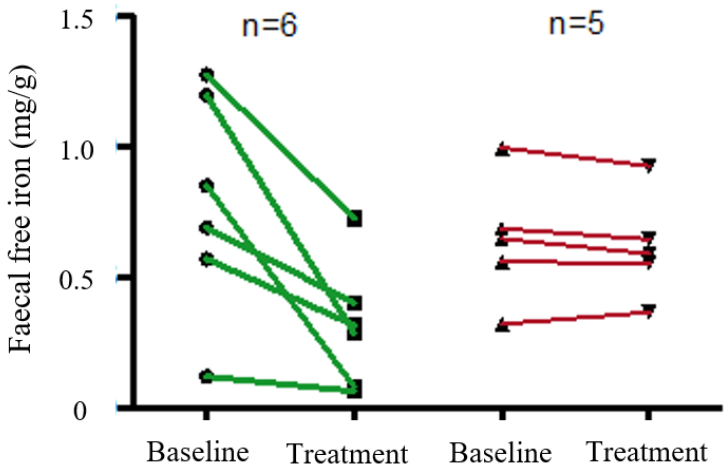


Figure 8.3 – Faecal free iron levels in HH patients before and after venesection. *Faecal free iron levels were measured in 11 HH patients. 6 patients (green line) presented with significant decreases in faecal free iron levels ($p<0.001$), whilst faecal free iron levels remained unchanged in 5 patients (red line).*

Overall, amongst the 11 HH patients, a variability in response to venesection is observed, with a majority displaying a reduction in iron and a minority showing no change (Figure 8.3). Upon venesection, 6 HH patients showed significant reductions in faecal free iron levels whilst faecal free iron levels remained unchanged in 5 HH patients. On average, baseline faecal free iron levels of those patients whose faecal free iron levels reduced upon venesection, was 0.78 ±0.4 mg/g. This was reduced to 0.31 ±0.2 mg/g after venesection. The 5 HH patients in which no reduction in faecal free iron was observed had faecal free iron levels of 0.64 ±0.2 mg/g and 0.62 ±0.2 mg/g at baseline and after venesection, respectively.

The patients in which iron was reduced after venesection, had significantly more iron removed by phlebotomy compared to those whose iron levels were unchanged [2.7 g (+/-0.8) vs. 1.1 g (+/-0.7), respectively], and experienced significant reductions in ALT [43 (+/-11) IU/mL to 28 (+/-4) IU/mL, vs. 39 (+/-28) IU/mL to 22 (+/-9) IU/mL respectively] and HbA1c levels [33 (+/-4) mmol/mol to 27 (+/-4) mmol/mol, vs. 33 (+/-4) mmol/mol to 34 (+/-1) mmol/mol, respectively] (Table 8.2). Patients who had significantly more iron removed by phlebotomy presented a positive correlation with faecal free iron levels, which was also observed to decrease (Figure 8.3).

Table 8.2 – Alterations in various observed parameters for 11 HH patients

Parameter[^]	**Patients in which iron reduced post treatment (n=6)**	**Patients in which iron remained unchanged post treatment (n=5)**	**p value**
Age (y)	50 (6)	54 (13)	0.35
Female (%)	2 (40)	4 (80)	0.1
Weight (kg)	86 (15)	67 (14)	0.15
HFE C282Y/C282Y (%)	6 (100)	2 (40)	0.09
Baseline ferritin (μg/L) [*]	747 (4367)	441 (484)	0.24
Treatment ferritin (μg/L) [*]	156 (3939)	116 (460)	0.75
Baseline faecal free iron (mg/g)	0.78 (0.4)	0.64 (0.2)	0.42
Treatment faecal free iron (mg/g)	0.31 (0.2)	0.62 (0.2)	0.08
Total venesections (n)	10 (3)	6 (3)	**0.048**
Iron removed (g)	2.7 (0.8)	1.1 (0.7)	**0.03**
Baseline ALT (IU/mL)	43 (11)	39 (28)	0.69
Treatment ALT (IU/mL)	28 (4) [£]	22 (9)	[£]**0.03**
Baseline HbA1c (mmol/mol)	33 (4)	33 (4)	0.97
Treatment HbA1c (mmol/mol)	27 (4) [£]	34 (1)	[£]**0.03**

^Mean+/- standard deviation in brackets unless otherwise indicated. [£] baseline vs. treatment

[*]median (range). Alanine aminotransferase (ALT); international units per litre (IU); haemoglobin A1c (HbA1c). Significant findings highlighted in bold

8.4.2 Effects of venesection on the human gut microbiota

The effect of treatment on the gut microbiota was compared between the patients who showed lower levels of faecal free iron and those whose levels did not change after treatment in order to assess the impact of changes in faecal free iron on gut bacteria. While no difference in phylogenetic diversity was evident between baseline and treatment in both sets of patients, significant changes in bacterial genera and species were noted, but only in those who presented with lower faecal free iron levels upon venesection. Specifically, these patients experienced significant increases in the bacterial genus *Faecalibacterium* and decreases in the genera *Finegoldia*, *Adlercreutzia* and *Bacteroides*. Furthermore,

qPCR revealed that the levels of the bacterial species *Faecalibacterium prausnitzii*, *Dorea formicigenerans* and *Collinsella aerofaciens* (Figures 8.4a, c and e) were significantly increased in these patients but remained unchanged in patients with unaffected faecal free iron levels post-treatment (Figures 8.4b, d and f).

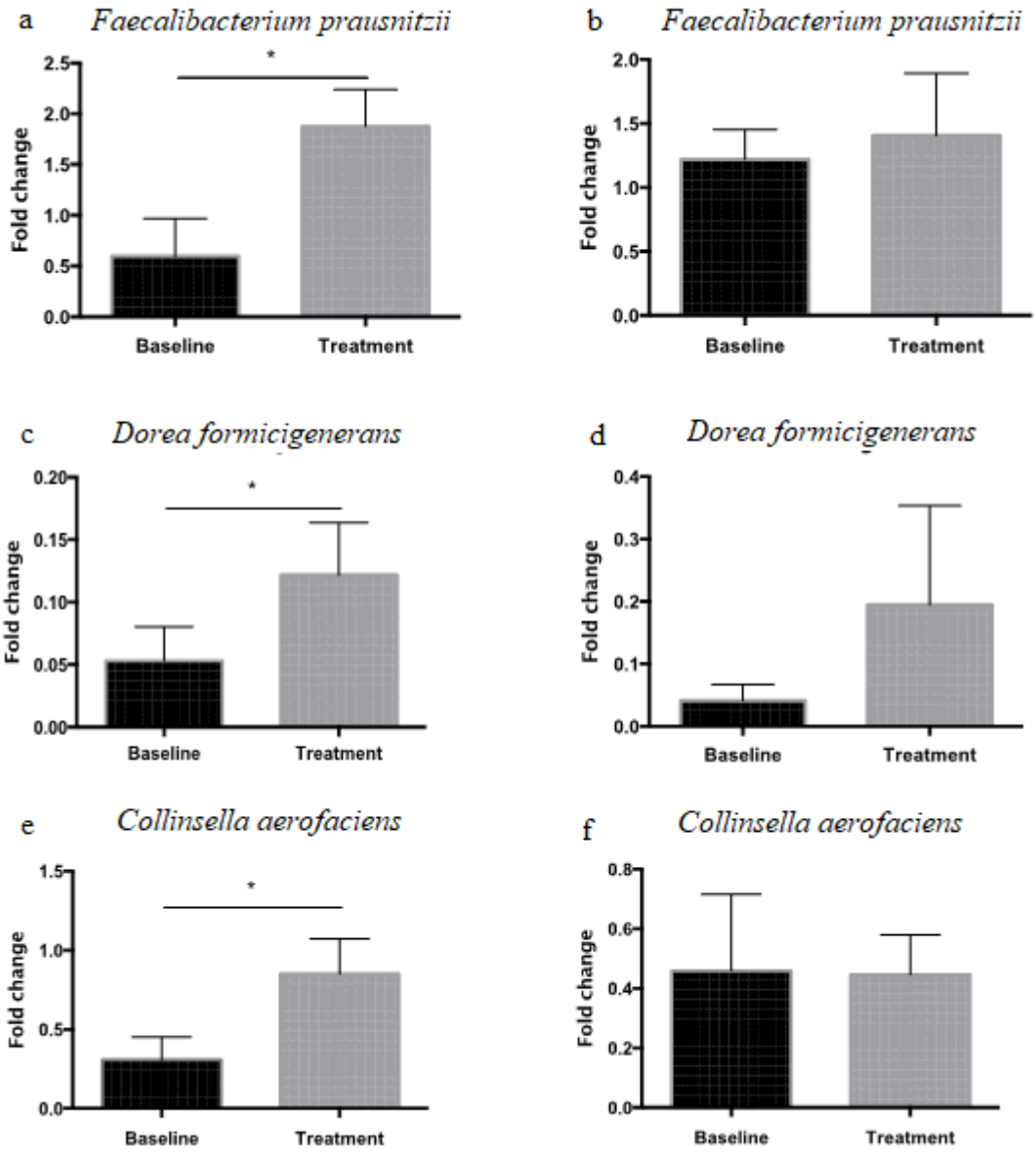


Figure 8.4 – Metagenomic profile of HH patients. *Alterations in different bacterial species for patients presenting with lower faecal free iron levels post-venesection (n=6; a, c and e) and those whose faecal free iron levels remained unaffected post-venesection (n=5; b, d and f).*

Metabolomic profiling of the patients was also carried out in tandem to metataxonomic analysis. Although significant changes were not observed between patients who had higher levels of iron removed through phlebotomy compared to those with lower levels of iron

removal, slightly raised concentrations of acetate and butyrate were noted in the former group (Figure 8.5a and b, respectively).

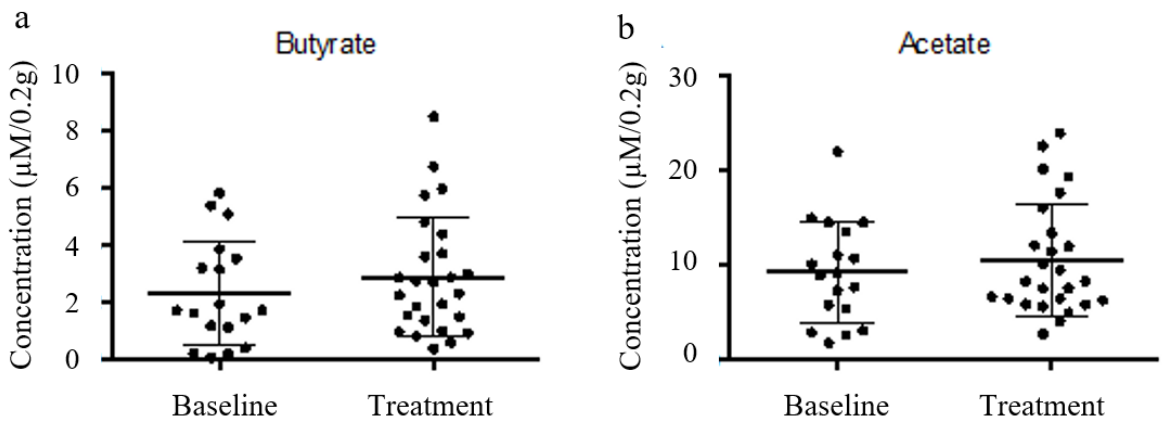


Figure 8.5 – Metabolite levels from patients with higher levels of iron removal post-phlebotomy. *Figure a shows levels of (a) butyrate in patients who presented with higher levels of iron removal through phlebotomy, whilst figure (b) displays levels of acetate.*

Furthermore, a visible shift in the metabolome of those patients presenting with higher iron removal post-treatment was observed. For these patients, if individual patient data is investigated, a greater separation in the metabolome is illustrated, where a shift is observed after treatment compared to baseline (Figure 8.6a). A less distinct shift in the metabolomic profile is observed for patients with lower levels of iron removal post-treatment when comparing baseline profiles to those with treatment (Figure 8.6b).

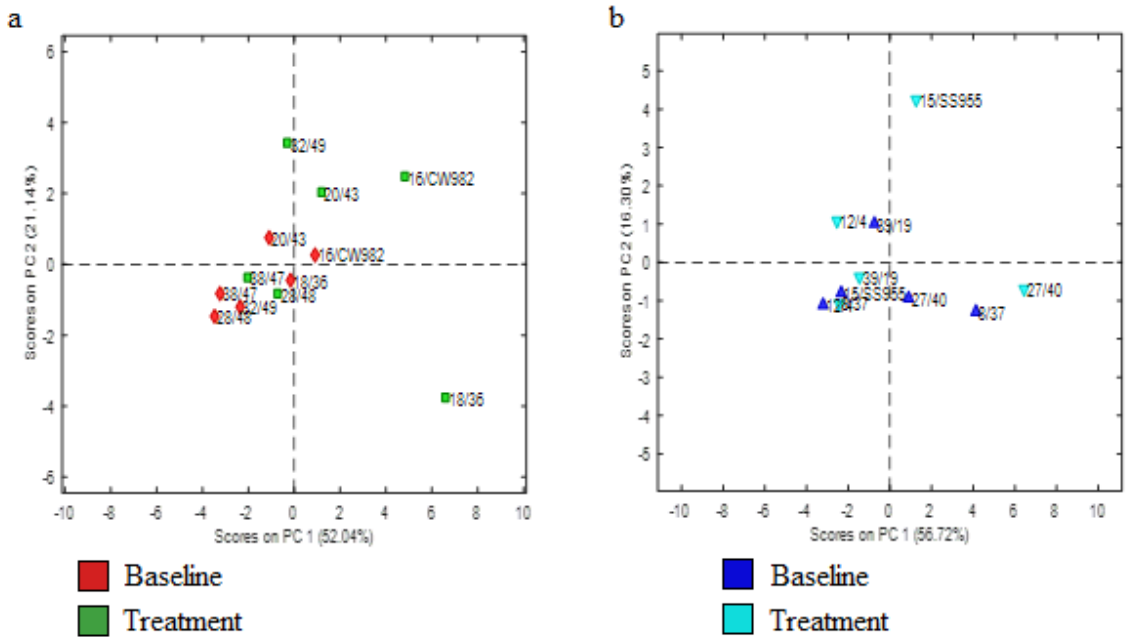


Figure 8.6 – Metabolomic profile of HH patients. *Figure (a) represents the metabolomic profile of HH patients (n=6) who presented with higher levels of iron removal post-phlebotomy, whilst figure (b) represents the metabolomic profile of HH patients (n=5) who presented with lower levels of iron removal post-phlebotomy.*

8.5 Discussion

Haemochromatosis is a disease which is caused by excessive iron absorption, which leads to iron overload. The majority of hereditary HH cases are associated with mutations in the *HFE* gene. Generally, this becomes clinically apparent during adulthood and can cause damage to many organs, including the skin, pancreas, liver and heart. HH patients who displayed higher levels of iron removal post-venesection had more iron removed at the time of analysis- this indicates that they were more overloaded at baseline and therefore tolerated phlebotomy better, and all were C282Y/C282Y HFE homozygotes, in whom the benefit of phlebotomy is most apparent.

Strikingly, in the study by Lee *et al.*, (2017) examining the effect of oral and intravenous iron supplementation on patients with inflammatory bowel disease, oral iron was associated with decreased abundances of the bacterial species *Faecalibacterium prausnitzii*, *Dorea formicigenerans* and *Collinsella aerofaciens.* Similarly, in the present study the reduction in colonic iron was associated with an increase in these species. Lee *et al.*, (2017) demonstrate a decrease in the relative abundance of *Faecalibacterium prausnitzii* in response to iron addition through oral administration of iron sulphate [349]. Our study supports this finding and illustrates an inverse correlation between iron and

Faecalibacterium prausnitzii whereby its relative abundance was observed to increase in patients who presented with higher levels of iron removal post-phlebotomy. *Faecalibacterium prausnitzii* is one of the main inhabitants of the human gut microbiota and has been reported to be one of the major butyrate producing species in the human colon. Literature so far shows that this species of bacteria behaves as a bioindicator of human health as in the context of disease, such as inflammatory bowel disease, this species decreases [350, 351].

An interesting observation was that of the genus *Collinsella*. We saw a significant increase in the relative abundance of *Collinsella* in patients who had higher levels of iron removed through phlebotomy. *Collinsella* is the dominant genus of the group *Coriobacteriales* and is frequently detected in the human colonic microbiota [301-303]. Moreover, *Collinsella* have been illustrated to ferment a vast range of different carbohydrates, such as glycogen, resulting in the production of metabolites such as butyrate and acetate. This may be reflected in the metabolite analysis performed in this study where concentrations of both these SCFAs, although not statistically significant, are observed to increase in patients who had higher levels of iron removed through phlebotomy. From these data, we can speculate that the increase observed in acetate and butyrate could result from the rise in relative abundance of *Collinsella*.

8.6 Conclusions

Results from this study highlight the importance of iron not only systemically but also in the gut microbiota. Overall, a general shift towards a healthier systemic and metabolic profile was observed within HH patients who had more iron removed through phlebotomy. This was accompanied with an increase in beneficial bacterial species in the large intestine as well as subtle changes in metabolomic profiles, suggesting the removal of iron led to an increase in potentially beneficial bacteria. Tightly regulating the availability of iron in individuals presenting with HH may represent a novel therapy and merits further investigation.

CHAPTER NINE

9 General discussion

9.1 Summary of findings

The overall aim of the research presented in this thesis was to investigate the effects of iron on the function and composition of the human gut microbiota, using *in vitro* colonic batch fermentation models and a human intervention study. It has already been reported that the presence or absence of iron is able to alter the composition of the human gut microbiota. The work presented in this thesis aimed to utilise both chemical and dietary iron chelators to the impact of iron on the composition of the bacterial community present in the human colon either at the level of individual bacteria or in a more complex gut microbial community level. These results formed the basis of a human study investigating what effects an encapsulated dietary iron chelator (phytin) may produce *in vivo*, with a focus on changes to the composition of the gut microbiota.

9.1.1 Bacterial growth under iron-supplemented conditions

The growth of pure bacterial cultures has been reported to be affected by different levels of iron. The data presented in chapter 3 indicated that when supplemented with iron, in the form of $FeSO_4$, the growth of pure cultures of *E. coli* and *S.* Typhimurium significantly increased in comparison to the non-supplemented control. These findings were further investigated through the addition of iron to the same bacteria grown under iron-chelated conditions. Results indicated that the growth of *E. coli* and *S.* Typhimurium were significantly impaired when cultured in iron-chelated media, but growth resumed once an external source of iron was added to the culture.

9.1.2 Bacterial growth under iron-chelated conditions

The removal of iron via iron chelators from the media in which bacteria are cultured resulted in the reduction of bacterial growth, including those that have the potential to display pathogenic phenotypes (chapter 4). These bacterial species include *E. coli, S.* Typhimurium, *C. perfringens* and *B.thetaiotaomicron.* Some chelators inhibited the growth of the bacteria to a greater extent, however, in general, the pattern remained the same. Beneficial species such as *B. longum* and *L. rhamnosus* were unaffected by all the iron chelators tested (BPDS, 22D, Lf, TA and PA), except for Manucol LD, a form of sodium alginate. This was speculated to be down to the toxic effects of sodium, as has been previously reported [271]. The removal of iron has been reported to decrease the growth of some of these bacteria in previous studies [248] and our results show that for all the bacterial species examined, iron concentrations were observed to decrease in the presence

of the iron chelator tested. This indicates that the reduction in growth is potentially related to the lower levels of iron.

9.1.3 Effect of iron on the human gut microbiota composition examined in vitro

The latter part of chapter 3 determined the effect of iron supplementation on a mixed community of bacteria, derived from the faecal microbiota of healthy volunteers through *in vitro* colonic fermentations. Previous studies have examined the effect of iron fortification on the human gut microbiota, but varying results were observed [148, 229, 230], as also observed in our *in vitro* studies. The viable counts of common bacterial families, *Enterobacteriaceae* and *Bifidobacteriaceae*, differed between individual donors, suggesting that the effect of iron on gut microbial composition is highly variable.

The effects of iron chelation on the composition of the human gut microbiota, however, are slightly more pronounced. *In vitro* colonic fermentations (chapter 5) showed a decrease in viable counts of three bacterial groups, when the microbiota belonging to three healthy donors were cultured in the presence of BPDS a chemical chelator. These three groups were total anaerobes, lactobacilli and *Enterobacteriaceae*. Compositional analysis further illustrated a decrease in the relative abundance of *Escherichia* in two out of three donors, presumably reflecting the reduction of viable counts of *Enterobacteriaceae*, to which *Escherichia* belongs. Similarly, a reduction in the counts of the beneficial bifidobacteria in the faecal microbiota of all the donors was observed, and this was further confirmed with the decrease of *Bifidobacterium* relative abundance estimated via 16S-metataxonomic analysis.

The culturing of human faecal microbiota in the presence of phytin also led to similar results (chapter 5). The biggest difference observed between phytin and BPDS was the increase in relative abundance of *Bifidobacterium* and viable counts of bifidobacteria in the presence of phytin. The positive impact of phytin on the beneficial genus bifidobacteria led to the design of a human trial, outlined in chapter 7, in which encapsulated phytin was consumed by participants.

9.1.4 Influence of iron chelation on SCFA production

Metabolite analysis via ^{1}H NMR spectroscopy indicated a variation in the concentrations of SCFAs produced by the cultured human faecal microbiota (chapter 5). Although correlations of metabolite levels with the relative abundance of bacterial taxa did not

provide a causal relationship, it may still provide some indications as to which taxa are responsible for the observed differences. When cultured with BPDS, a decrease was observed for three metabolites, acetate, butyrate and propionate, which are usually associated with positive effects on human health, [142, 213, 217, 290, 352, 353], suggesting low iron levels could potentially have a negative effect on the host. Decreases in relative abundances of *Bifidobacterium*, *Ruminococcus* and *Bacteroides* could potentially be linked with the decreases in acetate, butyrate and propionate concentrations, respectively, since members of these genera have been associated with the production of these SCFAs.

Propionate and formate levels were increased in the presence of phytin. The increase in formate concentrations could be linked to the rise seen in the relative abundance for *Collinsella*, a genus well-recognised to ferment glycogen to produce formate [285].

9.1.5 Effects of iron on the human gut microbiota in vivo

Dynamic dissolution assays were used for investigations into the optimal conditions for a colonic delivery system. Capsules were sprayed with a coating suspension with a dual-action mechanism whereby the release of the content within the capsule was triggered by the colonic luminal pH and colonic starch-fermenting properties of the gut microbiota. To find the optimal coating thickness of the suspension, static and dynamic dissolution assays were implemented to record time of capsule content release under physiological conditions. Capsules with the optimal thickness were then prepared for use in a human dietary intervention trial investigating the effect of phytin on the gut microbiota.

Chapter 7 presented a double-blinded, randomised, 2-phase crossover, human dietary intervention study, in which the effects of encapsulated phytin on the gut microbiota of 14 participants was investigated.

Serum ferritin was measured as a marker of capsule release and the results from the human trial indicated stable serum ferritin levels in all participants throughout the entire duration of the trial. This suggests that phytin was not released before it reached the colon and that the Phloral® coating kept the capsule intact and therefore withstood the gastrointestinal conditions.

Results from the human trial also indicated stable C-reactive protein levels in all participants, and therefore confirming the absence of systemic inflammation.

When aliquoting faecal samples for analysis, white, powdered clumps were present in the faeces of those consuming encapsulated phytin. Further analysis revealed that the outer shell of the capsule had dissolved, leaving only the content in the faeces. This observation indicated that phytin was not dispersed in the colonic lumen and was therefore unable to implement its iron-chelating properties in the colonic environment. Therefore, it was not possible to make any conclusions regarding the iron chelating properties of phytin. Due to this, as expected no treatment effect was observed and results were comparable to the control, when examining water-soluble iron concentrations, faecal metabolite profiles and bacterial composition.

Although any conclusions on the iron chelating abilities of phytin cannot be made due to unsuccessful dispersion, community analysis of the faecal microbiota showed that samples tended to cluster based on the participant, and that there was variation in the abundances of bacteria within the communities over time.

It was also confirmed that acetate levels were present in much higher concentrations in comparison to propionate and butyrate regardless of study arm, suggesting the presence of numerous acetate-producing bacterial species. Higher levels of acetate in comparison to other SCFAs have previously been reported in other studies, where one reports that acetate, propionate and butyrate are produced in approximate molar ratios of 60:20:20 [354, 355].

9.2 Limitations of the research

The bacterial species investigated in chapters 3 and 4 for the impact of changes in iron concentration on growth could have been further investigated by the measurement of potential siderophore activity. A well-defined protocol has already been established, known as the chrome azurol sulphonate assay. This would have helped to understand the underlying mechanism of growth behaviour in different bacterial species when cultured under various concentrations of iron.

A limitation of the *in vitro* studies using human faecal microbiota to assess the effect of iron chelation on microbial composition was the small number of donors used. This made it difficult to draw any conclusions due to the high level of inter-individual heterogeneity in the faecal microbiota.

One limitation in the iron chelation experiments carried out in chapter 5 was the lack of isolation of bacterial species enumerated from the *in vitro* batch fermentation model. Although the *in vitro* batch fermentation models provided good representation of bacterial

groups affected by the removal of iron, through viable counts data, isolation of bacterial species would have given information on the species that were most affected. Further analysis could then have been carried on bacterial species that were more sensitive to iron bioavailability and those that were more robust. Subsequently, as mentioned in the paragraph above, these isolates could have been further targeted for siderophore activity to confirm that the effects observed were linked to iron bioavailability.

The sequencing data from the human trial did not identify the presence of bacteria belonging to the *Enterobacteriaceae* family in proportions higher than 4.8%. This is surprising since *Enterobacteriaceae* is a large family of Gram-negative bacteria, with many of its members being present in the core gut microbiota in humans. Some of these members include *Salmonella, Escherichia, Klebsiella* and *Shigella.* Microbial profiling data detected levels of *Enterobacteriaceae* in all donors tested and therefore it is surprising that only low levels of identification of this family was made from the human faecal microbiota of the human trial participants.

As mentioned earlier, the lack of dispersion of phytin in the faecal material meant it was not possible to make any conclusions. However, the next section suggests ways in which this trial could have been carried out successfully in relation to phytin dispersion.

Regardless of the lack of phytin dispersion in the human trial, one limitation of this trial was the diet consumed by participants. It was originally thought to impose a diet restriction on participants whereby phytin-rich foods would be restricted, however, it was later decided that this restriction could negatively affect the trial and therefore participants were asked to maintain their habitual diet. Not taking habitual diet into consideration when recruiting participants and the lack of dietary control during the study were weaknesses of the study.

9.3 Future research

The data presented in this thesis identified many different groups of bacteria that were altered in their growth patterns when cultured in the presence of an iron chelator in *in vitro* batch fermentation studies. However, it is unclear which bacterial species are mainly affected by the change in iron bioavailability. For the purpose of future research, specific species and strains of bacteria could be isolated from these *in vitro* colonic batch fermentation models, and using molecular methods, investigated further for the presence of iron regulatory genes. The identification of these genes would help elucidate further the

impact of iron bioavailability on the cellular mechanisms of various bacterial species. Furthermore, this could be followed by gene knockout experiments to identify which gene(s) may be responsible for the change in growth of the bacterial isolates.

As mentioned in the previous section, siderophore activity of bacterial species would add invaluable information on the interrelationship between bacterial activity and iron bioavailability. The identification of genes involving siderophore biosynthesis is predictive of high or low virulence activity and therefore targeting these genes through knockout experiments could help observe if bacterial virulence is attenuated when these specific genes are removed. Once identified, competition assays could further categorise which bacterial isolates have stronger iron-scavenging properties when cultured in the presence of host siderophore proteins.

For pure culture growth assays using iron chelators, a range of phylogenetically diverse human gut isolates could be used, which would be associated with the colonic lumen or mucosa. This could potentially help to elucidate whether the ability to scavenge iron by bacteria is widespread amongst the human gut microbiota or restricted to a single phylogenetic group, as presently, many of the colonic bacteria have been shown to utilise iron in one way or the other for growth purposes.

A three-stage continuous culture system could be used to culture human faecal microbiota. The data presented in chapters 4 and 5 used an *in vitro* model which is only suitable for short-term and experiments usually last a maximum of 24 h before nutrients are exhausted. A continuous fermentation model would allow for the constant replenishment of nutrients, a longer duration of fermentation and therefore be more physiologically representative of the human colon. This model could also be used to further investigate and identify prebiotics, which encourage the growth of probiotic bacteria, such as lactobacilli and *Bifidobacterium* strains, in iron-chelated media. Should probiotic bacteria bloom upon the addition of prebiotics, the potential administration of a prebiotic and an iron chelator could be tested in human intervention studies with the possible aim of commercialisation.

The white, powdered clumps that were present in the faeces of those consuming encapsulated phytin indicated lack of phytin dispersion and therefore suggesting that a better form of phytic acid should be used in future studies. A liquid form of phytic acid could be encapsulated to allow for better mixing with the faecal material.

If phytin dispersion was indeed successful, many other lines of investigations could have then been carried out. For example, high performance liquid chromatography (HPLC) analysis of phytin could be used to determine the form of phytin that is active in the colon. Phytic acid can be present in lower inositol forms than inositol hexakisphosphate. The lower forms (tetra-, tri-, di-, and mono) do not have iron chelating abilities. This would then confirm any observations seen.

Another potential line of research involves the identification of any phytase activity that could take place in the gut microbiota of participants when consuming encapsulated phytin. The presence of phytase activity would indicate the breakdown of phytin and therefore the release of iron from the phytin-iron complex.

In line with the previous suggestion, identification of bacterial species which are known to possess phytate-degrading properties could also prove useful. Species belonging to *Bifidobacterium* and *Lactobacillus* have been reported to have the ability to degrade phytic acid. To confirm this, specific species of bacteria could be isolated from the stool samples of participants from both treatment arms and examined for genes related to phytase activity.

9.4 Conclusion

In conclusion, the research presented in this thesis provides evidence that the removal of iron through iron chelators could reduce potentially pathogenic bacteria and increase those that are deemed beneficial, such as *Bifidobacterium*, which have been reported to represent a more healthy gut microbiota [140]. The use of an *in vitro* model, where phylogenetic and metabolite analysis can be extrapolated, provided interesting findings in the importance of iron on the behaviours of mixed bacterial communities, especially when compared to pure cultures of bacteria. Phytin was shown to clump in the faeces of the human trial participants and was therefore unable to bind iron. Due to this, it was not possible to make any conclusions on the iron chelating abilities of phytin. The human intervention trial, therefore, highlighted that further research is required in implementing a better form of phytin to achieve successful dispersion in the colon.

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Appendix

A human intervention trial investigating the effects of Phytin on the human gut microbiome

Short title: Effect of Phytin on Human Gut Microbiome (EPoM Study)

Protocol
Version 4
21.Nov.18

Chief Investigator:
Professor Arjan Narbad

Investigators:
Study Manager: Miss Bhavika Parmanand
Study Scientist: Dr Lee Kellingray
Study Advisor: Professor Susan Fairweather-Tait

PROTOCOL SIGNATURE PAGE

Study Title: A human intervention trial investigating the effects of Phytin on the human gut microbiome

Version 4; 21st November 2018
IRAS number 251932

Sponsor's Approval:

This protocol has been approved by The Quadram Institute Bioscience Human Research Governance Committee (HRGC)

Signature 

Name Dr Antonietta Melchini

Role QIB Human Studies Coordinator

Date 4th December 2018

I have fully discussed the objectives of this trial and the contents of this protocol with the Sponsor's representative. I understand that the information in this protocol is confidential and should not be disclosed other than to those directly involved in the execution or ethical review of the trial.

I agree to conduct this trial according to this protocol and to comply with its requirements, subject to ethical and safety considerations and guidelines, and to conduct the trial in accordance with International Conference on Harmonisation (ICH) guidelines on Good Clinical Practices (GCP) and with the applicable regulatory requirements.

Chief Investigator name and address: Arjan Narbad, Quadram Institute, Norwich, NR4 6UQ

Signature:  Date: 04/12/18

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[1] Annex 14 - Please refer to separate booklet, titled "Annex 14, Catalogue's A-C"

Annex 24 Funding Body ISP Award Letter

List of Catalogues in Annex 14 referred to in document:

Catalogue A Raw Material

A1. Phytin

A1.1 Phytin Food Grade Certificate
A1.2 Phytin Certificate of Analysis
A1.3 Phytin GMO Statement
A1.4 Phytin Material Safety Data Sheet
A1.5 Phytin Shelf Life
A1.6 Phytin Heavy Metals Testing
A1.7 TSUNO Classification Accreditation
A1.8 TSUNO ISO 9001
A1.9 MHRA Medicinal Status

A2. Microcrystalline Cellulose (MCC)

A2.1 MCC Process Flow Sheet
A2.2 MCC Product Information
A2.3 MCC Material Safety Data Sheet
A2.4 MCC GMO Statement
A2.5 MCC Food Statement
A2.6 MCC Ingredient Declaration
A2.7 MCC Allergen List
A2.8 MCC Residual Solvents Statement

A3. Coating Formulation (Phloral®)

A3.1 Amylomaize Starch SDS
A3.2 Eudragit S100 SDS
A3.3 PlasACRYL T20 SDS
A3.4 Reagent Alcohol SDS
A3.5 Triethyl Citrate Specification
A3.6 Triethyl Citrate GMO Statement
A3.7 Triethyl Citrate Food Statement
A3.8 Phloral® Food Statement

Catalogue B Uncoated Capsules

B1. QualiCaps

B1.1 QualiCaps Certificate of Analysis
B1.2 QualiCaps Technical Brochure
B1.3 QualiCaps Technical Information

Catalogue C Coated Capsules

C1. Microbial testing of trial capsules

Background

During recent years it has become apparent that the gastrointestinal tract (GIT) plays a crucial role in the metabolism of dietary compounds. This is due, in part, to the complex microbial ecosystem in the human intestine and its major role in human health, largely related to its metabolic activity. The human colon contains approximately 200 g of living microbial cells, at a concentration of roughly 10^{11}-10^{12} cells/mL [1]. However, the large number of bacteria present in the colon is not a direct reflection of microbial diversity, as >90% of the intestinal microbiome belongs to members of the Firmicutes and Bacteroidetes phyla [2]. In spite of this, it is estimated that the microbial gene catalogues existing in the colon consists of approximately 3 million genes, which is approximately 100 times greater than those encoded by the host genome [3].

The bacterial genes present in the gut are highly diverse, in particular the genes that code for metabolic enzymes [4]. This is largely due to the range of non- or partlydigested food components that reach the colon, including carbohydrates, proteins and phytochemicals. Undigested carbohydrates undergo bacterial fermentation in the colon, generating bacterial products, such as short-chain fatty acids. This bacterial community relies on the continuous provision of micronutrients for metabolism and growth [5]. As a result, there is constant competition for essential micronutrients, which is reflected in their requirements and uptake mechanisms [6]. One of these micronutrients, iron, is highly abundant in colonic contents, and is an essential nutrient for virtually all organisms, including most bacterial species [5]. Iron is a component of haemoglobin, and is also necessary for cellular growth, development, normal functioning, and synthesis of some hormones and connective tissue [7]. Dietary iron has two forms: haem and non-haem [8]. Meat, seafood, and poultry contain both forms of iron, whereas plants and iron-fortified foods contain only non-haem iron. On average, 15 mg of iron is consumed daily in the diet, however, iron absorption is strictly regulated because there is no mechanism for excreting iron that is surplus to requirements. The quantity that is required to maintain iron balance is absorbed in the small intestine, and this is generally approximately 15% of the total dietary intake [9]. Due to the low absorption, relatively large quantities of iron reach the colonic lumen, and this is illustrated by the relatively high concentrations of iron that is recovered in the faeces of British adults on a standard Western diet [5, 10].

However, the iron supply is typically very limited to the micro-organisms as a result of the host iron-withholding mechanisms that can help stop pathogenic organisms from growing [11]. Therefore, despite high quantities of dietary iron in the gut lumen, many micro-organisms of the gut have developed mechanisms to acquire iron, even when available iron is sparse. A well-known example of these mechanisms is the production of bacterial siderophores, molecules which are able to scavenge both ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms of iron, even in iron-limited environments. On average, bacteria need 10^{-7} – 10^{-5} M iron for optimal growth [5]. *E. coli*, a potentially pathogenic species of bacteria belonging to the Enterobacteriaceae family, for example can take up both forms of iron. Pathogenic bacteria use iron to promote growth and, conversely, these bacteria decrease in numbers when iron is limited [12, 13]. *In vivo* knockout studies using mice have shown that specific iron uptake mechanisms are essential in the survival and virulence of some bacterial species, such as *E. coli* [14, 15]. Unlike most bacteria, members of the Bifidobacteriaceae and Lactobacilliaceae families (two families that are seen as beneficial to the host) have a very limited need for iron, if any at all [16]. Lactobacilli do not produce siderophores to sequester iron, and their growth is similar in media with and without iron. It is known that Lactobacillus does not require iron for growth as there is lack of haem-containing enzymes but can substitute other metals for iron, such as cobalt and manganese [17, 18], which may give these bacteria a competitive advantage in low-iron conditions. Bifidobacterium breve, a crucial Bifidobacterium species in breast-fed infants, can acquire luminal iron with the help of a divalent metal permease, however, many of the Bifidobacterium species do not make siderophores or other forms of iron-carriers.

The host has developed numerous mechanisms to curb the acquisition of iron by pathogenic species of bacteria, such as the sequestering of iron by lipocalin-2, a protein belonging to the innate immune system. Dietary components also have a large influence on iron availability. Organic acids, such as citrate, have been shown to form a weak soluble chelate with iron which potentially prevents the precipitation of iron, keeping it in its soluble form, once it has left the acidic conditions of the stomach and entered the duodenum at a higher pH [19]. Ascorbic acid, (Vitamin C), is able to chelate iron and also initiate the reduction of iron, and is a well-known enhancer of iron absorption [20, 21].

Together with dietary components that increase the availability of iron, there are several compounds which decrease iron availability. Phytic acid (Figure. 1), also

known as inositol hexakisphosphate (IP6) or phytate (when in salt form), is the principal storage form of phosphorus in many plants, such as legumes, seeds, nuts and cereals [22]. Phytic acid content varies greatly among plants and is due to different factors, such as the type of seed, climate, and environmental conditions.

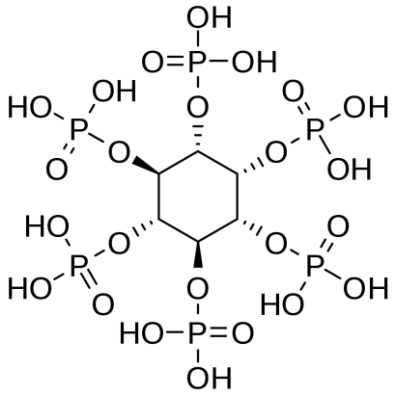


Figure 1. Structure of phytic acid

Studies in humans report that between 37-66% of dietary phytate is degraded during digestion in the stomach and small intestine when the diet is rich in plant food phytases [23-25], a type of phosphatase enzyme that catalyses the hydrolysis of phytic acid.

Beneficial properties of phytic acid have been proposed, including antioxidant [26] and anticancer [27, 28] activities, but phytate is generally regarded as an antinutrient. *In vitro* and *in vivo* studies have demonstrated that phytic acid forms insoluble complexes with several divalent minerals, thereby preventing absorption, and can potentially result in zinc and iron deficiencies [29-32]. Once these insoluble complexes are formed, the mineral cannot be absorbed in the small intestine and therefore pass into the colon. Although phytic acid also binds the metals that the beneficial bacteria use, at pH 6-7 (representative of the colon), phytic acid preferentially binds iron, suggesting a protective role of phytic acid in preventing iron acquisition by potentially pathogenic bacteria.

Several published studies have found that the degradation of phytate varies between individuals and is largely based on the type of diet consumed. As most plant foods such as legumes, cereals and whole grain products, are processed or heat-treated during food production and the preparation of meals, many of the phytases present in these foods are likely to be inactivated. In individuals whose diets consist of high

amounts of wheat or rye bran i.e. foods that contain native phytases, strong phytate hydrolysis occurs in the stomach, with the remaining small portion of non-degraded phytate being hydrolysed in the colon [33].

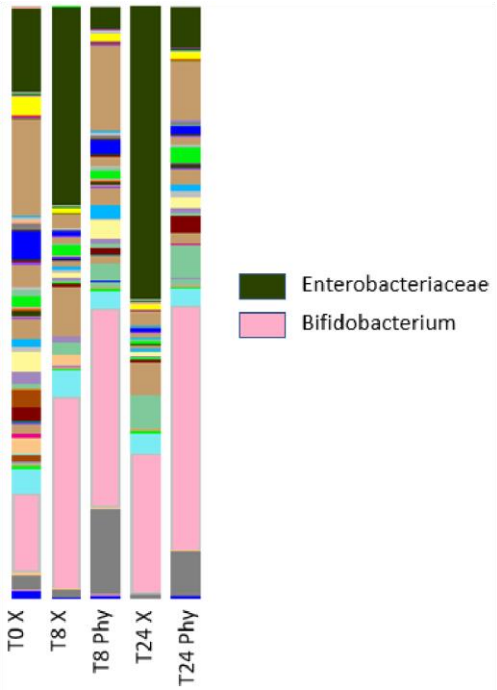
Certain gut micro-organisms (Bifidobacteriaceae and coliforms), have been shown to be able to break down phytates. One study showed that the highest phytate degrading activity belonged to *Lactobacillus reuteri*, *Lactobacillus salivarius* and *Bifidobacterium dentium* [34]. Notably, phytate-bound iron found in the colon is present in the insoluble form making it difficult to degrade [35, 36] suggesting a potential role for phytate in the withholding of iron from potentially pathogenic bacteria.

As well as playing a major role in human nutrition, our gut bacteria also have a profound influence on human physiology and immunology. It is believed that changes in the composition of our gut bacteria, known as gut dysbiosis, may be associated with some diseases, such as inflammatory bowel disease and colon cancer, as well as metabolic disorders such as obesity [37, 38]. Currently, it is believed that preserving the appropriate compositional balance of the gut microbiome may help to maintain the health of the host. Consequently, there has been an increase in research relating to probiotic bacteria. The World Health Organization defines probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit on the host”, the most common of which are *Lactobacillus* and *Bifidobacterium* strains [39]. A link between iron and the gut microbiome has been observed. Several studies have investigated the effects of iron-fortified foods on the infant microbiome, and have reported increased relative abundances of potentially pathogenic bacterial taxa associated with higher concentrations of iron [40-42]. Other studies have examined the role of iron in the gut microbiome in individuals with iron disorders and have speculated that there is a link between high iron levels and increased severity of disease.

Anaemia (iron deficiency) is a severe problem in lesser developed countries, such as Africa, particularly amongst children. Alongside anaemia, many children in Africa suffer from a disrupted gut microbiome due to lack of clean water and food. This unfortunately results in gut microbiomes that tend to skew towards a more pathogenic profile, such as high abundances of Enterobacteriaceae, and less of the beneficial bacteria, such as Bifidobacterium. In an attempt to resolve or significantly diminish the issues related to anaemia, many children are given iron supplements, in

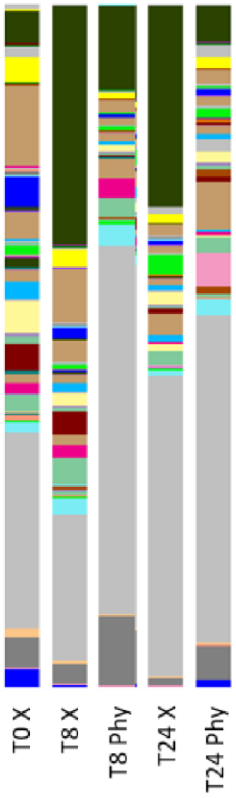
the form of iron-fortified foods. These are foods that contain a bioavailable form of iron to allow for better iron absorption in the small intestine. However, as discussed earlier on, absorption of iron itself is relatively poor. Therefore, providing these children with more iron is in fact counter-productive as more iron is now available for the already pathogenic-heavy microbiome of these children. This, in turn, exacerbates the severity of their already compromised guts [42-44]. This scenario is a good example of where the use of iron chelators in the colon may help alleviate gut microbiome dysbiosis.

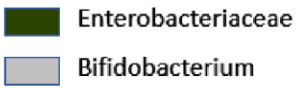
However, despite numerous studies measuring the effects of iron supplementation, only a limited number of studies have investigated the effects of iron chelation on the gut microbiome of healthy individuals. Our *in vitro* colonic fermentation experiments (data not published, however, data has been included in a manuscript for resubmission in 1 week) have indicated that limiting the availability of iron to gut bacteria by means of iron-chelating compounds found in foods (e.g. phytin; a calcium magnesium salt of phytic acid, and dominant form of phytate in plants). The relative abundance of potentially pathogenic bacterial taxa, such as *Escherichia* and *Bacteroides*, decreases, whilst simultaneously increasing the abundance of beneficial bacterial taxa, such as *Bifidobacterium* (Figure 2, 3 individual experiments with relative abundances defined in the adjacent bar chart).



Relative Abundance (%)

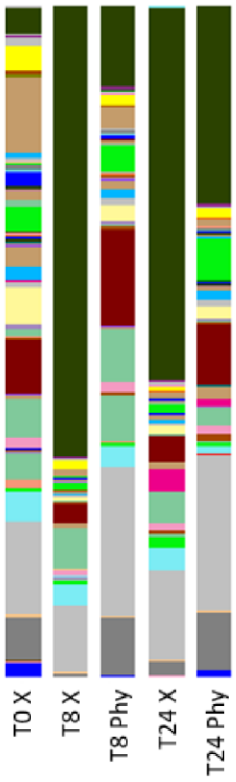
genus	TO X	T8X	T8 Phy	T24X	T24 Phy
Enterobacteriaceae	14.23	33.49	3.72	49.41	6.90
Bifidobacterium	13.21	32.32	33.65	23.48	41.26

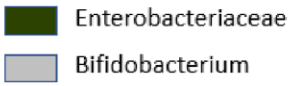




Relative Abundance (%)

Genus	TO X	T8X	T8 Phy	T24X	T24 Phy
Enterobacteriaceae	4.93	34.89	12.27	29.64	5.20
Bifidobacterium	28.68	21.37	53.94	43.83	47.94





Relative Abundance (%)

genus	TO X	T8X	T8 Phy	T24X	T24 Phy
Enterobacteriaceae	3.98	67.09	11.79	55.44	29.23
Bifidobacterium	13.84	9.90	22.08	13.06	22.98

Figure 2. The effect of phytin (Phy) on Enterobacteriaceae and Bifidobacterium.

'X' denotes control, 'Phy' denotes phytin, 'T0/8/24' denotes time in hours. This diagram illustrates the effects of phytin (an iron-chelator) on the relative abundance of

Enterobacteriaceae (pathogenic bacteria) and Bifidobacterium (beneficial bacteria). In the presence of phytin, the relative abundance of Enterobacteriaceae decrease whilst that of Bifidobacterium increases, suggesting the importance of iron for pathogenic bacteria

Hypothesis

Consumption of encapsulated phytin will cause a change in the composition of the colonic gut microbiome, and specifically it will decrease the proportions of potentially harmful Enterobacteriaceae, compared to the faecal microbiome after consuming the placebo capsule as well as compared to the baseline faecal samples of individuals.

Objectives:

Primary

To investigate whether consuming phytin for two weeks will cause a proportional decrease in human gut Enterobacteriaceae compared to the number of Enterobacteriaceae present in the participants' gut microbiome after consuming the control capsule.

Secondary

- To investigate whether the delivery of phytin to the colon for a period of two weeks will be associated with an increase in human gut bifidobacteriaceae through the chelation of iron, compared to the number of bifidobacteriaceae present in the participants' baseline gut microbiota, as determined by faecal bacteria phylogenic analysis
- To ascertain whether consuming phytin modulates the gut microbial community as a whole, as compared to the consumption of a placebo capsule
- To determine whether the consumption of phytin causes a change in short chain fatty acid levels in the faeces, via changes in the gut microbiome function
- To determine whether the consumption of phytin causes a change in the available iron present in the faeces
- To ascertain the extent of phytin degradation that takes place in the colon based on the known concentration of phytin administered via the capsule
- To determine levels of calprotectin as a marker of gut inflammation

- To determine levels of C-Reactive Protein (CRP) as a marker of systemic inflammation[2]
- To measure serum ferritin levels as a marker of the time of capsule release

Study design

The study will be led by Professor Arjan Narbad (QIB Research Leader). All aspects of the study will be managed by Miss Bhavika Parmanand (QIB PhD student) with assistance from Dr Lee Kellingray (QIB scientist) and Professor Susan FairweatherTait (UEA Professor). A delegation log will be used for recording the roles and responsibilities of the local research team and the authorisation of the Principal Investigator. The study will be carried out in collaboration with the Clinical Research Facility (CRF) at the Quadram Institute (QI). The QI CRF is an NHS-governed facility and all clinical procedures for this study will be carried out by the QI CRF team following NNUH standard operating procedures. Clinical assessment and procedures will be performed by two members of the CRF team when research participants are attending the CRF. This will include a registered nurse and another member of staff who is trained in NNUH emergency procedures. When no clinical assessment or interventions are to be performed (for example in the case of an appointment for consent), two members of the CRF team will also be present. This will include a Healthcare professional who is trained n NNUH emergency procedures and a second designated member of staff to provide support.

Participants (n=14) will be recruited into a randomised, double-blind, two-phase crossover dietary intervention. The study team will recruit male and female aged between 18 and 50 years, as this was the number deemed sufficient to observe changes in Enterobacteriaceae and Bifidobacteriaceae within the gut microbiome based upon power calculations (detailed on pages 34-35 of this protocol. An age range of 18-50 was decided upon as it is believed that the gut microbiota shows a stable form throughout the adulthood (classified as 18-50, which include young and middle-aged adults), but it accumulates changes during the aging process [45, 46].

[2] We will be measuring serum ferritin to test whether the phytin affects iron status through reducing iron absorption. In order to do this, it's important to rule out inflammation as this will increase ferritin levels and make it impossible to interpret whether phytin has affected body iron status. Without it we cannot test for an effect of phytin on iron absorption. CRP has to be measured at the same time as serum ferritin as the latter will increase rapidly in response to infection or inflammation, so baseline values alone will not be informative.

Recruited participants (n=14) will be asked to maintain their habitual diet throughout the length of the study. Following randomisation (see 'Randomisation process'), 7 participants (dependent on randomisation results) will consume 2 capsules, each containing phytin (test capsule), 3 times a day with a meal for a period of 2 weeks. The remaining 7 participants will consume 2 placebo capsules, each containing microcrystalline cellulose, 3 times a day with a meal for a period of 2 weeks. Phase 1 will be followed by a 2-week washout period, where all participants will cease capsule consumption. After the washout period, Phase 2 will begin where participants that were initially consuming the test capsule will instead consume the placebo capsule, and vice-versa. Again, for both groups, 2 capsules will be taken 3 times a day with a meal for a total period of 2 weeks. A summarised diagram of this study design can be found in Figure 3.

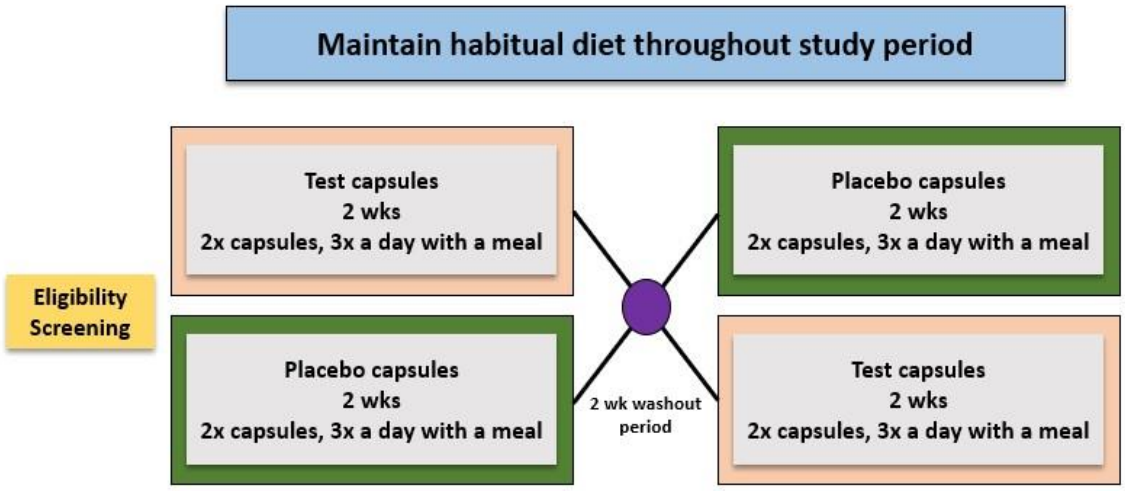


Figure 3. Summary of study design

The participants will be asked to provide blood and faecal samples, and complete a capsule checklist, stool charts (noting the frequency and consistency of their bowel movements) and food frequency questionnaires at various stages throughout the intervention. A flowchart summarising this approach can be found below (Figure 4). Participants who do not have a BMI between 19.5 (underweight) and 30 (obese) kg/m^2 will be excluded on the grounds that their gut microbiome may be affected, as studies have shown that the gut microbiome of individuals with a BMI between 18.5 and 30 kg/m^2 are significantly different to obese or underweight individuals [37, 47].

Figure 4: Flow diagram of EPoM study

Pre-study talk (Visit 1, QI CRF, ~1 hr)

- Member of study team will discuss Participant Information sheet and answer any questions
- Potential participants supplied with container for urinalysis, and a copy of Bristol Stool Chart - Minimum 72 hr consideration period, participant to contact study team if interested

Eligibility screening (Visit 2, QI CRF, ~1.5 hr)

- Participant to sign 2 consent forms (study participation and sample storage), and a medical declaration form - Copies of each given to participant
- Screening questionnaire completed with participant by QI CRF Research Nurse
- Measurements for pulse, blood pressure, height, weight and BMI taken, Multistix® urine dipstick test performed
- Blood test for serum ferritin, HbA1c, full blood count and CRP

Faecal sample collection kit pick-up (Visit 3, QI CRF, ~20 min)

- If successful at eligibility screening, participant will be asked to collect a faecal sample collection kit

Faecal sample drop-off (Visit 4, QI CRF, ~10 min)

- Participant to drop off faecal sample in kit provided during Visit 3; date and time arranged for start of study

Phase 1 (Visit 5, QI CRF, ~1 hr) Days 1-14

- If participant has not yet provided a faecal sample, opportunity will be given here to do so
- Confirm participant is happy to proceed with the study and assessment day questionnaire filled
- QI CRF to take 15 mL blood sample to assess CRP and serum ferritin
- 2x capsules to be consumed 3 times a day with a meal for a period of 14 days
- First 2 capsules (either study arm AB or BA) must be consumed at the QI CRF (food provided)
- Participant will be provided with the rest of the capsules required for Phase 1 (bottle will include extra capsules)
- Participant will be provided with new faecal collection kit, along with a Bristol Stool Chart, food frequency questionnaire and capsule checklist

Mid-Phase 1 faecal sample drop-off (Visit 6, QI CRF, ~10 min) Day 7

- Participant to drop off faecal sample in kit provided during Visit 5, new faecal collection kit provided

End of Phase 1 (Visit 7, QI CRF, ~30 min) Day 14

- Participant to drop off faecal sample in kit provided during Visit 6
- Blood sample taken by QI CRF Research Nurse (bloods used for iron and CRP measurements)
- Participants to return the following: *(i) stool chart; (ii) food frequency questionnaire; (iii) capsule checklist and (iv) capsule bottle, including any capsules that have not been consumed*
- New faecal collection kit provided

Washout Phase (Days 15-28)

- Normal diet continued with no capsule consumption
- Stool sample to be provided at end of this phase, delivered when the participant next visits the QI CRF

Phase 2 (Visit 8, QI CRF, ~45 min) Days 29-42

- Participant to provide faecal sample in kit provided during Visit 7, QI CRF Nurse to take blood sample (iron and CRP)
  - Refer to Phase 1 for remaining details
- Exceptions: *(i) Alternative capsules to Phase 1 provided*

Mid-Phase 2 faecal sample drop-off (Visit 9, QI CRF, ~10 min) Day 35

- Same as 'Mid-Phase 1 faecal sample drop-off'

End of Phase 2 (Visit 10, QI CRF, ~30 min) Day 42

- Participant to drop off faecal sample in kit provided during Visit 9, blood sample taken by QI CRF Research Nurse (bloods used for iron and CRP measurements)
- Participants to return the following: *(i) stool chart; (ii) food frequency questionnaire; (iii) capsule checklist and (iv)* 16 *capsule bottle, including any capsules that have not been consumed*IRAS ID 251932
- End of study

Recruitment strategy

The study population will consist of men and women between the ages of 18 and 50 who meet the study inclusion criteria, and recruitment will continue until 14 participants complete the study.

The QIB Volunteer Database will be accessed by the QIB Volunteer Database Manager, Wendy Hollands, and it is anticipated that this will be the prime source of participant recruitment for the study. Apparently healthy potential participants from the QIB Volunteer database, who meet the basic inclusion criteria, will be sent a letter of invitation (Annex 1) to participate in the study. This will be supported by the Participant Information Sheet (PIS, Annex 2) and a response slip, with a pre-paid envelope included for returning the slip if they are interested and wish for further information. The QIB Volunteer database contains names and contact details of approximately 1271 people above the age of 18 years who have registered an interest for participating in human studies at QIB (The QIB Volunteer Database complies with the new EU General Data Protection Regulation (GPDR), which came into force in the UK on 25 May 2018, and the UK Data Protection Act (DPA) 2018). Identification of potential participants will be carried out by the QIB database manager (Wendy Hollands), with initial contact with potential participants from the database, including the posting of the invitation letter and PIS, carried out solely by Wendy Hollands. The available numbers for recruitment on the database are subject to variation as a result of the recruitment of participants by other studies.

Along with using the database as a source of recruitment, advertisements (Annex 3a & 3b) will be placed around the Norwich Research Park (University of East Anglia (UEA), John Innes Centre (JIC), Earlham Institute (EI) and Quadram Institute (QI)) and other appropriate locations for example, supermarkets, social clubs, church newsletters, golf and other sporting clubs, gymnasiums and leisure facilities within the local area (a 40-mile radius of QI). The posters advertising this study may have tear off contact slips attached to the poster to facilitate participant recruitment. If required, we will also obtain radio (and television) coverage. Social networking sites like Facebook and Twitter may also be used to either display the ethically approved

advert for the study, or to direct potential participants to a QI website containing the ethically approved advertisement and contact details of members of the study team. A member of the study team will send interested responders a letter of invitation (Annex 4) and a copy of the PIS (Annex 2) with accompanying response slip and pre-paid envelope in which to return the completed response slip to the study manager, if interested in taking part in the study.

If a potential participant registers an interest in taking part in the study, and 14 participants are currently recruited onto the study, the potential participant may be asked if they would be happy to be placed on a standby list. Those placed on the standby list will neither be consented nor screened until they are recruited onto the study and will be notified if their participation is not required. Those on the standby list may ask to be removed from the list at any time.

Study Talk at QI CRF – Visit 1

Following an expression of interest, potential participants will be contacted by telephone or e-mail by a member of the study team (study manager or study scientist) to arrange an appointment for a study talk at the QI CRF. The QI CRF is an NHS facility located within the QI building. This talk will be carried out by a member of the study team and all aspects of the study will be discussed. The potential participants will be encouraged to ask questions at this point, prior to making any commitment. At the end of the talk all potential participants will be given a minimum of 72 h to consider whether they wish to participate in the study, and during this consideration period they will not be contacted. If, following this period of consideration, the participant still wishes to take part they will be asked to contact the investigator named on the PIS. If, however, potential participants have decided after the talk that they are keen to take part in the study and request to book their eligibility screening appointment, the screening appointment will be made for a minimum of 72 h after the study talk. Before leaving the QI CRF, all potential participants will be supplied with a small clean container and a copy of the Bristol Stool Chart. Should the potential participant wish to take part in the study, the container will be used for a midstream urine sample from within 2 h of their eligibility screening appointment. The potential participant will be told that should they decide to take part in the study, the QI CRF Research Nurse will go through the Bristol Stool Chart with them at their eligibility screening appointment and ask them what their typical stool type is using the Bristol Stool Chart as a guide. Thereafter, the potential participants will be

advised that they will need to assess their typical stool type, referring to the Bristol Stool Chart, prior to their eligibility screening appointment. The potential participant will be informed that should they decide against taking part in the study, they may dispose of these in general waste. On booking an eligibility screening appointment, a member of the study team will post an appointment card (Annex 5) to the potential participant.

Eligibility screening at QI CRF – Visit 2

All those responding positively following this period of consideration will be invited to attend the QI CRF for an eligibility screening with a member of the QI CRF team, and the screening will be carried out following relevant QI CRF standard operating procedures. When using the QI CRF, if clinical assessments are to be performed, two members of the CRF team will be present when any study participants are attending. Participants will be reminded to bring a midstream sample of urine in the container supplied to them following their pre-study talk. Participants will need to collect their urine sample from within 2 h prior to the screening appointment as this is a required specification for the validity of the urine dipstick test (this will not be tested until after the consent forms for study participation and sample storage has been signed). Participants will also be reminded to bring with them details of any prescribed medication, herbal remedies or dietary supplements (i.e. name of medication, dose taken).

On arrival at the QI CRF the participant will be taken into a confidential room where a member of the study team (study manager or study scientist) or the QI CRF Research Nurse will go through 2 consent forms, one for written informed consent for study participation, and another for the long-term storage of samples at the Norwich Biorepository (Annex 6 and Annex 7, respectively) with the participant and encourage any questions they may have at this stage. Participants will then be asked to sign both the consent forms outlined above. The participants will also be asked to sign a medical declaration form (Annex 8) agreeing to inform the study team of any medication they may have to take, illnesses suffered, or if they become pregnant during the study. A signed copy of both the consent forms and medical declaration form will be given to the participant to keep.

Following consent, the QI CRF Research Nurse will perform a Multistix® urine dipstick test. The urine dipstick results will be known immediately. In the event of a flagged urinalysis indicating a re-screen is appropriate, the QI CRF Research Nurse

will speak to the participant regarding their results and they will be advised to visit their GP/practice nurse prior to returning for a re-screen. The re-screen will be postponed until results from the GP are known. If the urinalysis results are flagged on the second occasion the participant may be excluded depending on the tests flagged, and this decision will be made at the discretion of the QI medical advisor. If blood is flagged in the urine sample of female participants they will be asked if they are menstruating or have just finished menstruating, if they answer yes to either they will be asked to provide a second urine sample for testing 5 days after finishing menstruation. In the event of a flagged result on the second occasion, which indicates they may be re-screened, the QI CRF Research Nurse will speak to the participant and they will be advised to speak to their GP regarding their results prior to coming back to the QI CRF for a re-screen. Again, the re-screen will be postponed until results from the GP are known. All abnormal results will be referred to the QI medical advisor. In an event of flagged urinalysis result, the decision to exclude the participant from the study will be taken by the QI medical advisor.

The QI CRF Research Nurse will then complete a screening questionnaire with the participant (Annex 9), measure and record blood pressure (BP), pulse, height (cm) and weight (kg) and calculate Body Mass Index (BMI, kg/m^2). The screening questionnaire contains specific questions pertaining to stools, the QI CRF Research Nurse will explain the Bristol Stool Chart and should the participant indicate that their typical stool type is abnormal (type 1, 2, or 7), they may be referred to their GP and will be excluded from the study.

A blood test (20 mL volume) will also be performed to confirm serum ferritin and HbA1c (this test will give an indication of the participant's average glucose levels over a period of approximately 2-3 months). A full blood count and CRP (a marker of systemic inflammation) measurements will also be performed (bloods taken for eligibility screenings will be sent to NNUH for analysis).

If the flagged urine and blood results indicate exclusion from the study is appropriate (refer to inclusion and exclusion criteria), the decision to exclude the participant from the study will be taken by the QI medical advisor and the participant will be advised to speak to their GP regarding their results. Copy of all the eligibility screening results (blood test, blood pressure, pulse, weight, BMI, and urinalysis results) will be

sent to the GP (Annex 10), and this will be supported with a letter to their GP detailing their clinical results (Annex 11).

The GPs of those successfully recruited onto the study will be informed of their patient's participation in the study by letter (Annex 12) along with a study description (Annex 13). It is expected that all participants who successfully pass the screening, and who wish to continue, will commence on the study within three months of their screening appointment otherwise a re-screen will be necessary.

Note: *Participants who do not commence the study within three months of a successful eligibility screening will need to be re-screened if they wish to partake in the trial.*

Randomisation Process – allocation of test or placebo group

It is important that the groups are balanced in number because of possible order effect, and so given the small sample size, block randomization will be used to allocate participants to the two treatment arms, with 2 blocks of 4 participants and 3 blocks each of 2 participants making 14 participants in total. Blocks will be ordered at random. The allocation sequence will be generated using randomization.com, with the seed recorded for replicability.

Blinding process

This process will be carried out by a QIB scientist who is not part of the study team. Wendy Hollands (QIB FIH scientist) will be responsible for this process, and therefore ensures the trial remains blinded to the study participants, Chief Investigators and study scientists/advisors. Below is a summarised overview of how the blinding process will be implemented:

Step 1: Test capsules will be filled with phytin and placed in a HDPE bottle labelled "Phytin"

Step 2: Placebo capsules will be filled with MCC and placed in a HDPE bottle labelled "Placebo"

Step 3: Each participant will receive either a batch of test or placebo capsules, therefore the capsules will be divided into 14 sets (as n=14 for this study). Again, HDPE bottles will be used (these bottles will NOT be labelled at this stage)

Step 4: The 14x "Phytin" HDPE bottles will be placed in a box labelled "Phytin"

Step 5: The 14x “Placebo” HDPE bottles will be placed in a box labelled “Placebo”

Step 6: Both boxes will be given to Wendy Hollands and she will assign both boxes with the letters, “A” or “B”

Step 7: Boxes “A” and “B” will be given back to Miss Bhavika Parmanand and each bottle subsequently labelled with the details below

EPoM study: **Treatment A/Treatment B**

Volunteer code: Date opened:

Two capsules to be taken three times a day, with a meal

Store at room temperature
Keep out of reach of children/pets
For food trial use only Date of manufacture:

Quadram Institute, James Watson Road, Norwich, NR4 6UQ. Tel: 01603 255000

Figure 5: Labelling for capsule containers

Next, Miss Bhavika Parmanand will allocate the participants to either study arm “AB” or “BA”.

Participants of the study, Chief Investigators and Study Scientists/Advisors will remain blind to this allocation throughout the trial as well as during analyses of samples taken from participants to ensure an unbiased approach is adopted to the evaluation of results. However, the code may be broken in the event of a medical emergency as deemed appropriate and necessary by the QI CRF Research Nurse. All personal information will be kept confidential and known only to the chief investigator, members of the study team, QI CRF Research Nurse and the participant’s GP.

Note: to ensure uniformity of phytin dosage with capsules, as and when the capsules are being divided into individual HDPE pots, a subset of capsules will be removed and quantified for phytin concentration levels.

Inclusion criteria

- Men and women aged between 18 and 50
- Non-smokers (e-cigarette/vape users are able to participate)

- Those with a body mass index (BMI) between 19.5 and 30 kg/m^2 • Those that live within a 40-mile radius of Norwich

Exclusion criteria

You will not be able to take part if you(r):

- results of our screening test indicate you are not suitable to take part in this study
- are pregnant, have been pregnant in the last year or are lactating and/or breast feeding
- are currently suffering from, or have ever suffered from, any diagnosed gastrointestinal disease, gastrointestinal disorders including regular diarrhoea and constipation (excluding hiatus hernia unless symptomatic), and/or have undergone gastrointestinal surgery, or the study intervention/procedure is contraindicated
- have been diagnosed with any long-term medical condition that may affect the study outcome (e.g. cancer, diabetes, haemophilia, cardiovascular disease, glaucoma, anaemia). These will be assessed on an individual basis
- have been diagnosed with any long-term medical condition requiring medication that may affect the study outcome
- regularly taking over the counter medications for digestive/gastrointestinal conditions
- are on long-term antibiotic therapy. You may be able to participate if 4 weeks has passed from the end of a course of antibiotics (this will be assessed on an individual basis)
- regularly take laxatives (once a month or more)
- take certain dietary supplements or herbal remedies and are unwilling to stop taking them for one month prior to and during study period. This will be assessed on an individual basis
- take pre- or pro-biotic drinks &/or yoghurts on an occasional basis, unless willing to abstain for one month prior to and during the study period. (if you regularly take pre-&/or pro biotics (3+ times a week, and for more than one month) and will continue throughout the study then you will not be excluded) • are on or plan to start a diet programme that may affect the study outcome (e.g. 5:2 fasting diet) unless willing to abstain for 1 month prior to and during study period. This will be assessed on an individual basis

- recently returned to the UK following a period abroad, and who have suffered gastric symptoms during the period abroad or on return to the UK. These will be assessed on an individual basis
- regular/recent (within 3 months) use of colonic irrigation or other bowel cleansing techniques
- are involved in another research project that includes dietary intervention or involving blood sampling
- record blood in your stools or have two or more episodes of constipation or diarrhoea (type 1, 2, or 7 stools) during the study
- are unwilling to provide GPs contact details
- are unable to provide written informed consent.
- regularly consume more than 15 units (women) or 22 units (men) of alcohol a week
- Regularly taking iron supplements
- Those unable to swallow capsules
- Those with abnormal blood pressure measurements (160/100 will be regarded as an exclusion value)
- Are related to someone in the study (e.g. spouse, partner, immediate family member)

Study Intervention

Participants will receive both phytin-rich and placebo capsules as part of their normal diet for four weeks during the study. The order in which they will consume these capsules will be randomly assigned.

Placebo capsules

Microcrystalline cellulose (MCC) is a purified, partly depolymerised cellulose with shorter, crystalline polymer chains. Its strong binding performance make MCC one of the most commonly used fillers and binders in drug formulations. The product is manufactured by controlled partial hydrolysis of high purity wood pulp, followed by purification and drying. Pharmaceutical MCC will be purchased from DFE Pharma (https://www.dfepharma.com/en/excipients/mcc.aspx) and all related documents can be found in Annex 14, Catalogue A2, documents A2.1-2.8.

Phytin capsules

Dose of phytic acid (phytin)

The dose of phytin to be administered was calculated on the basis that it would be sufficient to chelate most of the iron that enters the colon. Phytin (MW=847 g/mol) is a salt form of phytic acid (MW=660 g/mol). Literature data refers to phytic acid and this MW has been used in calculations. On average, an estimated 15 mg of iron is consumed by men via the diet on a daily basis [19, 48]; intakes in women are lower (8-9 mg/day). Of this amount, approximately 15% is absorbed in the small intestine. Therefore, on average, 13 mg (0.233 mmoles; MW of iron – 55.8 g/mol) of iron travels through to the colon and is found in daily faecal matter [5].

A significant molar excess of phytin is required to ensure that a substantial fraction of the iron in the colon is bound to phytin and not available for use by pathogenic colonic bacteria. We used data from human studies that investigated how increasing phytic acid : Fe ratios affected the absorption of iron from the small intestine to determine the effective dose of phytin. The report of Tuntawiroon *et al*., [49] shows that increasing the phytic acid : Fe ratio from 3:1 to 14:1 substantially reduced iron absorption from 22% to 7%. Taking an average phytic acid daily ingestion in the UK of 700 mg, assuming 50% is degraded during digestion in the upper intestine, 350 mg (=0.530 mmoles) will reach the colon, along with an average of 13 mg (=0.233 mmoles) of Fe, giving a molar ratio of 2.27 phytic acid : Fe. To increase the ratio to >14:1, we will supplement with an amount of phytin that alone achieves a PA : Fe ratio of 12 : 1. Therefore, the quantity of phytic acid in the supplement will be 12 x 0.233 = 2.8 mmoles, which is 1845 mg of phytic acid (MW-660 g/mol), and 2368 mg of phytin (MW=847 g/mol), rounded up to 2.4 g phytin (equivalent to 1870 mg of phytic acid).

Preliminary work has shown that a maximum of 0.4 g phytin can be filled into 1x size 00 capsule. Therefore, participants will be required to consume two capsules (00) containing 0.4 g phytin 3x a day in conjunction with any 3 meals. Therefore, the total daily intake of phytin delivered is 2.4 g, and the total daily intake of phytic acid delivered is 1.87 g.

Safety considerations

The product to be administered is TSUNO Rice Fine Chemicals IP6-phytin (phytin) which is a salt form of phytic acid ($C_6H_6O_{24}P_6Mg_4CaNaKCa$). There are no studies of the safety of consuming this particular product. The quantities of Mg, Na, K, and Ca

delivered by 2.4 g phytin are not considered toxic. The phytin has been tested for heavy metals by Eurofins Food Testing UK, with no concerning levels identified and the results are provided in Annex 14, Catalogue A1. The safety of consuming 1870 mg per day of additional phytic acid was assessed.

First, the proposed 1870 mg per day dose was compared with estimates of average daily phytic acid consumption from the scientific literature. A review on phytic acid by Schlemmer *et al.,* [33] provides an extensive list of estimates of phytic acid intakes. Phytic acid intakes vary according to differences in diets, and intakes in African and Asian countries are higher than those in the UK and other countries in Europe, and intakes in vegetarians and vegans are higher than those in omnivores. Schlemmer *et al.,* [33] reports estimates of mean phytic acid daily intakes varying from 504 to 844 mg for adults in earlier studies [50], while a recent study calculated the mean daily phytic acid intake in men (aged 40 years) at 1436 ± 755 mg [51]. The more recent estimate (1436 ± 755, mean ± SD) is about twice the mean from earlier studies, but similar to that reported for Swedish vegetarians (mean = 1146 mg/day, range = 5002927 mg/day), US male vegetarians (1550 ± 550 mg / day), males and females aged 20-45 in India (1560-2500 mg / day) and Nigerians (mean = 2200 mg / day). Here we use the more recent UK estimate (1436 ± 755). The proposed dose of 1870 mg phytic acid per day is approximately 1.3-fold greater than the estimated average intake in UK males, and about 2.7-fold higher than the 700 mg / day reported in earlier studies. Inputting the 1436 ± 755 (mean ± SD) data into a normal distribution calculator (http://onlinestatbook.com/2/calculators/normal_dist.html) and calculating the proportion of the population ingesting >1870 mg phytin per day gives 28.3% (i.e. 28% of men in the UK consume 1870 mg / day phytic acid or more). The 99th percentile is around 3192 mg / day.

If we then consider that we might recruit a participant with average phytic acid consumption, then total phytic acid daily intake would be 3306 mg (1870 + 1436). Considering a male participant at the high end of phytate intake from their normal diet (2000 mg / day), then total phytic acid intake would be 4270 mg / day (2400 + 1870). It is therefore possible that a subject may consume more phytic acid than is normal in the UK, but not more than has been reported as normal intakes in several vegan/vegetarian populations around the world.

Second, we reviewed the evidence for toxicity of consuming high doses of phytic acid. We could find no reports that phytic acid ingested in normal diets has caused toxic effects in humans, with reported consumption as high as 5.6 g / day. We found a number of Safety Data Sheets from suppliers of phytic acid (e.g. Santa Cruz, Carbosynth, TCI America) and none of these indicated adverse effects associated with oral consumption. Statements include “no significant acute toxicological data identified in literature”. Phytic acid has been approved as *‘generally regarded as safe (GRAS) when used in accordance with good manufacturing or feeding practice’*. No reports of studies to investigate possible toxicity of phytic acid in humans were found. There are a few reports looking at toxicity in animal models and some LD_{50} (dose causing 50% death), but none of these are for oral ingestion (they all involve injection, e.g. intraperitoneal), and they were not considered relevant.

Finally, phytic acid is a metal chelator and is especially effective at chelating iron but also other minerals like zinc. Phytic acid-bound minerals such as iron and zinc are not available for absorption from the gut and phytate is therefore regarded as an anti-nutrient. However, as the phytin is being administered in specially prepared capsules that resist stomach / small intestinal digestion and release their contents only once they reach the colon, and significant mineral absorption only occurs in the small intestine (not colon), the intervention is not expected to reduce mineral uptake in the participants.

In summary, phytic acid is an approved food additive and there are no known/reported toxic effects in humans. The intended dose is above the mean daily intake in the UK population, but at a level reported in several population sub-groups around the world such as vegetarians and vegans, and in people consuming some African and Asian diets. Considering the available evidence, we do not anticipate the intended dose of phytin causing adverse effects in the target group of study participants. However, as the dosage used for this trial has not been investigated before, this trial is testing this specific dose of phytin for the first time.

Study Materials

IP6, also known as phytin mineral salt, will be purchased from TSUNO Rice Fine Chemicals Company Limited, in Japan (https://www.tsuno.co.jp/en/products/finechemicals/ip6-biz/). IP6 from TSUNO is of food grade standard and therefore safe for human consumption. All factories

received ISO 9001 in 1998 followed by ISO 14001 in 2001 (Annex 14). A list of accreditation scopes can also be found in the documentation (Annex 14). TSUNO Rice Fine Chemicals obtained ISO 22000 in 2012 (https://www.tsuno.co.jp/en/aboutus/history/).

All other documentation regarding phytin can be found in Annex 14, Catalogue A1.

Phloral® Coating for Targeted Colonic Release

To ensure that phytin degradation does not occur in the stomach or small intestine, the study team has decided to encapsulate phytin with Phloral®. Phloral® is a new technology invented for the precise and consistent delivery of compounds to the colon, and has successfully completed Phase 3 clinical studies, registered at Clinicaltrials.gov (NCT01903252) [52]. It exploits changes in gastrointestinal pH in combination with the enzymatic activity of the microbiota as independent but complementary release mechanisms to guarantee site-specific release (Figure 6).

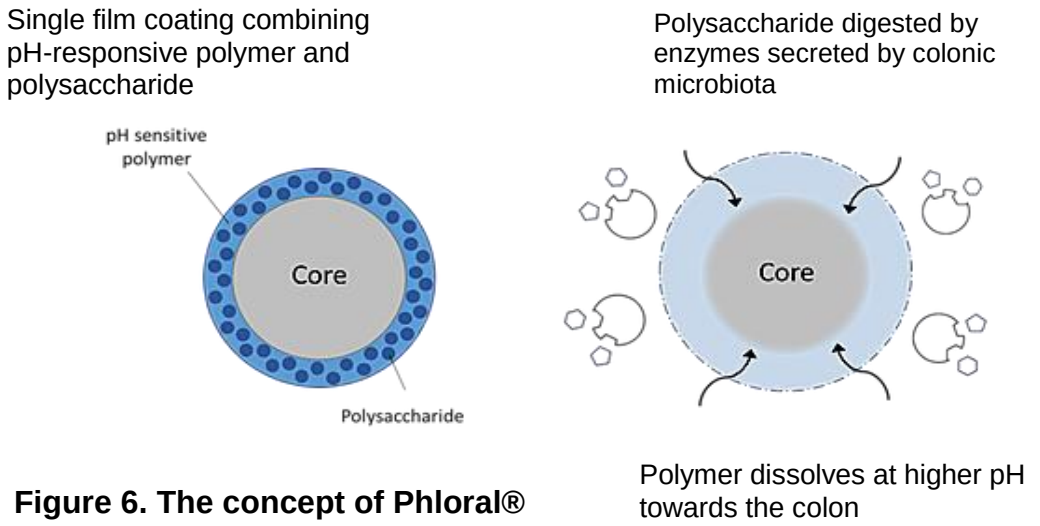


Figure 6. The concept of Phloral®

Even though the pH in the GI tract varies, the polysaccharide component is independently digested by enzymes secreted by the trillions of bacteria naturally residing in the colon. This additional fail-safe mechanism overcomes the limitations of conventional polymer coatings. Furthermore, Phloral® was evaluated against a widely used conventional pH sensitive coating in 8 human subjects. Radiolabelled tablets were administered under various feeding regimens. Transit and disintegration was tracked by gamma scintigraphy, and results demonstrated that all Phloral® coated tablets were successfully released in the colon, whereas 3/8 conventional pH sensitive polymer coated tablets failed to release and were excreted intact (Figure

7). Further information on the raw materials used for this formulation can be found in Annex 14, Catalogue A3, documents A3.1-3.7. A signed document regarding the safety of Phloral® formulation can also be found in Annex 14, Catalogue A3, document A3.8

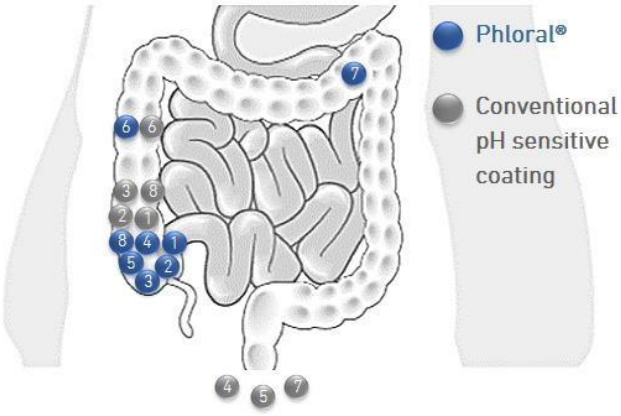


Figure 7. Testing of Phloral® in 8 human subjects. Tablets illustrate the site of disintegration in individual subjects. Data reported in the fed state. Conventional tablets remained intact in subjects 4, 5 and 7 and were excreted via the stools. https://www.intractpharma.com/phloral

Encapsulation of phytin/MCC with Phloral®

A fully organic coating suspension will be used to coat capsules to be consumed during the human trial. Empty, size 00 Hydroxypropylmethylcellulose (HPMC) capsules will be purchased from Qualicaps, Spain (Annex 14, Catalogue B, documents B1.1-1.3). HPMC capsules are derived from vegetable cellulose and are 100% natural. HPMC capsules contain no gelatine, wheat, gluten, preservatives, animal by-products or starch and are made from pure cellulose of either poplar or pine, therefore allowing vegans, vegetarians and those that are intolerant to wheat or gluten, to participate in the study.

A mini coating machine (Caleva Mini Coater Drier) located at Intract Pharma (London) will be used to coat 2500 size 00 HPMC capsules. These capsules will be separated so that the caps and bodies are coated separately to the required coating thickness. Once the bodies and caps of a total of 2500 capsules have been coated, the capsules will be left overnight to dry.

To ensure the Phloral®-coated capsules are safe for consumption, these capsules will be prepared by Miss Bhavika Parmanand under sterile conditions, with fresh ingredients used for the coating suspension. A subset of the trial capsules will then

be sent to Eurofins Food Testing (Wolverhampton, UK) for toxicology tests and microbial testing to ensure coated capsules are free from bacterial contamination and other contaminants, therefore safe for human consumption (Annex 14, Catalogue C). Once results from toxicological and microbial testing are available, capsules (bodies and caps separated) will be delivered to the QI CRF directly in appropriate containers. The bodies will then be filled with either phytin or MCC and capped when complete. This process will be carried out at the QI CRF. Once all bodies have been filled with either phytin or MCC and capped, the closed capsules will be stored in the containers outlined in the next section and kept at the QI CRF. Again, a subset of the trial capsules will then be sent to Eurofins Food Testing for toxicology tests and microbial testing to ensure filled capsules are free from bacterial contamination and other contaminants, therefore safe for human consumption. To ensure uniformity of the phytin dose within the capsules, a subset of the filled capsules will be analysed to quantify and confirm phytin concentrations.

Capsule Management and Distribution

During the course of the study, the capsules that will be provided to the participants will be kept at the QI CRF, accessed only by the QI CRF Research Nurse. The capsules will be stored in HDPE containers.

Two boxes (one each for test and placebo capsules) will be labelled “A” and “B” (please refer to section “Randomisation process” for details). Once the test and placebo capsules have been placed in the containers, the containers will be stored at the QI CRF at room temperature, food grade room.

The QI CRF Research Nurse will be responsible for dispensing the capsules, randomly allocated to the participants. The QI CRF Research Nurse will be provided with the list generated by Miss Bhavika Parmanand, which links the participant code number to either “AB” or “BA”. An inventory log will be kept by the QI CRF Research Nurse to ensure that capsule numbers are recorded. This, in conjunction with dispensing records will allow the QI CRF Research Nurse to keep track of how many capsules have been distributed and to which participant.

Assessment of Compliance

Although participants have not been asked to make any changes to their habitual diets throughput the entirety of this study, each participant will be asked to complete

a Food Frequency Questionnaire (Annex 15) upon completion of each of the test phases. This will be used to assess participant's habitual diet over the trial period and whether their food habits correlate with, or are reflected in, the outcome. It will also give an indication of any participants with a naturally high, phytin-based diet.

Furthermore, to assess compliance with the phytin-rich intervention, participants will be provided with a capsule checklist (Annex 16) and asked to mark down each time they ingest the capsules. This will help them to remember if they have taken the dose at the appropriate times points as well as serving as a measure of compliance. Also, at the end of each treatment phase, participants will be asked to return the bottle containing any unused capsules. These will be counted and then stored for reanalysis should any issue arise in the future.

Study procedures after Study Talk (Visit 1) and Eligibility Screening (Visit 2):

Faecal sample collection kit pick-up (Visit 3)

Participants will be invited to take part in this study if the blood and urine tests from the eligibility screening are satisfactory, and they meet all the listed participation criteria.

Participants will be asked to collect a faecal sample collection kit, along with a detailed sheet on how we would like them to collect and store their faecal sample buntil they can deliver it to the QI CRF. Everything the participant needs to collect the sample will be provided in the kit.

Faecal sample drop-off (Visit 4)

Participants will be asked to bring their faecal sample to the QI CRF in the collection kit that they were given during Visit 3. Alternatively, if need be, it can be arranged for the sample to be collected by a member of the study team. This faecal sample will provide a baseline gut microbial profile of the participant. After this faecal sample has been provided, the study team will arrange a time and date for the participant to start the trial.

Phase 1 (Visit 5, Days 1 – 14)

When the participant visits the QI CRF, they will be asked to provide a faecal sample if they have not already done so during Visit 4. The participants will be asked

whether they are happy to continue with the study, and whether they have had any changes to their health in the form of a follow-up health questionnaire. A QI CRF Research Nurse will take a 15 mL blood sample (to test for ferritin and CRP levels). The participant will receive either “AB” or “BA”, dependent on what group they have been randomly assigned to for Phase 1. The first 2 capsules must be consumed at the QI CRF and participants must remain here for a minimum of 20 minutes. Food will be provided for participants on this occasion. After the participants have consumed the first 2 capsules, those randomly assigned to the test capsule will receive a bottle of capsules (this takes into account for any capsules that may be lost/misplaced by the participant), each containing 0.4 g phytin. Those randomly assigned to placebo capsule will also receive a bottle of capsules (accounting for lost/misplaced capsules), each containing 0.4 g microcrystalline cellulose. Participants will be informed that capsules must be stored in a cool, dry place dry place. The participants will be reminded that they are to consume 2x capsules 3 times a day, in conjunction with a meal, for a period of two weeks.

All participants will also be provided with the following forms: (i) food frequency questionnaire, (ii) stool chart and (iii) capsule checklist. The first two forms will need to be filled out for a consecutive 7 days; the final form is to remind participants to consume the capsules and tick the relevant box to show that they have taken the capsules.

Finally, before they leave the QI CRF, they will be provided with a new faecal sample collection kit for the next stool sample.

Mid-Phase 1 faecal sample drop-off (Visit 6, Day 7)

Once the participants are halfway through Phase 1, they will be asked to provide a faecal sample and drop it off at the QI CRF in the faecal collection kit they were given at the end of Visit 5. Once this sample has been dropped off, they will be provided with a new faecal collection kit and asked to continue with the remaining of Phase 1.

End of Phase 1 (Visit 7, Day 14)

At the end of Phase 1, participants will be asked to deliver a faecal sample to the QI CRF in the faecal collection kit provided during Visit 6. When they are at the QI CRF, a QI CRF Research Nurse will take a 15 mL blood sample (to test for ferritin and CRP levels). Participants will be asked to return the bottle containing the capsules that was given to them at the beginning of Phase 1, including any capsules that have

not been consumed. Also, the participants will be asked to return the completed forms (food frequency questionnaire, stool chart and capsule checklist). Finally, a new faecal collection kit will be provided to the participants for the next stool sample.

Washout Phase (Days 15-28)

A wash-out phase lasting for 2 weeks was decided upon based on previous human studies carried out looking at various dietary interventions. An average of 2 weeks was deemed sufficient for the gut microbiota to normalise to its baseline levels before the start of the treatment [53, 54]. Participants will be asked to provide a faecal sample (as described in Study procedures: Faecal collection) after a minimum of two weeks following the end of Phase 1, i.e. the end of the washout phase. They will be asked to deliver this sample on Day 1 of Phase 2, in the collection kit provided during Visit 7. If, shortly after this 2-week period, the participant has not contacted the study team, a member of the study team will contact the participant to ascertain whether the participant would like to continue with the study.

Phase 2 (Visit 8, Days 29-42)

Participants will be asked to deliver a faecal sample before starting Phase 2. As before, they will be given an opportunity to produce this sample at the QI CRF if they were unable to do so before. Participants will be asked if they are happy to continue on with the study, and whether they have had any changes to their health since the end of Phase 1, and this will be addressed in the form of a follow-up health questionnaire. A QI CRF Research Nurse will take a 15 mL blood sample for ferritin and CRP analysis. For Phase 2, participants will be given a bottle containing the alternative set of capsules to what they consumed during Phase 1. All other aspects of Phase 2 are identical to Phase 1 – please refer to ‘Phase 1’ for these details.

Mid-Phase 2 (Visit 9, Day 35)

Please refer to section titled ‘Mid-Phase 1 Faecal sample drop-off’ for more details.

End of Phase 2 (Visit 10, Day 42)

At the end of Phase 2, the participants will be asked to deliver a final faecal sample to the QI CRF in the faecal collection kit provided during Visit 9. When the participants are at the QI CRF, a QI CRF Research Nurse will take a final 15 mL blood sample to test for ferritin and CRP levels. The participants will be asked to return the bottle containing the capsules that was given to them at the beginning of

Phase 2, including any capsules that have not been consumed. Also, the participants will be asked to return the completed forms (food frequency questionnaire, stool chart and capsule checklist), and this will mark the end of the study.

Note: *if a participant is unexpectedly unable to attend a pre-arranged assessment at the end of a 2-week treatment period, depending on their next availability, they will be asked to come in either a couple days before/after the scheduled visit. If the participant is not available till after more than 3 days of the scheduled visit, they may be asked to repeat the treatment. Furthermore, if the participant intends to go on holiday/travel whilst taking part in this study, we will require them to remain in the UK during both Phase 1 and 2. They may travel during the washout phase.*
Below is a brief breakdown of the overall samples/forms that will be collected from the participant through the course of this study:

Blood samples (all taken at QI CRF by QI CRF Research Nurse) Total number: 5 samples, amounting to 80 mL (16 teaspoons)

- **Sample 1**: Eligibility screening, 20 mL, results sent to GP. Analysed by NNUH Pathology Laboratories. Testing for ferritin, CRP, full blood count and HBA1c.
- **Sample 2**: Start of Phase 1, 15 mL, results analysed in QIB laboratories. Testing for ferritin and CRP
- **Sample 3**: End of Phase 1, 15 mL, results analysed in QIB laboratories. Testing for ferritin and CRP
- **Sample 4**: Start of Phase 2, 15 mL, results analysed in QIB laboratories. Testing for ferritin and CRP
- **Sample 5**: End of Phase 2, 15 mL, results analysed in QIB laboratories. Testing for ferritin and CRP

Faecal samples
Total number: 6 samples

- **Sample 1:** Before starting Phase 1
- **Sample 2:** Midway Phase 1
- **Sample 3:** End of Phase 1
- **Sample 4:** End of washout phase/before starting Phase 2
- **Sample 5:** Midway Phase 2
- **Sample 6:** End of Phase 2

Stool charts

Total number: 2 charts, each recording 7 consecutive days from Phase 1 and 2

- **Stool chart 1:** During Phase 1
- **Stool chart 2:** During Phase 2

Capsule checklist

Total number: 1 checklist for entire study

Food frequency questionnaires

Total number: 2 questionnaires, each recording 7 consecutive days from Phase 1 and 2

- **Questionnaire 1:** During Phase 1
- **Questionnaire 2:** During Phase 2

Sample/Form collections

Blood collection

For the eligibility screening, a venous blood sample (20 mL) will be taken by a QI CRF Research Nurse for assessment of a full blood count, HbA1c, CRP and ferritin levels. Eligibility screening bloods will be sent to NNUH Pathology Laboratories for analysis. All eligibility screening results will be sent to the participant's GP. The blood samples taken during the course of the study (4 x 15 mL samples) will be used for research purposes. These blood samples will be used to ascertain iron status (serum ferritin) and to confirm lack of chronic inflammation/infection (CRP). These samples will be analysed at QIB laboratories.

Faecal collection

The faecal collection kit will consist of a bag containing a faecal collection pot, an autoclavable sample bag for collecting the faeces in, a plastic clip (for sealing the autoclavable bag), nappy sack and an insulated container for transporting the sample securely back to QI CRF. The participant will also be provided with study specific instructions (Annex 17) as to how to collect and store the faecal sample and how to contact members of the study team or QI CRF Research Nurse regarding arrangements for collection or delivery of the faecal sample to the QI CRF. These samples will be analysed at QIB laboratories

Detailed instructions will be provided as to how to collect their faecal sample directly into the labelled plastic autoclavable sample bag in the collection pot. Once the sample has been collected the autoclavable sample bag should then be sealed closed with the plastic clip, removed from the collecting pot and placed into the nappy sack which should be closed by tying the handles in a double knot. The tied nappy sack, containing the sample should then be sealed shut in the insulated container. Participants will be advised to wash their hands after collecting the samples. Participants will be asked to write the date and time of sample collection on a label on the lid of the insulated container and contact a member of the study team to arrange delivery or collection of the sample. Should the participant forget to bring the sample with them, they will be given the opportunity to produce one at the QI CRF. If they are unable to deliver the faecal samples themselves on assessment days, arrangements will be made for a member of the study team to collect the sample from the participant at a convenient time.

All containers used for faecal collections will be sterilised prior to being given to the participant, to prevent contamination and minimise infection risk. The importance of hand washing after sample collection by participants will be reiterated in the faecal collection instruction sheet.

It is hoped that the participants will be able to bring their faecal sample to a member of the study team at the QI CRF, however it may be that a participant requires a member of the study team to collect their faecal sample, and this will be arranged on an individual basis.

When receiving a faecal sample, a member of the study team will collect the entirety of the faecal collection kit from the participant, except for the sample collection pot which will have been disposed of.

Identical faecal collection kits and instructions will be provided to the participants prior to the collection point of all further faecal samples, and participants will be asked to collect the faecal samples in an identical manner to the first faecal sample. Should any participant suffer from diarrhoea during the study, they will be required to contact a member of the study team and will be asked to refrain from collecting a faecal sample for a minimum of 48 h after the last episode of diarrhoea. Should the diarrhoea persist for more than 72 h, the participant may be advised by the QI CRF

Research Nurse to speak to their GP. The participant may be excluded from the study and this decision will be at the discretion of the QI medical advisor.

Stool chart

During the study the participants will be asked to keep a record of the frequency and consistency of their bowel movements using the Bristol Stool Chart as a guide (Annex 18). The participants will be asked to keep this record for a period of seven consecutive days during each of the two Phases. These scores will be used as an estimate of gut function. If the participant's stool chart indicates an abnormal stool pattern, they may be advised by the QI CRF Research Nurse to speak to their GP, who will receive a copy of the participants stool chart. Any participant who records two or more episodes of type 1, 2 or 7 stools, or the presence of blood in their stools, on their stool chart, will be excluded and a copy of their stool chart will be sent to their GP. The participant may be excluded from the study and this decision will be at the discretion of the QI medical advisor.

Food Frequency Questionnaires

The participants will be asked to complete 2 food frequency questionnaires, one during each treatment Phase. They will need to complete both these questionnaires for a period of 7 consecutive days. This will be used to assess participant's habitual diet over the trial period and whether their food habits correlate with, or are reflected in, the outcome. It will also give an indication of any participants with a naturally high, phytin-based diet.

Capsule Checklists

The participants will be asked to complete a capsule checklist for the entirety of the study. This will (i) help them to remember to take the capsules at the appropriate time-points and (ii) serve as a measure of compliance.

Completion of the study

Upon completion of the study, the general findings of the study will be reported back to the participants in the form of a basic summary.

Withdrawal from the study

Participants will be withdrawn from the study if the participant, for any reason, loses capacity to consent during the study, or their medical situation changes. Identifiable

data or samples already collected with consent would be retained and used in the study. No further data or sample would be collected, or any other research procedures carried out on or in relation to the participant. If at any point during the study the participant wishes to withdraw, they may do so without giving a reason and their clinical care and participation in future studies at QIB will not be affected. The participant will be sent a withdrawal letter (Annex 19) explaining this and thanking them for their participation so far. Again, any samples or data collected up to the point of withdrawal will be kept and used in the study if possible.

Adverse event (AE) and Serious adverse events (SAE)

This study will comply with the NNUH Trust system for reporting adverse events and will adhere to the NNUH SOP 206 (Adverse Events: Identifying, Recording and Reporting adverse events for Non-CTIMP Healthcare Research Studies). AEs will be evaluated in terms of seriousness, relatedness and expectedness by QI CRF nurse and QI medical advisor. All adverse events/reaction that are not considered serious will be documented on the relevant case report forms (CRFs) (Annex 20). The completed form will be filed along with the other CRFs for the study and a copy provided to the Sponsor. SAEs/SARs will be reported on the NNUH SAE form (Annex 21). SAEs will be notified by the CI to the Sponsor within 24hrs of the CI becoming aware of the event. This will be followed within 48hrs of becoming aware of the event by a detailed, written report provided by the QI medical advisor. SAEs will be notified by the CI to the REC where in the opinion of the QI medical advisor it was possibly, probably or definitely related, within 15 days of the CI becoming aware of it. The CI will report all logged events to the REC annually as a Safety Report; a copy of this report will be provided to the QIB HRGC. The CI will report all logged events to the NNUH R&D. All SAEs will be followed up by the QI medical advisor until satisfactory resolution, and this should be recorded as a Follow Up report on the SAE form, and on the SAE log. At each stage of follow up the QI medical advisor will sign and date the form.

A serious adverse event (SAE) is defined by ICH GCP as an untoward occurrence that:

- Results in death
- Is life threatening
- Requires hospitalisation or prolongation of existing hospitalisation
- Results in persistent or significant disability or incapability

- is otherwise considered medically significant by the QI medical advisor

The participant will only be deemed to have suffered an adverse event/reaction or a serious adverse event/reaction if the participant has taken part in any stage of the intervention.

Methods

Gut microbiome analysis

The faecal samples produced by the participant will either be delivered to the QI CRF by the participant, or a member of the study team will arrange to go and collect the samples. Samples delivered at the QI CRF will be safely transported to the QIB laboratories facilities located within the same building. Upon receipt of a faecal sample, part of it will be divided up into aliquots totalling no more than 10g and stored at –80°C until analysis. The DNA will be extracted from faecal aliquots using the FastDNA spin kit for soil (MP Biomedicals) according to the method of Maukonen *et al* [55] and stored at –20°C. Part of the extracted DNA from the faecal samples will have the 16S rRNA genes amplified by PCR, followed by sequencing using a Next Generation platform such as Illumina, in-house at QIB. Faecal bacterial phylogenetic analysis will give both the bacterial genera present and the relative proportions of each genus within the sample. This will make it possible to observe shifts in the proportions of various bacterial taxa, namely Enterobacteriaceae and Bifidobacteriaceae, in the faecal microbiome of each participant, due to a) the consumption of the different capsules (either test or placebo) compared to one another, and b) the consumption of the different capsules (either test or placebo) compared to the baseline sample obtained at the start of the study. This information can then be compared with the iron quantification data and used to try and determine whether the faecal microbiome composition correlates to the concentrations of iron.

Faecal metabolite analysis

Short chain fatty acids are bacterial waste products that are beneficial to the host, and as such can be considered a biomarker of good gut health. Nuclear magnetic resonance spectroscopy (NMR) or MS-based techniques such as HPLC or LC-MS will be used to determine the profile of metabolites, such as short chain fatty acids, from faecal aliquots. Faecal aliquots of 0.2 g will be diluted using 12 x volume of NMR buffer and homogenised. This will then be centrifuged at 3200 xg for 15 mins at

4ºC, before 700 µl of the supernatant is added to an NMR tube for spectral acquisition. Data analysis will be performed at the QIB.

Faecal calprotectin analysis

The inflammatory status of the patient at the time of faecal sample collection will be assessed. Calprotectin is a biomarker that is present in the faeces when intestinal inflammation is present. An aliquot of the faecal sample provided by the participant will be used to determine calprotectin levels using a commercially available kit. Briefly, the faecal aliquot will be placed in disposable vials. After homogenisation and centrifugation, an extraction buffer will be added to the aliquots as per manufacturers instruction and analysed using a commercial ELISA kit at OD 405 nm against a standard curve. These samples will be analysed at QIB laboratories.

Faecal iron analysis

A 20 µL aliquot of the faecal water will be used to quantify iron concentrations of the faecal samples using the ferrozine assay as per manual instructions (Iron Assay Kit ab83366, Abcam, UK). Briefly, iron in the sample is reduced using an Fe reducer, provided by the kit, after which iron reacts with Ferene S (an iron chromogen) to produce a stable coloured complex. Absorbance measurements will be taken at 593 nm. These samples will be analysed at QIB laboratories.

Faecal phytin analysis

Faecal aliquots will be used to determine faecal phytin levels using a commercial kit (Total Phosphorus Assay kit; Megazyme). These samples will be analysed at QIB laboratories.

Blood analysis

A venous blood sample (20 mL) will be taken by a QI CRF Research Nurse and sent to NNUH Laboratory for assessment of iron status and to confirm lack of chronic inflammation/infection (haemoglobin, ferritin, and C-reactive protein) for the sample taken for the eligibility screening. For the remaining blood samples taken throughout the study, bloods (15 mL) will be analysed within QIB laboratories. A total of 80 mL (16 teaspoons) of blood will be taken from each participant throughout the course of this trial.

The data analyst will be blind to the conditions of the study until all analysis is fully completed.

Statistics:

Statistical analysis:

The primary outcome measure (relative abundance of Enterobacteriaceae) will be compared between placebo and treatment phases within each patient using a linear mixed model regression analysis, with appropriate transformations to ensure normality of residuals. The faecal samples collected during Phase 1 and 2 (mid and post samples for each phase) for each participant will be included as data points. Models will include the fixed effects of time period, (Phase 1 vs Phase 2) and treatment (placebo vs active), and the random effect of participant. The primary hypothesis will be tested with a two-sided test at $p<0.05$.

Power calculations:

Prior data from our lab using the same outcome measure and similar participant criteria suggests that the within-person variation in Enterobacteriaceae abundance has a standard deviation of 1.743 on the logit scale. Using two samples per-person per-treatment, this means that the within-participant difference between mean active vs mean placebo abundance will also have a standard deviation of 1.743 across participants. With a sample size of 14 completers there will be 80% power to detect a within participant difference of 1.41 on the logit scale. Although there are no directly comparable prior estimates of these effects, our *in vitro* data suggests the change in the abundance of Enterobacteriaceae upon phytin is around 1.91. Allowing for uncertainty in the precision and applicability of these estimates and the possible occurrence of unusable data due to technical failure we will aim to recruit 14 participants.

Power calculations were conducted using R statistical software version 3.5.0.

Data Protection and Participant Confidentiality

Participants who are successfully recruited onto the study will be assigned a unique code number which will be kept in a secure file. A lockable filing cabinet or cupboard will be used to keep paper documents that include the file linking the participant to

the code and personal information. All electronic data will be stored on a password protected shared data file. Confidential data will be accessed only by the study team. Participants' personal data will be held in a locked cabinet in QI CRF. Participants' personal data will also be held in a locked cabinet or password protected electronic file in QIB. Only the study team will have access to these data. The samples will be known only by their code number. All data collected will also be identified by code only. Data will be stored for 15 years after completion or discontinuation of the study. These data will not be used to contact research participants after the study is completed. The data will be stored in the QIB human studies archive. Access to archived data will be limited to the study scientist and Chief Investigators (CI) of the study or the CI's successor. The quality assurance auditors may also be allowed access with the permission, and in the presence, of the CI. The main computer storage will be on one main QIB computer, but as part of a password protected shared network. All QIB computers are individually password protected and the shared network access is limited to those working within the research area. Only the study scientists will have access to the file linking personal data to the participants' unique code. Manual files/folders will consist of separate named and numbered files for each participant. No data with the participants' name will be filed in the numbered file and vice versa.

Data Sharing and Access

The research protocols will be registered in a publicly accessible database after gaining favourable ethical opinion. Registration to ClinicalTrials.gov Protocol Registration and Results System (PRS) using QIB account will allow us to be transparent in our work.

Definition of End of Study

The end of the study is the date of the last visit of the last participant

Ethical considerations:

Informed Consent

Before participation in the intervention study, all participants will be asked to give written informed consent for study participation, and the long-term storage of samples at the Norwich Biorepository. Prior to consent being given, the participant

will be provided with the Participant Information Sheet by the QIB Volunteer Database Manager, which provides all the information about their involvement in the study and we will ensure that all their questions are answered. The ability of participants to give informed consent will depend on them receiving enough information about the study, the participant exercising their right to choose, the participants ability to understand the information and the ability of the participant to make a decision.

Use of samples in future research:
Participants will sign a consent form agreeing to store their samples long-term at the Norwich Biorepository (Annex 7), which holds a Human Tissue Authority licence (IRAS no: 130478, East of England - Cambridge East Research Ethics Committee 08/h0304/85+5). Once the study has ended, blood and stool samples will be transferred to the Norwich Biorepository by the study team. Participants will be able to take part in the study even if they do not want their samples to be stored at the Norwich Biorepository. Storage of samples at the Norwich Biorepository after this study has ended will enable further data collection from this study (if needed) and use of the samples in future research. It is important to stress that any further analysis will be carried out in compliance with ethical requirements.

Data Protection

This study will comply with the new EU General Data Protection Regulation (GPDR), which came into force in the UK on 25 May 2018 and the UK Data Protection Act (DPA) 2018, with regards to the collection, storage, processing and disclosure of personal information and will adhere to the GPDR and DPA core principles to maintain confidentiality.

Procedures for any harm experienced by participants

If, throughout the period of this human intervention trial, any participant is harmed by taking part, there are no exclusive compensation privileges. If, due to negligence, harm is caused to the participant and there are grounds for legal action, the participant will likely have to pay for these legal costs.
We appreciate that under specific circumstance, participants may still wish to file a complaint, and in this case, a confidential service which is designed to support patients, relatives and carers will be available to them. This service is the Patient Advice and Liaison Service (PALS) and the website can be found here:

http://www.nnuh.nhs.uk/patients-visitors/help-support/pals/. QIB has liability insurance (Annex 22) with regards to research involving human participants. Please note that the Institute will not fund any legal costs arising from any action unless awarded by a court.

Furthermore, as this study involves the QI CRF, which is an NHS facility, indemnity is provided through NHS schemes.

Participant wellbeing throughout the trial

Throughout the study, the participant will be frequently asked whether any health situations have risen since their participation in the study, in the form of an 'Assessment Day Checklist' (Annex 23), usually at the start and end of each Phase. If any participant becomes unwell at any stage of the study, the first point of action will be to see their GP or A&E. GPs will be informed about the participants's involvement in this study by letter and will receive copies of the PIS. The PIS will advise participants to contact the emergency service (via 999) in case of a medical emergency, and ensure that study team is contacted as soon as practically possible. The decision to exclude the participant from the study will be taken by the QI medical adsvisor.

Capsule safety

The QI CRF follows local Environmental Health Guidelines for the preparation and storage of capsules for study participants. All staff handling, preparing or delivering capsules for the participants will hold Level 2 Food Safety.

Toxicity

No cases of phytin toxicity has been reported, but please refer to 'Dosage and Toxicity' for more information regarding this.

Participant's expenses/inconvenience payments

Eligibility screening urine sample (x1)	£2
Individual stool collection (x6) □ 6 x £5	£30
14-day Stool chart □ 14 days at £2 per day completed	£28
168x capsule consumption □ 168 capsules at £1 per capsule consumed	£168
Blood samples (x5) □ 5 x £10	£50

TOTAL = **£278**

Participants will receive £278 as an inconvenience payment if the study is completed; if the participant withdraws or is excluded from the study, *payment will be pro-rata*. However, if the participant undergoes rescreening the inconvenience payment could total £280. Participants travelling by car will be reimbursed travel expenses to and from the QI CRF. This will be reimbursed at the QIB's current mileage rate. Those participants travelling by public transport will be reimbursed costs on production of a ticket or receipt. If participants require transport, the study can provide a taxi to and from QI CRF. This is paid for by the study.

Study partners

The study will be sponsored by the QIB and funded through the Biotechnology and Biological Sciences Research Council (BBSRC); this study was funded by the BBSRC Institute Strategic Programme Food Innovation and Health QIB.

The study will be led by Professor Arjan Narbad. All aspects of the study will be managed by Miss Bhavika Parmanand with assistance from Dr Lee Kellingray and Professor Susan Fairweather-Tait where necessary.

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[Insert Date]

Dear_____,

Thank you for your interest in research studies at the Quadram Institute Bioscience.

I have sent you the details of,

Short title: Effect of Phytin on Human Gut Microbiome (EPoM Study)

which is one of the studies in progress at present, as your details currently held on the database indicate that you may fit the criteria for this study. If you are interested in participating in this study, please complete and return the reply slip in the enclosed participant information sheet. If you have any further questions, please contact the study manager concerned, Miss Bhavika Parmanand on **01603 255021** or **bhavika.parmanand@quadram.ac.uk** as stated on the enclosed participant information sheet.

If, however, any of your details have changed or change in the future, or you would prefer to no longer remain on the database please could you inform the QIB Volunteer Database Manager on 01603 255051.

Thank you.

Yours sincerely,

Wendy Hollands
QIB Volunteer Database Manager
Wendy.hollands@quadram.ac.uk

Participant Information Sheet

Short title: Effect of Phytin on Human Gut Microbiome
(EPoM Study)

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve for you. Please take time to read the following information carefully. Talk to others about the study if you wish.

Part 1 tells you the purpose of this study and what will happen if you take part
Part 2 gives you more detailed information about the conduct of the study

Please ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. This information sheet is yours to keep.
Thank you for reading this.

This study is funded through Biotechnology and Biological Sciences Research Council (BBSRC); this study was funded by the BBSRC Institute Strategic Programme Food Innovation and Health QIB.

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Study Mobile number (to be included when available)

PART 1

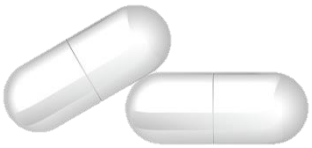
What is the purpose of the study?

Within many plants, such as seeds, nuts and cereals, there is a compound called phytic acid. Phytic acid has many beneficial properties, including producing molecules which slows down the damage that can be caused to other molecules within the body. Phytic acid has also been known to help in the treatment of cancer.

Phytic acid binds iron very strongly. Iron is an extremely important nutrient not only for humans, but also for a lot of bacteria. In humans, iron is absorbed in the small intestine. Unfortunately, iron does not get absorbed very well and so a lot of it travels into the large intestine. The large intestine contains trillions of bacteria and a lot of these bacteria use iron as food. However, not all bacteria in the large intestine are 'good bacteria'. Some bacteria, such as Enterobacteria, can be harmful to people's health. For this reason, if iron is kept away from these 'bad bacteria' through the binding of phytic acid and iron, it could prove to be beneficial to human health.

In general, our gut contains trillions of bacteria, many of which help us to unlock extra nutrients from the food we eat. Some bacteria, such as Bifidobacteria, are often referred to as 'good bacteria' and are added to foods such as yoghurts. Many 'good bacteria' are able to survive without iron and this makes it even more important to make sure the 'bad bacteria' have limited access to iron. Otherwise, we could find ourselves with a large intestine that has more harmful bacteria than beneficial bacteria.
samples to see if there is a relationship between iron and phytin levels.

In this study, we will ask you to consume either the test capsule, which contains phytin (a salt form of phytic acid), or a control capsule, which contains a powder resembling phytin but is actually an inactive substance. We are interested in whether consuming these capsules will decrease Enterobacteria (one of the 'bad bacteria' in the large intestine).



What we aim to do

Along with looking at whether these capsules cause a change in the number of Enterobacteria, we will also be looking to see whether this in turn increases the number of 'good bacteria', such as Bifidobacteria.

Furthermore, we want to make sure that the capsules only take effect in the large intestine. This is why we have made capsules that act in the large intestine only. As mentioned before, we know that phytic acid binds iron. Therefore, to double check that the phytic acid hasn't been released before the large intestine, we will take blood samples throughout the study to check your iron levels.

Finally, not only do we want to look at Enterobacteria and Bifidobacteria – we want to take a look at your gut bacteria as a whole community (like we said, there are trillions of bacteria!). So, to do this, we will ask you to provide us with faecal samples throughout the study. We will then extract the bacterial DNA from these samples (your own DNA will not be looked at, only bacterial DNA) and use this data to find out whether other types of bacteria in your large intestine are affected by the capsules. At the same time, we will also measure the levels of iron in your faecal

Why have I been invited?

You have received this information sheet because either you have responded to an advertisement about this study, or your details are currently held on the Quadram Institute Bioscience (QIB) volunteer database.

Who can take part in the study?

We are aiming to recruit a total of 14 volunteers (male and female) who meet the following requirements:

- Aged between 18 and 50
- Non-smokers (e-cigarette and vape users are able to partake in the study)
- Have a body mass index (BMI, kg/m^2) between 19.5 and 30
- Do not fall in the study exclusion criteria (found in the next section)

If you register an interest in taking part, and we are near to the 14-participant point, we may ask you if you are happy to be placed on a standby list. If you are put on the list, there will be no guarantee that you will be required to take part in the study, but we will keep you informed, and you are entitled to ask to be removed from the standby list at any time.

Who cannot take part in the study?

You will not be able to take part if you(r):

- results of our screening test indicate you are not suitable to take part in this study
- are pregnant, have been pregnant in the last year or are lactating and/or breast feeding
- are currently suffering from, or have ever suffered from, any diagnosed gastrointestinal disease, gastrointestinal disorders including regular diarrhoea and constipation (excluding hiatus hernia unless symptomatic), and/or have undergone gastrointestinal surgery, or the study intervention/procedure is contraindicated
- have been diagnosed with any long-term medical condition that may affect the study outcome (e.g. cancer, diabetes, haemophilia, cardiovascular disease, glaucoma, anaemia). These will be assessed on an individual basis
- have been diagnosed with any long-term medical condition requiring medication that may affect the study outcome.
- regularly taking over the counter medications for digestive/gastrointestinal conditions.
- are on long-term antibiotic therapy. You may be able to participate if 4 weeks has passed from the end of a course of antibiotics (this will be assessed on an individual basis).
- regularly take laxatives (once a month or more).
- take certain dietary supplements or herbal remedies and are unwilling to stop taking them for one month prior to and during study period. This will be assessed on an individual basis.
- take pre- or pro-biotic drinks &/or yoghurts on an occasional basis, unless willing to abstain for one month prior to and during the study period. (if you regularly take pre-&/or pro biotics (3+ times a week, and for more than one month) and will continue throughout the study then you will not be excluded)
- are on or plan to start a diet programme that may affect the study outcome (e.g. 5:2 fasting diet) unless willing to abstain for 1 month prior to and during study period. This will be assessed on an individual basis
- recently returned to the UK following a period abroad, and who have suffered gastric symptoms during the period abroad or on return to the UK. These will be assessed on an individual basis
- regular/recent (within 3 months) use of colonic irrigation or other bowel cleansing techniques
- are involved in another research project that includes dietary intervention or involving blood sampling
- record blood in your stools or have two or more episodes of constipation

or diarrhoea (type 1, 2, or 7 stools) during the study
- are unwilling to provide GPs contact details
- are unable to provide written informed consent
- are related to or living with any member of the study team
- regularly consume more than 15 units (women) or 22 units (men) of alcohol a week
- Regularly taking iron supplements
- Those unable to swallow capsules
- Those with abnormal blood pressure measurements (160/100 will be regarded as an exclusion value)
- Are related to someone in the study (e.g. spouse, partner, immediate family member)

Do I have to take part?

It is up to you to decide whether you would like to take part or not. We will describe the study in this information sheet and if, after reading it, you are interested in participating and meet the study criteria, please complete the response form using the pre-paid envelope enclosed. On receipt of the response form, a member of the study team will contact you by telephone to arrange a study talk with you at the QI CRF in Norwich and give you the opportunity to ask any questions you may have at this time.

You may also contact the study team by telephone, Bhavika Parmanand on 01603 255021; Dr Lee Kellingray on 01603 255070 or by email bhavika.parmanand@quadram.ac.uk or lee.kellingray @quadram.ac.uk if you have any questions before or during the study. Please feel free to say no simply by not responding to this letter. Do not worry, no one will contact you or try to persuade you to join the study. If you are on the QIB volunteer database, a decision to withdraw or not to take part will not affect your participation in future studies. Finally, an expression of interest does not commit you to taking part.

What happens if I decide to take part?

If you decide to take part, your involvement in the study will last about 8 weeks depending on your availability for the study visits. You will be required to visit the QI CRF on 10 separate occasions (4 visits before the study starts and 6 visits during the study). Where possible, appointments will be made at your convenience but will take place on a weekday during QI CRF opening hours.

Below are the QI CRF visits outlined in more detail, explaining the study along the way. The QI CRF is located at James Watson Road, Norwich, NR4 6UQ.

Pre-study talk (Visit 1)

This meeting will last approximately an hour. A member of the study team will go through this information sheet with you and answer any questions you may have. After this meeting, you will be given as long as you need to decide whether or not you wish to take part in the study. This period will be a minimum of 72 hours. If you decide to take part in the study, you will need to contact a member of the study team (details on front page of this information sheet) to arrange an appointment for the next visit.

After the talk, you will be given a copy of the Bristol Stool Chart and a small container for urinalysis. Should you decide not to take part in the study, you may dispose of these as you see fit. If you do join the study, we would like you to use the Bristol Stool Chart to identify what your typical stool type is, as you will be asked this at your screening visit by the QI CRF research nurse. The container will be used for a urine sample, which you will need to bring to your next visit.

Note: Where possible, for females, the screening visit will be arranged for 7 days after their last period.

Eligibility screening and Informed Consent
(Visit 2)

This visit can last up to 1.5 hours. You will need to bring a midstream urine sample ***in the container that we gave you during Visit 1***. If you use any other container, this could affect your results and therefore your eligibility to take part in the study. If you have lost the pot, we will provide you with another at your appointment and give you the opportunity to produce a urine sample during the visit.

Before we carry out the eligibility assessment, you will be asked to sign two consent forms agreeing to (i) participate in the study and (ii) have your samples stored in the Norwich Biorepository. These forms will also be signed by a member of the study team (study manager or study scientist) or the QI CRF Research Nurse. You will also complete a medical declaration form with the QI CRF Research Nurse, which you will be given a copy of. Once you have signed the consent forms you are still free to withdraw at any time without giving a reason.

After you have signed the consent forms, the QI CRF research nurse will carry out a urine dipstick test on your urine sample. The results of this will be known immediately and the CRF Research Nurse will discuss the results with you. Depending on your urine dipstick test results you may be excluded from the study or offered a re-screen and advised to speak to your doctor or surgery nurse about your results. All abnormal results will be referred to the QI CRF Medical Advisor. In an event of flagged urinalysis result, the decision to exclude you from the study will be taken by the QI medical advisor.
A QI CRF research nurse will then complete a brief eligibility screening questionnaire with you and also measure and record the following:

- Blood pressure
- Pulse rate
- Height
- Weight
- BMI

Your height and weight will be used to calculate your BMI. BMI is a measure of whether you are a healthy weight for your height. If your BMI is outside the range of 19.5 and 30 kg/m^2 you will not be able to take part in the study.

The QI CRF research nurse will then take a 20 mL (roughly 4 teaspoons) blood sample from a vein in your arm. This blood test will measure your full blood count, iron levels, CRP (a marker for inflammation) and blood sugar levels. These will be assessed for anything outside the standard reference ranges and ensure you are eligible to participate in the study. Bloods will be tested at the NNUH Pathology Laboratories. The turnaround time for the results is roughly a week.

We will send copies of all your clinical results (urine and blood results, blood pressure, pulse, BMI and weight) to your GP. If any of your clinical results are outside the standard reference ranges, we may recommend that you speak to your GP about the results. Any results outside the reference ranges will be checked by the QI medical advisor and will be in charge of making any decisions regarding blood/urine abnormalities. The QI medical advisor will also advise whether we will offer you the opportunity to re-screen in the event of abnormal results. If, on the second occasion, your results fall outside the standard reference ranges, you may be excluded from the study based on the results flagged. We cannot tell you what your results means as we are not medically trained to do so. Finally, please remember, these tests are performed to determine if you are suitable for the study, not to find out if you are healthy.

Please note, if you do not commence the study within 3 months of your eligibility screening, you will have to be re-screened should you wish to take part.

Faecal sample collection kit pick-up (Visit 3)
This visit will last for approximately 20 minutes. You will be invited to take part in this study if the blood and urine tests from the eligibility screening is satisfactory, and you meet all the listed criteria for participation. You will be asked to collect a faecal sample collection kit, along with a detailed sheet on how we would like you to collect and store your faecal sample until you can deliver it to us, or it can be collected by a member of the study team. Everything you need to collect the sample will be provided within the kit.

Faecal sample drop-off (Visit 4)
This visit will last for approximately 10 minutes. You will be asked to bring a faecal sample to the QI CRF in the faecal collection kit provided during Visit 3. After you have given us your first faecal sample, which will be used to give us an idea of the bacteria present in your large intestine, we will arrange a time and date for you to collect the first batch of capsules and commence the study.

The study
There are three phases in this study, and you do not need to be fasted on any days during any of these phases. Each phase will last for a period of 14 days. These phases are described below:

Phase 1: 2 capsules containing either 0.4 g phytin or control powder, 3 times a day. Therefore, a total daily intake of phytin consumed during Phase 1 is 2.4 g.
Washout phase: no capsules consumed during this phase
Phase 2: 2 capsules containing either 0.4 g phytin or control powder, 3 times a day. Therefore, a total daily intake of phytin consumed during Phase 2 is 2.4 g.

The order in which you consume either the phytin or control capsules will be 'randomly' assigned by a computer. By this, we mean that the order is assigned by a method similar to being picked out of a hat. Neither the study scientist nor you will know in which order you are consuming the capsules. Also, both types of capsules will look identical.

During Phase 1 and Phase 2, you will be asked to complete two forms, for a consecutive 7 days each. The first is a stool chart and the second is a food frequency questionnaire. The stool chart will help us gauge the frequency and consistency of the stools you produce. The food frequency questionnaire will be used to assess your habitual diet over the study period and whether your food habits correlate with, or are reflected in, the study outcome.

Phase 1 (Visit 5, Days 1-14)
This visit will last for approximately an hour. When you visit the QI CRF to begin Phase 1, you will be asked to provide a faecal sample if you have not done so already (Visits 3 and 4). You will be asked if you are happy to continue with the study, and whether you have had any changes to your health (this will be addressed in the form of a follow-up health questionnaire) or medication since your screening appointment which may affect the study data. A QI CRF research nurse will take a 15 mL blood sample (to test for iron and CRP).

As mentioned earlier on in "The Study", you will be required to consume 2 capsules (randomly assigned), 3 times a day with a meal. The first 2 capsules of each phase must be consumed at the QI CRF and remain at the QI CRF for a minimum of 20 minutes (food will be provided on this occasion). After you have consumed the first 2 capsules, you will be provided with a bottle containing the rest of

the capsules required for Phase 1. This bottle will include extra capsules in case any are lost/misplaced.

You will be given a new faecal collection kit before you leave the QI CRF for the next stool sample you will provide. You will also be given a form with the Bristol Stool Chart to record your stool frequency and consistency, along with a food frequency questionnaire. Please remember to fill both these forms for a consecutive 7 days during Phase 1 (these can be any 7 days you wish). Finally, you will be given a Capsule Checklist, where you need to tick the relevant box to show you have taken the capsules.

Mid-Phase 1 Faecal Sample Drop-off (Visit 6, Day 7)
This visit will last for approximately 10 minutes. Once you are halfway through Phase 1, we will need you provide us with a faecal sample. The faecal collection kit given at the end of your last visit (Visit 5) should be used to collect this sample. Once you have dropped this sample off, you will be provided with a new faecal collection kit for the next sample.

End of Phase 1 (Visit 7, Day 14)
This visit will last for approximately 30 minutes. At the end of Phase 1, you will be asked to deliver a faecal sample to the QI CRF in the faecal collection kit provided during Visit 6. When you are at the QI CRF, a QI CRF research nurse will take a 15 mL blood sample (to test for iron and CRP). You will also be asked to return the bottle containing the capsules that was given to you at the start of Phase 1, including any capsules that were not consumed during Phase 1. The completed food frequency questionnaire, capsule checklist and stool charts will be collected from you and finally, a new faecal collection kit will be provided.

Washout Phase (Days 15-28)
During the washout phase, you will continue your normal diet with no capsule consumption. At the end of this phase, you will be asked to provide us with a stool sample, delivered when you next visit the QI CRF.

Phase 2 (Visit 8, Days 29-42)
This visit will last for approximately 30-45 minutes. You will be asked to deliver a faecal sample before starting Phase 2. As before, you will be given an opportunity to produce a faecal sample at the QI CRF if you were unable to do so before. You will be asked if you are happy to continue with the study, and whether you have had any changes to your health (this will be addressed in the form of a follow-up health questionnaire) or medication since the end of Phase 1 which may affect the study data. A QI CRF Research Nurse will take a 15 mL blood sample (to test for iron and CRP). For Phase 2, you will be given a bottle containing the alternative set of capsules to what you consumed during Phase 1. All other aspects of Phase 2 are identical to Phase 1. Please refer to the section titled 'Phase 1' for details.

Mid-Phase 2 Faecal Sample Drop-off (Visit 9, Day 35)
This visit will last for approximately 10 minutes. Please refer to section titled 'Mid-Phase 1 Faecal Sample Drop-off' for more details.

End of Phase 2 (Visit 10, Day 42)
This visit will last for approximately 30 minutes. At the end of Phase 2, you will be asked to deliver a faecal sample to the QI CRF in the faecal collection kit provided during Visit 9. When you are at the QI CRF, a QI CRF research nurse will take a 15 mL blood sample (to test for iron and CRP). You will also be asked to return the bottle that was given to you at the start of Phase 2, including any capsules that were not consumed during

Phase 2. Finally, the completed food frequency questionnaire, capsule checklist and stool charts will be collected from you and this will mark the end of the study.
Please note, if you intend to go on holiday/travel whilst taking part in this study, we will require you to remain in the UK during both Phase 1 and 2. You may travel during the washout phase. If you are unexpectedly unable to attend a pre-arranged assessment at the end of a 2-week treatment period, depending on their next availability, you will be asked to come in either a couple days before/after the scheduled visit. If you are not available till after more than 3 days of the scheduled visit, you may be asked to repeat the treatment.

Below is a brief breakdown of the overall samples/forms that will be collected from you through the course of this study:

Blood samples
Total number: 5 samples, amounting to 80 mL (16 teaspoons)

- **Sample 1**: Eligibility screening, 20 mL, results sent to GP. Analysed by NNUH Pathology Laboratories. Testing for iron, CRP, full blood count and blood sugar.
- **Sample 2**: Start of Phase 1, 15 mL, results analysed in QIB laboratories. Testing for iron and CRP
- **Sample 3**: End of Phase 1, 15 mL, results analysed in QIB laboratories. Testing for iron and CRP
- **Sample 4**: Start of Phase 2, 15 mL, results analysed in QIB laboratories. Testing for iron and CRP
- **Sample 5**: End of Phase 2, 15 mL, results analysed in QIB laboratories. Testing for iron and CRP

Faecal samples
Total number: 6 samples

- **Sample 1:** Before starting Phase 1
- **Sample 2:** Midway Phase 1
- **Sample 3:** End of Phase 1
- **Sample 4:** End of washout phase/before starting Phase 2
- **Sample 5:** Midway Phase 2
- **Sample 6:** End of Phase 2

Stool charts
Total number: 2 charts, each recording 7 consecutive days from Phase 1 and 2

- **Stool chart 1:** During Phase 1
- **Stool chart 2:** During Phase 2

Capsule checklist
Total number: 1 checklist for entire study
Food frequency questionnaires
Total number: 2 questionnaires, each recording 7 consecutive days from Phase 1 and 2

- **Questionnaire 1:** During Phase 1
- **Questionnaire 2:** During Phase 2

A flowchart summarising the outline of this study can be found on page 14.

What happens if I become unwell during the study?

If you become unwell during the study, we may ask you to stop taking the capsules and repeat the Phase that you are in once you are better. This will, of course, depend on the nature of the illness and whether or not it will affect the study outcome.
In a medical emergency, you should contact the emergency service (via 999) and please ensure that your GP and the study team are informed as soon as practicably possible.

Access to your personal information

When you are screened for the study, you will be given a code number. This code number is unique to you and will be used to protect your identity and make your samples anonymous. Access to any information about you will be restricted to the research team, nurses at the QI CRF and your GP. There is more information about this in Part 2.

Expenses and payments

Participating in these studies is on a voluntary basis. However, we do recognise that taking part can cause some inconvenience and there are associated travel costs. Thus, you will receive £268 as an inconvenience payment; if

you withdraw or are excluded from the study, *payment will be pro-rata*. This means that you will be paid up until the point of withdrawal/exclusion from study. Travelling expenses to and from the QI CRF will be reimbursed on presentation of a receipt for buses or trains, or at the current QIB mileage rate for private cars. If you require transport to and from the QI CRF, please let us know and we will arrange and pay for a taxi.

All payments are liable to tax and you are responsible for declaring your own payments for tax purposes. Members of staff at QIB are free to participate in this study provided they meet the study criteria; however, we would like to point out that their inconvenience payment will be taxed at source in accordance with BBSRC and QIB rules and HM Revenue and Customs (HMRC). If you are in receipt of benefits this payment may affect your benefits.

What are the risks/side effects of participating in this study?

As with any pressure measurement (like blood pressure) the inflation of the blood pressure cuffs may cause slight discomfort and a reddening of the arm where the cuff is placed, but this affects some people more than others.

There can be a small amount of discomfort when taking blood samples. As mentioned before, this may affect some people more than others but generally, any discomfort occurs on insertion of needle. You may develop a small bruise at the site of injection, but as with any bruise, this will fade.

What are the potential benefits of taking part?

For you, there are no direct benefits however, your participation and subsequent results will help us to understand if there are any effects of iron limitation on the gut bacteria.

Who will carry out the medical procedures?

The study will be carried out in collaboration with the QI CRF. The QI CRF is an NHS-governed facility and all clinical procedures for this study will be carried out by the QI CRF team following NNUH standard operating procedures. Clinical assessment and procedures will be performed by two members of the CRF team when you are attending the CRF. This will include a registered nurse and another member of staff who is trained in NNUH emergency procedures. When no clinical assessment or interventions are to be performed (for example in the case of an appointment for consent), two members of the QI CRF team will also be present. This will include a Healthcare professional who is trained n NNUH emergency procedures and a second designated member of staff to provide support.

Will my taking part be kept confidential?

Yes – we follow Good Clinical Practice (GCP) and strict ethical and research governance rules. All information about you will be handled in confidence. More details about this are included in Part 2.

Data will be managed by the study team in compliance with EU General Data Protection Regulation (GPDR) and the UK Data Protection Act (DPA; 2018).

This completes Part 1 of the information sheet. If the information in Part 1 has interested you and you are considering taking part, it is important that you read the additional information in Part 2 before making any decision.

PART 2

What if relevant new information becomes available or changes to the study are made?

If there are changes to the study or new information becomes available, we will tell

you. If these changes are significant, you may be asked to sign another consent form.

What will happen if I don't want to carry on with the study?

You are free to withdraw from the study at any time without giving a reason. However, a study team member will need to be informed of your decision to withdraw. If you withdraw from the study, we will analyse your samples collected up to the point that you leave the study with those we obtain from all the volunteers, unless you decide otherwise. You will receive payment pro-rata for any samples, diaries etc. you have contributed.

How safe is it for me to ingest these capsules?

Phytic acid has been approved as a food additive and has been given 'generally regarded as safe' (GRAS) status for human consumption. There are no known reports of phytic acid toxicity via oral consumption in humans. The particular form of phytic acid (phytin) in the capsules that we will provide has not been directly tested for its safety, and is therefore the first trial, but it is regarded as just one of several forms of phytic acid and similar to numerous closely related forms that are widely consumed.

The daily dose of phytic acid you are being asked to consume is above the average daily intake in the UK population, which is around 0.7 g/day, although some people consume much more than this. The dose is similar to the amounts consumed by specific population groups such as vegans and vegetarians, and in other parts of the world (e.g. regions of Africa and Asia). The highest reported average intakes were 5.6 g/day.

What if there is a problem?

If you have any concerns about the study, you should ask to speak to the study manager, Bhavika Parmanand on 01603 255021 who will do her best to answer your questions. If you are still unhappy, and wish to complain formally, you can do this through the chairperson of the QI Human Research Governance Committee (HRGC) – Dr Antonietta Melchini on 01603 255030.

What if something happens to me while I am on the study?

QIB accepts responsibility for carrying out trials and as such will give consideration to claims from participants for any harm suffered by them as a result of participating in the trial, with the exception of those claims arising out of negligence by the participant. QIB has liability insurance in respect of research work involving human volunteers. Please note that the Institute will not fund any legal costs arising from any action unless awarded by a court.

If you wish to complain or have any concerns about the way you have been treated whilst taking part in this study at the QI CRF, there will be a local hospital complaints procedure that you can follow. If you wish to complain you should contact the Patient Advice and Liaison Service (PALS) at the NNUH on 01603 289036 (email: pals@nnuh.nhs.uk). Their offices are located next to Kimberley Ward, East Block Level 2 or please ask at the main reception desks at the Inpatient and Outpatient NNUH hospital entrances. The office has an answerphone which is available 24 hours a day and messages will be responded to as quickly as possible. As this study involves the QI CRF, which is an NHS facility, indemnity is provided through NHS schemes.

Will my taking part in this study be kept confidential?

All information collected about you during the course of the study will be kept strictly confidential. Any information leaving QIB, such as bacterial DNA extracted from stool samples for phylogenetic analysis, will be

anonymous. Study information will be stored in locked filing cabinets at the QIB. Personal data collected will be processed by computer, however only personal information that is essential for the study will be collected.

When you are screened for the study, you will be given a unique code number (volunteer code number). This number will be used to identify your samples and prevents anyone from working out whose samples are whose. Access to your personal records is restricted to the study team, the QI CRF Research Nurse and your GP.

The Quadram Institute Bioscience is the sponsor for this study based in the United Kingdom. We will be using information from you in order to undertake this study and will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly. QIB will keep identifiable information about you 15 years after the study has finished.

Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To safeguard your rights, we will use the minimum personally-identifiable information possible.

You can find out more about how we use your information by contacting the QIB Data Protection Officer [Mr Mohamed Imran, mohamed.imran@nbi.ac.uk] or QIB Human studies coordinator [Dr Antonietta Melchini, antonietta.melchini@quadram.ac.uk].
The only people in QIB who will have access to information that identifies you will be people who need to contact you to under emergency unblinding procedures or audit the data collection process. The people who analyse the information will not be able to identify you and will not be able to find out your name or contact details.

QIB will keep identifiable information about you from this study for 15 years after the study has finished.

Data will be managed by the study team in compliance with EU General Data Protection Regulation (GPDR) and the UK Data Protection Act (DPA; 2018).

All research is subject to inspection and audit. Although your records may be accessed for this purpose, any personal information remains confidential. Please note, QI has CCTV cameras in use for security purposes.

Will my GP be informed?

Yes, it is routine practice to inform your GP that you are taking part in a study at the QIB and we will send them:

- Details of the study (including the PIS)
- Your eligibility screening results including the dipstick urine test, blood test, blood pressure, pulse, weight and BMI
- Any stool charts that indicate abnormal stools or the presence of blood

This is one of the things you agree to when signing one of the consent forms (study participation). Any screening results which fall outside standard reference ranges will be assessed by the QI medical advisor. We are unable to discuss test results with you; however, you will be advised to speak to your GP about the results if deemed necessary.

What will happen to the samples I give?

The urine sample at QI CRF during the screening will be used immediately for a urine dipstick test and then discarded. The 20 mL blood sample you provide at the eligibility screening (Visit 2) will be sent to NNUH Pathology Laboratories for a full blood count,

iron levels, CRP levels and blood sugar levels. The purpose of this is to check for anything outside the reference ranges which may affect your well-being if you took part but also to make sure you fit the criteria for the study.

The blood and faecal samples that you provide during the course of the study (Phase 1 and Phase 2) will be used for research purposes. The blood samples taken throughout the study will be used to measure your iron levels. These measurements will be used as a baseline sample to ensure that iron levels are not affected by early release of capsules. CRP levels will also be analysed to ensure no systemic inflammation is present. The bacteria from your faecal sample will be collected and the different types of bacteria present will be determined by extracting bacterial DNA. As mentioned earlier on, your own DNA will not be looked at, only bacterial DNA. The faecal samples will also be used to examine changes in gut metabolites (these are substances that the body produces that are needed for cell survival) using nuclear magnetic resonance spectroscopy (NMR). NMR is a method that is able to separate the components of a mixture.

Once all the samples have been analysed, samples will be put into long-term storage at the Norwich Research Park Biorepository (this is the second consent form you would have signed at screening) ; which holds a Human Tissue Licence for this purpose (IRAS no: 130478, East of England - Cambridge East Research Ethics Committee 08/h0304/85+5). Samples may then be extracted from the Biorepository for use in future research projects where approval is ethically sought.

What will happen to the results of the research study?

As a volunteer you are valuable to us, but we are unable to tell you any of your individual results. The data resulting from the study may be published in scientific journals or presented at meetings with our funders. At the end of the study we will provide you with some feedback about what we have found as a result of your help and what it may mean for future research. Please note that the data is presented as a whole and is anonymous. Your name will not appear anywhere in any of the results presented, shared or published.

Who is organising and funding this study?

This study is funded through Biotechnology and Biological Sciences Research Council (BBSRC); this study was funded by the BBSRC Institute Strategic Programme Food Innovation and Health QIB.

Who has reviewed this study?

At QIB this research project has been reviewed by the QIB Human Research Governance Committee (HRGC), as well as an external Local Research Ethics Committee (REC). These are groups of independent people who review research to protect your safety, rights, well-being and dignity. This study has been reviewed by all committees and given a favourable opinion. Following ethical approval, the study protocol will also be registered at Clinicaltrials.gov.

Further information-what we need you to tell us

We need you to tell us some things for your safety and for the success of the study.

Please tell us if you:

- Have any episodes of illness, even if it is just a headache
- Are injured in any way
- Feel unwell during or after a visit to the unit
- Become pregnant

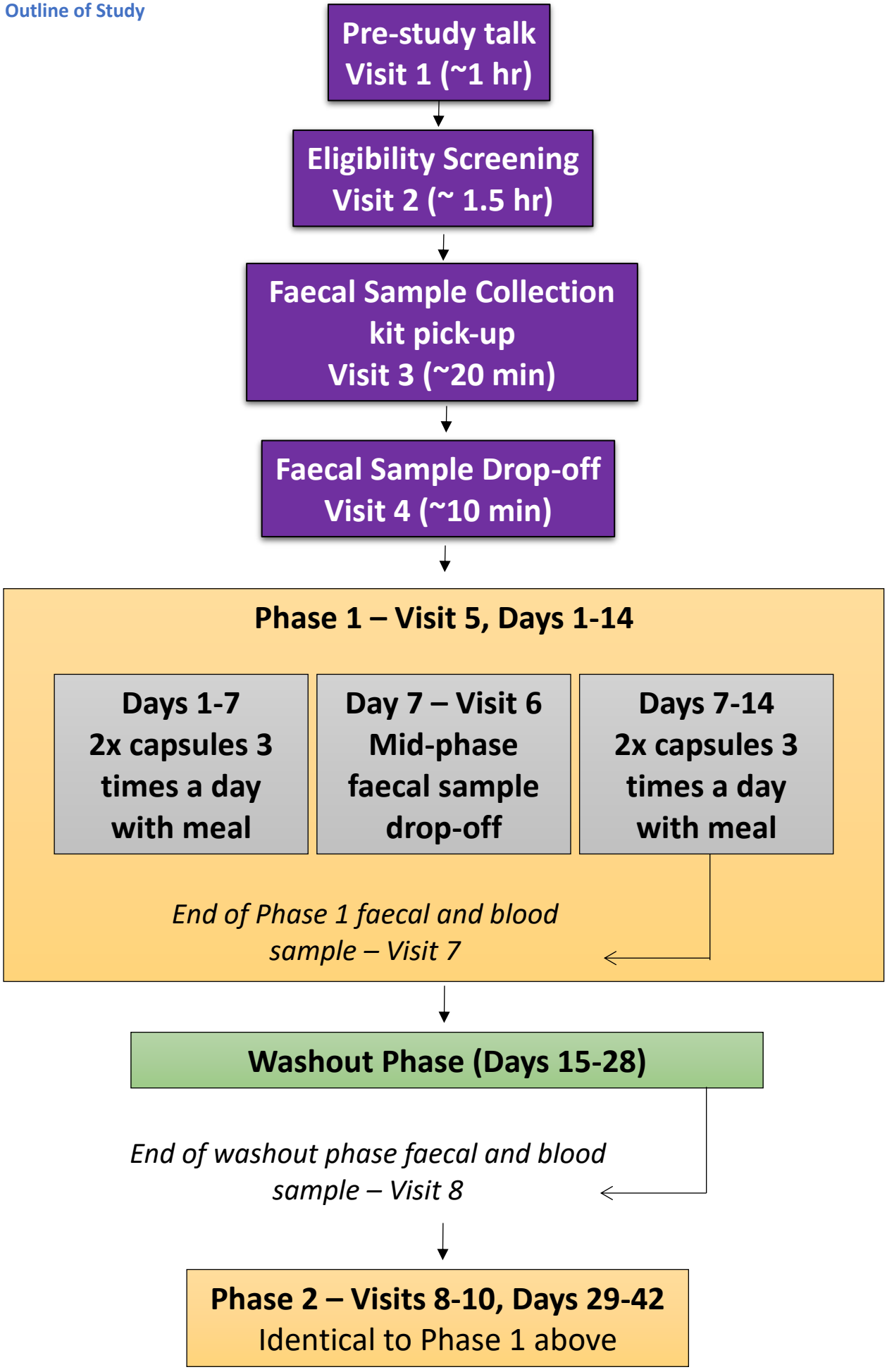
Some medicines affect the information we are collecting. Please tell us if you take any medication including those you purchase at the chemist or supermarket (e.g. paracetamol).

You should bring details of any medication (i.e. name of the medicine and the dose taken) you are taking when you come for your screening visit (Visit 1).

Taking part in the research is entirely voluntary! You are free to withdraw from the study at any time without giving a reason.

For further information or to arrange a study appointment, please contact a member of the study team or complete the attached response slip and return to us using the prepaid envelope enclosed. Thank you!

Outline of Study



Short title: Effect of Phytin on Human Gut Microbiome
(EPoM Study)

I am interested in taking part and/or finding out more information about this study (please complete the personal details below).

Name: ……

Address: ……

……

……

……

Daytime telephone ……

Evening telephone ……

Mobile ……

I am happy for a message to be left via my daytime/evening/mobile number: **YES/NO**
**please circle as applicable*

Preferred number/time to call: ……

E-mail address ……

Please return this form in the **FREEPOST** envelope provided, to:

Miss Bhavika Parmanand
Quadram Institute Bioscience
FREEPOST XXX
Norwich Research Park
Colney
Norwich
NR4 7UA

Expressing an interest does not commit you to taking part in the study

WE NEED YOUR HELP ON

Effect of Phytin on Human Gut Microbiome

The EPoM Study

There is evidence that regularly eating foods containing phytin, such as cereals and legumes, may decrease the number of 'bad' bacteria in your gut.

We need to recruit:

Men and women aged between 18 - 50 years old for an 8-week study

You would have to:

☑ **Consume capsules containing phytin**
☑ **Provide biological samples such as urine, stools and blood**

We will: **Reimburse your expenses**
Provide recompense for taking part in the study

If you live within 40 miles of Norwich and would like further information on the study, please contact:

Miss Bhavika Parmanand
01603 255021
Bhavika.parmanand@quadram.ac.uk

Dr Lee Kellingray
01603 255070
Lee.kellingray@quadram.ac.uk

The study will be sponsored by the QIB and funded through the Biotechnology and Biological Sciences Research Council (BBSRC); this study was funded by the BBSRC Institute Strategic Programme Food Innovation and Health ISP.
An expression of interest does not commit you to participation.

WE NEED YOUR HELP ON

Effect of Phytin on Human Gut Microbiome

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01603 255070
Lee.kellingray@quadram.ac.uk

The study will be sponsored by the QIB and funded through the Biotechnology and Biological Sciences Research Council (BBSRC); this study was funded by the BBSRC Institute Strategic Programme Food Innovation and Health ISP.

An expression of interest does not commit you to participation.

Please take a tear off slip

EPoM Study Bhavika.parmanand@quadram.ac.uk Tel: 01603 255021	EPoM Study Bhavika.parmanand@quadram.ac.uk Tel: 01603 255021	EPoM Study Bhavika.parmanand@quadram.ac.uk Tel: 01603 255021	EPoM Study Bhavika.parmanand@quadram.ac.uk Tel: 01603 255021	EPoM Study Bhavika.parmanand@quadram.ac.uk Tel: 01603 255021	EPoM Study Bhavika.parmanand@quadram.ac.uk Tel: 01603 255021	EPoM Study Bhavika.parmanand@quadram.ac.uk Tel: 01603 255021

Quadram Institute Bioscience
Norwich Research Park
Colney
Norwich NR4 7UA
UK

www.quadram.ac.uk

[Insert Date]

Dear_____,

Thank you for your interest in the following research study:

Short title: Effect of Phytin on Human Gut Microbiome (EPoM Study)

at the Quadram Institute Bioscience, study location Quadram Institute Clinical Research Facility (QI CRF).

I have sent you the details of this study, which is in progress at present, as you have responded to an advert about the study and you may fit the criteria for this study. If you have any further questions about the study, please contact, Miss Bhavika Parmanand on **01603 255021** or **bhavika.parmanand@quadram.ac.uk** as stated on the enclosed participant information sheet.

If you are interested in taking part or getting more information about the study, please fill out the reply slip on **page 14** of your participant information sheet, return it to the Quadram Institute in the freepost envelope provided and a member of the study team will be in touch.

Thank you.

Yours sincerely,

Miss Bhavika Parmanand,
EPoM study manager

IRAS ID 251932

Quadram Institute

EPoM Appointment Card

Screening/Rescreening at QI CRF Bring with you a midstream urine sample from within 2 hours of your appointment time. Also bring with you the name and address of your GP and details of any medication and/or supplements taken	Date: Time:
Start of Phase 1 at QI CRF Bring with you a faecal sample	Date: Time:
Start of Phase 2 at QI CRF Bring with you a faecal sample	Date: Time:

If you are unable to make the appointment, please contact Miss Bhavika Parmanand on 01603 255021 or the QI CRF Research Nurse (number to be included when available). Thank you

NHS Norfolk and Norwich University Hospitals NHS Foundation Trust

Quadram Institute

EPoM Appointment Card

Screening/Rescreening at QI CRF
Bring with you a midstream urine sample from within 2 hours of your appointment time. Also bring with you the name and address of your GP and details of any medication and/or supplements taken
Date:
Time:

Start of Phase 1 at QI CRF
Bring with you a faecal sample
Date:
Time:

Start of Phase 2 at QI CRF
Bring with you a faecal sample
Date:
Time:

If you are unable to make the appointment, please contact Miss Bhavika Parmanand on 01603 255021 or the QI CRF Research Nurse (number to be included when available). Thank you

NHS Norfolk and Norwich University Hospitals NHS Foundation Trust

INFORMED CONSENT FORM FOR RESEARCH STUDY

IRAS ID: [251932]
Study Number: [19/EE/0005]
Participant Identification Number for this trial:

Study Title: A human intervention trial investigating the effects of phytin on the human gut microbiota
Chief Investigator: Professor Arjan Narbad

Volunteer please initial each box

I confirm that I have read the information sheet dated..... (version.....) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

With who have you discussed the information for this research study?

Name: …… Role: EPoM Study Manager/Study Scientist/Research Nurse

I understand that my participation is voluntary and that I am free to withdraw at any time (i) without giving any reason, (ii) without my medical care or legal rights being affected, and (iii) without my withdrawal affecting future participation in other research studies at QIB and at the QI CRF and NNUH hospital ☐

I agree that I do not fall within the basic exclusion criteria listed for this research study ☐

I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from QIB, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

I understand that my personal information and data will be held confidentially at QIB and that it will be destroyed after 15 years. ☐

I understand that the information collected about me will be used to support other research in the future and may be shared anonymously with other researchers. ☐

I agree to my General Practitioner being involved in the study, including any necessary exchange of information about me between my GP and the research team. ☐

Name and address of your General Practitioner: ……

……

I understand that all research is subject to inspection and audit.
NB: although your records may be accessed for this purpose your personal information remains confidential ☐

I agree to take part in the above study.

Signed: …… (Name in BLOCK letters)

Date: …… Date of Birth: ……

INFORMED CONSENT FORM FOR RESEARCH STUDY

IRAS ID: 251932
Study Number: [to insert after HRA submission]
Participant Identification Number for this trial:

I confirm that the volunteer above has been given a full verbal and written explanation of the study.

Signed: …… (Name in BLOCK letters)

Role: …… (in BLOCK letters) Date: ……

1 copy of the signed consent form must be given to the volunteer to keep.
1 copy of the signed consent form must be kept in the study records at QIB and QI CRF notes

Our Vision
To provide every patient with the care we want for those we love the most

Norfolk and Norwich University Hospitals NHS
NHS Foundation Trust

The Norwich Biorepository

The donation, collection, storage and use of samples of tissue and/or fluids and/or other material from a healthy adult donor for research

Information sheet for healthy donors - Version 15 (21 February 2014)

Thank you for considering giving a sample for biomedical research. This information sheet provides a brief summary to help you to understand what this means and involves.

There is a consent form after the information sheet. It is important that you complete and sign it, if you decide to give a sample. Please complete all parts of the consent form.

Doctors and other health professionals may take samples (which may be blood, other fluids, small biopsies, or something else) from patients to help make a diagnosis and decide how best to treat them.

They may also ask to take similar samples from healthy donors, like you, specifically for research purposes. They use those samples to learn more about health and/or illness, how disease happens and how to treat it, and sometimes to help develop new medicines.

For the purposes of medical research you do not need to be in perfect health. We may need to ask you some questions to confirm that you do NOT suffer from the medical condition which is being studied.

Samples from healthy volunteers, like you, will include only those that can be obtained externally, or by normal routes (e.g., saliva, urine, faeces), or by minimal invasion involving very little risk to the donor (e.g., blood taken from a vein close to the surface, or a throat swab). Such minimally invasive procedures include those that might be undertaken during a routine visit to a general practitioner. Nothing more invasive than that is permitted using this consent form.

Samples donated (given) to the Norwich Biorepository are not:

- Normally used in animal research. It will be made clear to you if animal research is an integral part of the project for which we are seeking a donation.

Continued……

- Used in cloning experiments. However, the Biorepository would consider the use of donations in non-reproductive cloning experiments based on their scientific value and in the context of prevailing law and ethical standards. It will be made clear to you if cloning experiments are part of the research project for which we are seeking a donation.

To undertake research on the sample(s) that you are considering donating, we need your permission and signed consent.

If you give permission for a sample to be taken –

- The Hospital will own the sample

- The sample may be stored, usually in a deep freezer, until it is used. The freezer is referred to as a tissue bank in the consent form

- Nobody involved in the research will know where the sample has come from.

- The sample will be used only in experiments that are ethical and to help other people. Please see the section entitled 'Scientific and ethical approval' below to understand what we mean by *ethical*.

- Your donated sample(s) and any genetic material derived from it (them) may be stored indefinitely for future research projects, which may include whole genome sequencing. *Whole genome sequencing* means reading your total DNA code (your genetic blueprint) in a single assessment.

- We might give some or all of the sample to other doctors or researchers for their experiments, if they are ethical and to help other people. Some of these people might work in companies in this country or abroad

- Data derived from your sample(s) may be placed anonymously in an international database for future research. While we will take all possible steps to maintain your anonymity and protect your privacy, there is a very small risk that genetic information produced in the research and stored on databases could lead to your identification by being linked to other stored information.

- We will keep some facts about you on our Biorepository database

- Although these facts might be given to the research doctors or scientists to help their experiments, we will NOT tell them your name or other details that would let them know who you are

- Doctors in the Hospital might also read your hospital records to help them understand what the doctors or researchers find out in the experiments. This is possible because your hospital records can be linked to the anonymous research sample without loss of confidentiality as far as the researchers are concerned. If the research results are important for you, it might be possible using this linkage to feed back the information to your doctor, so that any appropriate action can be considered.

The next sections give more detailed information. If you have any questions, please ask the person who is asking for your consent.

Continued……

WHAT WILL HAPPEN

Tissue, blood or other samples taken from you will be sent either to the Norwich Biorepository or to a research laboratory. **Only as much sample as is needed for research will be removed.**

The donation of a sample for research is designed to be as safe as possible. It is most unlikely that you will come to any harm as a result of this donation (though we cannot give any guarantees). Potential physical problems depend on the type of donation. They might include, for example, discomfort or pain, bleeding, or infection. **The person collecting the donation will be properly trained in the procedure. You will be asked to give your written consent before donation.**

While **we will take all possible steps to maintain your anonymity and protect your privacy**, there is a very small risk that genetic information produced in the research and stored on databases could lead to your identification by being linked to other stored information.

MEDICAL RESEARCH AND WHY THIS PROGRAMME IS IMPORTANT

Some of your sample, or material extracted from it, will be stored in a local tissue bank. This is part of a research programme which now includes the Norfolk and Norwich University Hospitals NHS Foundation Trust, the James Paget University Hospitals NHS Foundation Trust, the University of East Anglia (UEA), and the Institute of Food Research (IFR). Your donation may be used by ourselves or by researchers from other centres at a later date. Some of this research may involve an assessment of genetic material (DNA and/or RNA) to help us understand the genetic basis of health and disease.

The purpose of this research is to understand more about human health and disease. It may also allow us to develop new methods of disease prevention or new treatments for the benefit of future patients. Some of these research programmes could lead to the development of new products and processes, which may be developed commercially for the improvement of patient care. In that case, there would be no financial benefit to you.

Medically qualified doctors or other suitably qualified staff at the hospital may need to review your hospital case records, including hospital notes, if any, to help understand how the research findings made by other doctors or researchers using your donated samples fit with what is known of your medical history. It may be important to be able see how their research findings relate to past events in your health record. The hospital doctors will not give your name to those doing the research.

The research may also involve training doctors and scientists in scientific medicine, and may lead to higher qualifications for them (e.g., PhD or MD degrees). This is important for future research into diseases and for looking for new, more effective, treatments for them.

LINKS WITH OTHER ORGANISATIONS

If you agree, we may send stored samples or products derived from them to other approved tissue banks or companies in this country or abroad. This would be to support their research programmes or the research programmes of those companies' clients.

Continued……

Such outside organisations will provide financial support for the Norwich Biorepository (our tissue bank), to help it recover its operating costs. We are not, however, allowed to sell the stored samples in order to make any financial profit from these commercial links.

SCIENTIFIC AND ETHICAL APPROVAL

The Norwich Biorepository acts as a custodian of the samples it holds. It releases them only to individuals or organisations that have an acceptable scientific background and work to high ethical standards. We require that all such medical research has been approved by a properly constituted Research Governance Committee before it starts. It must also be approved by a Research Ethics Committee or on behalf of the Research Ethics Committee that oversees the work of the Norwich Biorepository under the terms of the Biorepository's own Research Ethics Committee approval. That committee is the Cambridge East Research Ethics Committee. These committees look particularly at the purpose and validity of the research proposal, the welfare of any participants and issues of consent and confidentiality. We will release samples to commercial companies only if they work to appropriate ethical and scientific standards.

YOUR RIGHTS

When your samples are obtained, some information about you will be kept on a computer in the Norwich Biorepository. This will help us understand how what we find in the laboratory relates to you as a person. You are entitled to ask to see what is recorded about you by applying to the Chairman of the Norwich Biorepository Committee, Norfolk & Norwich University Hospital, c/o Dept. of Histopathology, The Cotman Centre, Colney Lane, Norwich, NR4 7UB. No one other than you has the right to see these records. Any information needed for research purposes will be made anonymous before it is given to the researcher.

The researchers will not be able to find out your name or any personal details about you from the information that they receive.

You will have the opportunity to discuss with a doctor or researcher issues relating to the possible use of your samples for research purposes now or at the time the sample(s) is (are) collected. He or she will answer any questions you may have.

MAKING A DONATION (GIFT) OF A SAMPLE FOR RESEARCH

If you decide that you want your sample to be donated for research purposes, you will be asked to sign a special consent form. This will confirm your decision and state that you have read and understood this information sheet. When you sign the form you will give the ownership of the sample(s) to the Norfolk & Norwich University Hospitals NHS Foundation Trust. The sample(s) will then belong to the Trust. It will store the sample(s) for an indefinite period of time and will able to decide how it (they) should best be used for research. It will also have the right to dispose of unused stored material in an appropriate legal and ethical manner following normal procedures.

If you do not want to donate a sample to be stored in the tissue bank or used for research, please tell us and do NOT sign the special Consent Form. If you do not sign this form, the donation will not proceed.

Continued……

Complete the following details:

Donor's name……

Date of birth……

Address ……

……

……

The Norwich Biorepository

Consent for the collection, storage and release of human samples for research

I agree (Please initial small box) that the following sample(s) may be used for research, including genetic (DNA and/or RNA) studies and for the possible development of commercial products for the improvement of patient care, from which I would receive no financial benefit:

List sample(s) for research:	
	☐

I also agree that (Please initial small boxes, as appropriate):

These samples become the property of the Norfolk & Norwich University Hospitals NHS Foundation Trust ("the Trust") ☐

The Trust may store these samples in a tissue bank / biorepository ☐

The Trust may use these samples at its discretion in properly approved research programmes ☐

The Trust may pass on these samples to other approved tissue banks and/or companies, which may be in this country or abroad, in properly approved research programmes

Yes / **No** ☐

My genetic material and donated sample(s) may be stored for an indefinite amount of time for future research projects, which may include whole genome sequencing ☐

Information about my case may be kept on the Norwich Biorepository database ☐

Anonymous data derived from my sample(s) may be placed in an international database for future research ☐

Such information may be passed in an anonymous form to persons outside the Trust In connection with research and may be published with any research findings ☐

I agree that appropriately qualified staff employed by the Trust may review my hospital records, including case notes, as appropriate, for the purposes of research using the donated samples ☐

Continued……

Complete the following details:

Donor's name……

Date of birth……

Address ……

……

……

The Norwich Biorepository

Consent for the collection, storage and release of human samples for research

I also agree that (Please initial small boxes, as appropriate):

These samples may be used in ethically approved animal research

Yes
No

These samples may be used in ethically approved cloning research

Yes
No

I confirm that:

1) **I have read and understand the Information Sheet for healthy donors, Version 15, dated 21 February 2014**

2) **The issues have been explained to me, and that I have had the opportunity to ask questions.**

Signed_____ **(Donor)** **Date**_____

I have explained the request for sample for research purposes and have answered such questions as the donor has asked.

Signed_____ **Print name** _____

Doctor / Nursing Practitioner / Researcher / ***Other***_____
(Please delete as appropriate / indicate Other status)

Date_____

MEDICATION/MEDICAL CONDITIONS DECLARATION AGREEMENT

Certain illnesses and medication may affect the outcome of research studies. Therefore, we would like you to inform the study organisers if you

- start taking medication
- suffer from any illness

OR

- become pregnant

Please sign below to confirm that you have agreed to this request.

I…….consent to inform the study organiser of the commencement of any medication/medical changes whilst participating in the study

OR

If I think I may be pregnant whilst participating on the study.

Signature of volunteer…….

Date……..

Signature of scientist/member of QI CRF team …….

CONFIDENTIAL Participant Eligibility Screening Questionnaire EPoM

Participant code number…….. **Sex**: Male / Female

Date of birth: …… **Age**: ……**years**

Height: ……**cms** **Weight**: ……**Kg** **Body Mass Index** (BMI, kg/m^2) ……

Blood Pressure: **Right arm**……..**Left arm**…….. **Pulse**: ……..

Urinalysis: see page 4

Have you ever had any of the following? If yes give details below each relevant section.

Angina/heart disease: **Y** **N**
……
……

Thrombosis: **Y** **N**
……
……

High Blood Pressure: **Y** **N**
……
……

High Cholesterol: **Y** **N**
……
……

Chest problems: **Y** **N**
……
……

Diabetes: **Y** **N**
……
……

Depression or anxiety: **Y** **N**
……
……
……

Digestive/Gastrointestinal disorders: **Y** **N**
……
……
……

Skin conditions: **Y** **N**
……
……

Inflammatory disease: E.g. rheumatoid arthritis **Y** **N**
……
……

Liver problems: **Y** **N**
……
……

Other medical conditions: **Y** **N**
……
……

Kidney/Renal problems: **Y** **N**
……
……

Anaemia: **Y** **N**
……
……

Are you currently on any of the following?
If yes, give details below each relevant section of brand, dosage, frequency, when started etc.

Prescribed medication: **Y** **N**
……
Exclude if on medication for gastrointestinal problems

Colonic irrigation/bowel cleansing/ laxative/diarrhoea treatments: Y N

……

……

Exclude if on medication/treatment for diarrhoea, constipation, bowel discomfort/disorder (includes over the counter medications).

Dietary Supplements: Y N **Herbal remedies: Y N**

…… ……

…… ……

…… ……

If taking supplements/herbal remedies which may affect the study data and the participant is not willing to discontinue use for one month before and during the study, please exclude. Check supplements/Herbal remedies with scientist

WOMEN ONLY SECTION

Are you/could you be pregnant? Y N

Have you been pregnant within the last 12 months? Y N

Are you breast-feeding? Y N

When was your last period? ……

End of women only section

Have you had a major physical injury/operation? If yes give details below: Y N

……

……

Are you currently suffering from any illness/injury? If yes give details below: Y N

……

……

SMOKING:

Are you currently a: **Non-smoker / Current smoker / Ex-smoker / Lifelong smoker (circle appropriate)**

What do/did you smoke? (E.g. cigarettes, roll ups, cigars, pipe etc.)……

If a non-smoker/Ex-smoker, have you ever smoked? Y N

If yes, how long since you stopped smoking?.....How many did you smoke each day?.....

If currently a smoker/lifelong smoker: How many years have you been smoking? ……..

DRINKING:

Do you drink alcohol: Y N How many units do you drink per week? ……

A unit of alcohol is approximately half a pint of beer or lager, a single pub measure of spirit e.g. gin/vodka or a small glass of wine (125mL).

Exclude participants who appear to binge drink or regularly consume >15 units (women) or >22 units (men) per week.

DIETARY QUESTIONS:

Are you a vegan or vegetarian? Y N

Do you have any special dietary requirements: Y N

If yes state:……

……

Have you any known allergies: Y N

Food: …… **Drugs**: ……

…… ……

Other: ……

……

Are you currently on/or plan to start a diet programme? Y N

If yes state which:……

If on a diet programme which may affect the study data and the participant is not willing to discontinue for one month before and during the study, please exclude. Check diet programmes with scientist

BLOOD DONOR:

Are you a regular blood donor? Y N

If yes, when did you last donate blood? ……

Do you take prebiotics or probiotics? These may be in a drink, yoghurt or other products.

If yes, which products?.....

How frequently?.....

If taking regularly (3+ times a week) and willing to continue throughout the study, then include.

If taking occasionally (<3 time a week), participant will have to discontinue use for at least 1 month before starting the intervention and throughout the study. If participant not happy to do so, please exclude from study.

GUT FUNCTION:

On average, how many times do you open your bowels per day? ……

Do you typically need to strain when opening your bowels? Y N

What is the typical colour of your stools? ……

What is your typical stool type? (refer to Bristol Stool Chart):

Type: **1 2 3 4 5 6 7** (**circle appropriate**)

If participant typically passes stool types 1, 2, or 7, exclude from study.

Do you typically suffer from any pain/discomfort when passing stools? Y N

If yes, would you describe it as typically being: **Mild / Moderate / Severe** (**circle appropriate**)

Have you ever seen blood in your stools? Y N

If yes, when did you last see blood in your stools? ……

If the participant did not seek medical advice on blood in their stools, exclude from study.

GENERAL PRACTITIONER:

Do you agree to us informing your General Practitioner of your participation in the study or of any results found?

Y N

If you have answered NO to this question then we are unable to accept you on this study.

Name and Address of your General Practitioner:

……

……

……Telephone number:……

As far as you know are you related to or living with any member of study team? Y N

Are you currently participating in another research study? Y N

If yes check with participant whether it involves dietary intervention– refer to QI CRF Research Nurse or scientist

Are you currently undergoing any GP/Hospital investigations? Y N

Form completed by (print name):……Signature:……

Designation:…… Date: ……..

URINE DIPSTICK TEST RESULTS
Attach to screening questionnaire to be kept at Study centre

Study Title:

Participant code number **(NOT NAME)**:…… Date of Birth:…… Male/Female (circle)

Date of sample:…… Time of sample:……..

Multistix Dipstick urine test reults:

Protein:…… Glucose:…… Ketones:…… Bilirubin:…… Urobilinogen:…… Blood:……

Specific Gravity…… pH: ……

Test performed by:

Signature:

Date:

Time:

Menstruating: Y N N/A (circle as appropriate) **If menstruating do not refer to GP repeat urine test 5 days after finishing Menstruation. If blood indicated on this occasion refer to GP as flagged urine.**

Repeat urine dipstick test results.

pH……Protein:……Glucose:……Ketones:…… Bilirubin:……

Urobilinogen:…… Blood:…… Specific Gravity……

Test performed by:

Signature:

Date:

Time:

Comments:……
……
……
……

Please note a copy of these results must be sent to the participant's G.P on the Eligibility Screening Results Form.

BLOOD TEST RESULTS

Attach to screening questionnaire to be kept at Study centre

Study Title:

Participant code number (**NOT NAME**):…… Date of Birth:…… Male/Female (circle)

Date of sample:…… Time of sample:……

Serum ferritin ……..

HbA1c ……..

Full blood count ……

CRP ……

Test performed by:

Signature:

Date:

Time:

Comments:……
……
……
……

Please note a copy of these results must be sent to the participant's G.P on the Eligibility Screening Results Form.

Study Title: **Effect of Phytin on Human Gut Microbiome (EPoM Study)**

Participant's Name:……

Date of Birth:……. Male/Female:……..

Multistix Dipstick urine test reults:

pH……Protein:…… ….Glucose:…… Ketones:…… Bilirubin:……..
60 seconds 60 seconds 30 seconds 40 seconds 30 seconds

Urobilinogen:……... Blood:…….. Specific Gravity……
60 seconds 60 seconds 45 seconds

Menstruating: Y N N/A (circle as appropriate)

Test performed by (print name):…….. Signature:……. Date:…… Time:……

Repeat test required at surgery prior to re-screen: Y N N/A (circle as appropriate)

Repeat urine dipstick test results (QI CRF). Tested using Urine dipstick test strips/guide times as above

pH…….Protein:……Glucose:……Ketones:…… Bilirubin:…….

Urobilinogen:…….. Blood:……. Specific Gravity…….

Test performed by (print name):…….. Signature:……. Date:…… Time:……

Observations:

Blood Pressure: Right arm:……Left arm:……. Pulse: Rt…… Lt…….

Weight(kg): …… BMI(kg/m^2):…… Reference range for study: ≥19.5 - ≤30

Copies of screening blood results attached: Yes No Not applicable (circle as appropriate)

The ……. (above/1st urine/repeat urine /BP/ BMI (kg/m^2) –insert as appropriate) result(s) will / will not exclude your patient from this study.

Date:…….. Signature:……
Designation:……..

Abnormal results are referred to the QI CRF medical advisor for comments regarding participation in the study.

Quadram Institute Bioscience
Norwich Research Park
Colney
Norwich NR4 7UA
UK

www.quadram.ac.uk

[Insert Date]

Dear_____,

Your patient, ……., date of birth …….. has consented to take part in a dietary intervention at the Quadram Institute Bioscience (study location within the Quadram Institute Clinical Research Facility, QI CRF) entitled,

Short title: Effect of Phytin on Human Gut Microbiome (EPoM Study)

Following consent, it is our standard practice to screen the volunteers to exclude any health factors which may affect the study data or whose screening results may indicate an issue which may require further investigation. We are looking for healthy people who have no chronic illness and are not taking any prescribed medication which may affect the study data.

Some/none of your patient's results fell outside the standard reference range on this occasion.

These results **will/will not** affect the study data.

Your patient **will/will not** be able to participate in the study.

Yours sincerely,

Miss Bhavika Parmanand
EPoM Study Manager

Quadram Institute Bioscience
Norwich Research Park
Colney
Norwich NR4 7UA
UK

www.quadram.ac.uk

[Insert Date]

Dear_____,

This is to inform you that your patient …….. date of birth……has consented to participate in a dietary intervention study at the Quadram Institute Bioscience (study location at the Clinical Research Facility at Quadram Institute). The study,

Short title: Effect of Phytin on Human Gut Microbiome (EPoM Study)

has been approved by a Local Research Ethics Committee and the EPoM Study Manager, **Miss Bhavika Parmanand,** can be contacted on **01603 255021** or **bhavika.parmanand@quadram.ac.uk** if you require further information.

It is our policy to forward to the participant's GP copies of all screening results obtained during the study as well as the participant information sheet (PIS).

We anticipate your patient will complete this study by……..

Yours sincerely,

Miss Bhavika Parmanand
EPoM Study Manager

Short title: Effect of Phytin on Human Gut Microbiome (EPoM Study)

- You are receiving this study summary as one of your patients has consented to participate in the EPoM study undertaken at the QI CRF and has undergone screening (results enclosed, which includes dipstick urine test results, blood test results, BP, pulse, weight and BMI). In addition, copies of stool charts used during the study will be sent to you, should any significant changes to your patients stool type be recorded throughout the study period.

- **The main aim of this study** is to examine whether consuming phytin can modulate the gut microbiota and cause a decrease in the abundance of Enterobacteria. We will also be observing whether phytin causes an increase in the Bifidobacteria in our gut.
- Additionally, we are interested in how the gut bacteria are affected by phytin-induced iron restriction.
- Phytic acid is the principal storage form of phosphorus in many plants, such as legumes, seeds, nuts and cereals.
- When phytic acid is bound to a mineral it is known as phytate (mostly in the form of phytin). Studies in humans report that between 37-66% of dietary phytate is degraded during digestion in the stomach and small intestine when the diet is rich in plant food phytases.
- *In vitro* and *in vivo* studies have demonstrated that phytic acid forms insoluble complexes with several divalent minerals, thereby preventing absorption, and can potentially result in zinc and iron deficiencies. Once these insoluble complexes are formed, the mineral cannot be absorbed in the small intestine and therefore pass into the colon.
- Notably, phytate-bound iron found in the colon is present in the insoluble form making it difficult to degrade suggesting a potential role for phytate in the withholding of iron from potentially pathogenic bacteria.
- We aim to assess whether the iron-restricted colonic environment via phytin can explain the differences in the gut microbiota populations by faecal bacteria phylogeny analysis.
- Your patient will be asked to maintain their habitual diet for the duration of the study. The patient will be asked to consume encapsulated phytin for 2 weeks and encapsulated placebo for 2 weeks, in a random order, with a 2-week washout period separating these diets. During their involvement in the study, your patient will be asked to provide a total of 5 blood samples and 6 faecal samples for serum ferritin & gut microbiota/metabolite analysis, respectively.
- This project is funded through the Biotechnology and Biological Sciences Research Council (BBSRC); Food Innovation and Health QIB ISP; QIB acting as study sponsor.

ANNEX 14

CATALOGUE'S A-C

A human intervention trial investigating the effects of Phytin on the human gut microbiome

Short title: Effect of Phytin on Human Gut Microbiome (EPoM Study)

Chief Investigator:
Professor Arjan Narbad

Investigators:
Study Manager: Miss Bhavika Parmanand
Study Scientist: Dr Lee Kellingray
Study Advisor: Professor Susan Fairweather-Tait

Quadram Institute
Science Health Food Innovation

NHS
Norfolk and Norwich University Hospitals
NHS Foundation Trust

Annex 14, Catalogue A Raw Material

A1. Phytin

- A1.1 Phytin Food Grade Certificate
- A1.2 Phytin Certificate of Analysis
- A1.3 Phytin GMO Statement
- A1.4 Phytin Material Safety Data Sheet
- A1.5 Phytin Shelf Life
- A1.6 Phytin Heavy Metals Testing
- A1.7 TSUNO Classification Accreditation
- A1.8 TSUNO ISO 9001
- A1.9 MHRA Medicinal Status

A2. Microcrystalline Cellulose (MCC)

- A2.1 MCC Process Flow Sheet
- A2.2 MCC Product Information
- A2.3 MCC Material Safety Data Sheet
- A2.4 MCC GMO Statement
- A2.5 MCC Food Statement
- A2.6 MCC Ingredient Declaration
- A2.7 MCC Allergen List
- A2.8 MCC Residual Solvents Statement

A3. Coating Formulation (Phloral®)

- A3.1 Amylomaize Starch SDS
- A3.2 Eudragit S100 SDS
- A3.3 PlasACRYL T20 SDS
- A3.4 Reagent Alcohol SDS
- A3.5 Triethyl Citrate Specification
- A3.6 Triethyl Citrate GMO Statement
- A3.7 Triethyl Citrate Food Grade Statement
- A3.8 Phloral Food Grade Statement

TSUNO RICE FINE CHEMICALS CO.,LTD.
2283, CHONOMACHI, KATSURAGI-CHO, ITO-GUN, WAKAYAMA, 649-7194, JAPAN
TELEPHONE : +81 - (0) 736-22-8000
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FACSIMILE : +81 - (0) 736-22-3943

Date: Nov. 9, 2017

REPORT

(ANALYSIS CERTIFICATE)

This is to certify that we, the undersigned, inspector authorized by the TSUNO RICE FINE CHEMICALS research laboratories, examined the commodity, and obtained the following results.

Commodity	:	PHYTIN(RICE BRAN EXTRACT)
Quantity	:	sample
Packing	:	
Lot No.	:	0269
Date of assay	:	Apr. 28, 2016
Manufacturing date	:	Apr. 23, 2016
Expiry date	:	Apr. 23, 2019

RESULT

Appearance	:	White powder
Solubility	:	Easily soluble in acid solution, and hardly soluble in water. Its suspention with water shows neutral to acid
Heavy metals	:	Not more than 40 ppm
Arsenic	:	Not more than 4 ppm
Loss on drying(105℃, 4hrs)	:	0.90 %
Residue on ignition	:	75.4 %
Total phosphor	:	20.7 %
Phytin contents	:	94.4 % (Total phosphor × 4.559)

TSUNO RICE FINE CHEMICALS CO., LTD.

Quality Assurance Division

TSUNO RICE FINE CHEMICALS CO.,LTD.
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FACSIMILE : +81-(0) 736-22-3943

Date: August 9, 2018

To whom it may concern:

Re: Certificate of GMO Free

We, Tsuno Rice Fine Chemicals Co., Ltd., certify that the all the raw material used and Phytin manufactured in our plant of Katsuragi, Ito, Wakayama, Japan are Genetically Modified Organisms (GMO) Free, and that the equipments used are not in contact with other GMO products.

Yours truly,

Takashi Yamanaka
General Manager of Quality Assurance Division
Tsuno Rice Fine Chemicals Co., Ltd.

MATERIAL SAFETY DATA SHEET

1.CHEMICAL PRODUCT AND COMPANY IDENTIFICATION

Chemical Product Name : IP6
Common Chemical Name : IP6
Product Code(Supplier) : 11-934
Supplier : Tsuno Rice Fine Chemicals Co.,Ltd.
2283,Chonomachi,Katsuragi-cho Ito-gun,Wakayama,Japan

Emergency Telephone : +81-736-22-0061,8000

2.COMPOSITION/INFORMATION ON INGREDIENTS

Substance/Preparation : None
Information on hazardous Ingredients

Chemical name	CAS Number	EC Number	Symbol		R-Phrases
Phytin	3615-82-5	N/A	N/A	N/A	

3.HAZARD IDENTIFICATION

Physical/Chemical Hazards : None
Environmental Hazards : Easily biodegradable
Human Health Hazards : None

4.FIRST AID MEASURES

Effects and Symptoms
Ingestion : None

Inhalation : None

Skin Contact : None

Eye Contact : In case of contact with eyes,rinse immediately with plenty of Water and seek medical advice.

First Aid Measures

Ingestion : Wash out mouth with water. Get medical attention.

Inhalation : None

Skin Contact : Wash off with water. If you feel unwell, seek medical advice.

Eye Contact : Wash out with plenty of water with the eyelid hold wide open for at least 15 minutes. Get medical attention.

5.FIRE FIGHTING MEASURES

Extinguishing Media

Suitable : None Not suitable : None

Special Firefighting Procedures : None

Unusual Fire/Explosion : None

Hazardous Thermal : None

Protection of Firefighters : No special protection is needed.

6.ACCIDENTAL RELEASE MEASURES

Personal Precautions : None

Environmental Precautions: No special precaution required.

Methods Cleaning Up : Wash with plenty of water.

7.HANDLING AND STORAGE

Handling : No special precaution required.

Storage : Keep container tightly closed and store in a cool place.

Packing Materials

Suitable : Fiber drum.

8.EXPOSURE CONTROLS/PERSONAL PROTECTION

Engineering Measures : No specific regulation required.

Hygienic Measures : No specific regulation required.

Occupational Exposure Limits

Chemical Name : Phytin

Personal Protective Equipment

Respiratory System : None Skin

and Body : None

Hands : Suitable gloves

Eyes : None

9.PHYSICAL AND CHEMICAL PROPERTIES

Physical State : Powder

Color : White or yellowish white

Odor : Odorless

Boiling Point : N/A pH

: N/A

Solubility in Water : Hardly soluble

Vapor Density(Air=1) : Not datermined

Flash point : N/A

Auto ignition Temperature : N/A
Lower Explosion Limit : N/A
Upper Explosion Limit : N/A

10.STABILITY AND REACTIVITY

Stability : Stable under normal condition.
Conditions to avoid : Open air and sunlight
Materials to avoid : None
Hazardous Decomposition
Products : None

11.TOXICOLOGICAL INFORMATION

Comment : There is no toxicological data available on the preparation.
Chemical Name : Phytin
Acute Toxicity
Oral : No oral toxicity is known.
Dermal : No dermal toxicity is known.
Inhalation : No inhalation toxicity is known.
Skin Irritation : No data available
Eye Irritation : No data available
Sensitization : No data available
Chronic Toxicity : No chronic toxicity is known.
Carcinogenicity : No carcinogenicity is known.

12.ECOLOGICAL INFORMATION

Comment : Easy biodegradable
Chemical Name : Phytin
Ecotoxicity : No ecotoxicity is known.

13.DISPOSAL CONSIDERATIONS

Methods of Disposal : Disposal according to the local legislation.

14.TRANSPORT INFORMATION

UN Number : None

Land Road/Railway

ADR/RID Class : N/A

ADR/RID Item Number : N/A Inland

Waterways

ADNR Class : N/A

Sea

IMDG Class : N/A

IMDG Page Number : N/A

Air

IATA-DGR Class : N/A National

Transport : N/A

Regulations

15.REGULATORY INFORMATION

EC Regulations : None

EC Classlfication : None

Label Name : Phytin(RICE BRAN EXTRACT)

Hazard Symbols : None

16.OTHER INFORMATION

HISTORY

Date first issue : Jul.13,2001

Date previous MSDS : Jul.13,2001

Date of issue : Jul.13,2001

Version : 1

MSDS prepared by : Tsuno Rice Fine Chemicals Co., Ltd 2283,Chonomachi,Katsuragi-cho,Ito-gun,Wakayama,Japan

TSUNO RICE FINE CHEMICALS CO.,LTD.
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FACSIMILE : +81 – (0) 736-22-3943

Date: August 10, 2018

To whom it may concern:

Re: Phytin

Shelf Life : 36 months from the manufacturing date

*Keep container tightly closed.
*Protect against sunlight.
*Store in cool and dark place

Yours truly,

Takashi Yamanaka
General Manager of Quality Assurance Division
Tsuno Rice Fine Chemicals Co., Ltd.

http://www.tsuno.co.jp

eurofins

PO Number **QIB0118056A**

Bhavika Parmanand
Quadram Institute Bioscience
Norwich Research Park
Colney
Norfolk
NR4 7UA

AR-18-UD-329004-01

Reported on **28/08/2018**
Reported by **Antony Bagshaw, ASM - Interim**

Certificate Of Analysis

Sample number	**400-2018-20105980**	Received on	**21/08/2018**
Your sample reference	**Phytin - Extract From Rice**	Your sample code	**BP_Phy_HM**

Test Code	Analyte	Result	SOP No.
Toxic Elements			
UD401	Arsenic (As)	0.013 mg/kg	ICPMS/010
UD033	Cadmium	0.664 mg/kg	ICPMS/010
UD032	Lead	0.026 mg/kg	ICPMS/010
UD579	Mercury	0.006 mg/kg	ICPMS/010

Unless stated, all results are expressed on a sample as received basis.
† Indicates that this test was subcontracted

Key: cfu colony forming units
< denotes less than
> denotes greater than

UKAS TESTING

* Indicates that this parameter is not included in the UKAS accreditation schedule for the laboratory.
Opinions and/or interpretations within this report are outside our accreditation scope.

~ estimated value

Eurofins Food Testing UK Ltd i54 Business Park Valiant Way
Wolverhampton
WV9 5GB

0342

www.eurofins.co.uk

Classification of accreditation scopes

Agriculture, forestry and fishing
Mining and quarrying
Food products, beverages and tobacco
Textiles and textile products
Leather and leather products
Wood and wood products
Pulp, paper and paper products
Publishing companies
Printing companies
Manufacture of coke and refined petroleum products
Nuclear fuel
Chemicals, chemical products and fibres
Pharmaceuticals
Rubber and plastic products
Non-metallic mineral products
Concrete, cement, lime, plaster, etc.
Basic metals and fabricated metal products
Machinery and equipment
Electrical and optical equipment
Shipbuilding
Aerospace
Other transport equipment
Manufacturing not elsewhere classified
Recycling
Electricity supply
Gas supply
Water supply
Construction
Wholesale and retail trade; Repair of motor vehicles, motorcycles and personal and household goods 30
Hotels and restaurants
Transport, storage and communication
Financial intermediation; real estate; renting
Information technology
Engineering services
Other services
Public administration
Education
Health and social work
Other social services

ISO 9001
CERTIFICATE OF REGISTRATION

Issue Date
2018/5/30

Registration Number
ＪＳＡＱ ３０６

Registered Organization and Address

Tsuno Food Industrial Co., Ltd.

94, Shinden, Katsuragi-cho, Ito-gun, WAKAYAMA, JAPAN

Related Company:
- Tsuno Rice Fine Chemicals Co., Ltd.
  2283, Chonomachi, Katsuragi-cho, Ito-gun, WAKAYAMA, JAPAN
- Tsuno Transportation Co., Ltd.
  2222-1, Chonomachi, Katsuragi-cho, Ito-gun, WAKAYAMA, JAPAN
- Tsuno Development Corporation
  94, Shinden, Katsuragi-cho, Ito-gun, WAKAYAMA, JAPAN

Management Systems Enhancement Department of the Japanese Standards Association (JSA) registers the Quality Management System of the above organization, which conforms to ＪＩＳ Ｑ ９００１:2015, ＩＳＯ ９００１:2015.

Condition of Registration

Described in Appendix to the Certificate of Registration No. ＪＳＡＱ ３０６－１４

Registration Date	Renewal Date	Expiry Date
1998/7/6	2016/7/6	2019/7/5

Senior Executive
Management Systems Enhancement Department
Japanese Standards Association

写

Japanese Standards Association Management Systems Enhancement Department 13-12, Mita 3 chome, Minato-ku, TOKYO, JAPAN Tel : 03-4231-8575 Fax : 03-4231-8681

ISO 9001

APPENDIX TO THE CERTIFICATE OF REGISTRATION

Issue Date
2018/5/30

Issue Number
JSAQ 306－14

Registered Organization

Tsuno Food Industrial Co., Ltd.

Condition of Registration is listed below.

Quality Management System Standard

JIS Q 9001:2015, ISO 9001:2015

The Scope of the Registration

Design, development and production of edible oil and fats, fatty acids, edible barley, feed, fertilizers, pharmaceutical ingredients, food additives, cosmetic ingredients and oil & fat chemical products.

History

Registration Date	Renewal Date	Revised Date
1998/7/6	2016/7/6	2018/5/28

The registered Organization shall abide by
the Rules Governing the Maintenance of registration.
Detailed information is described on QMS Registration Information Sheet.

Senior Executive
Management Systems Enhancement Department
Japanese Standards Association

写

Japanese Standards Association Management Systems Enhancement Department 13-12, Mita 3 chome, Minato-ku, TOKYO, JAPAN Tel : 03-4231-8575 Fax : 03-4231-8681

Medicines & Healthcare products Regulatory Agency

MHRA Regulating Medicines and Medical Devices

Quadram Institute
Norwich
NR4 7UA
UK
Bhavika.parmanand@quadram.ac.uk

MHRA

10 South Colonnade
Canary Wharf
London
E14 4PU
United Kingdom

www.gov.uk/mhra

Our Ref: 2018/000735

25 September 2018

Dear Miss Parmanand,

Product: Phytin Mineral Salt

I have reviewed the product listed above based on the information provided. Advice regarding this product is detailed below.

Medicines legislation

In the UK, as in the rest of the EC, medicinal products which are placed on the market are required to have marketing authorisations (formerly product licenses) in accordance with Regulation 46 (1) of the regulations. Amongst other things these provide that, unless exempt, no medicinal product shall be placed on the market unless an appropriate authorisation has been granted in accordance with Community provisions by the licensing authority or the European Commission.

It is an offence to sell or supply or to advertise a medicinal product which does not have authorisation.

A relevant “medicinal product” is defined in Regulation 2 of S.I. 2012/1916 as:

a) any substance or combination of substances presented as having properties of preventing or treating disease in human beings; or

(b) any substance or combination of substances that may be used by or administered to human beings with a view to-

(i) restoring, correcting or modifying a physiological function by exerting a pharmacological, immunological or metabolic action, or

(ii) making a medical diagnosis.

If it satisfies either of the above criteria, it may be classed as a medicinal product. In broad terms, when classifying a product, the Agency looks at the way it is presented and its actual or perceived function, that is, its effects (when administered) on human physiology.

Advertising

Regulation 279 of the Human Medicines Regulations 2012 states:

“A person may not publish an advertisement for a medicinal product unless one of the following is in force for the product-

(a) a marketing authorisation;
(b) a certificate of registration;
(c) a traditional herbal registration; or
(d) an Article 126a authorisation.

"Advertisement" is defined in Regulation 277 to cover "every form of advertising whether in a publication or elsewhere." Unfortunately, what constitutes a "medicinal claim" is not closely defined in the legislation but as a rough guide unacceptable medicinal claims include the following:

- references to medical conditions such as colds, headaches, cuts and bruises, spots, skin disorders, headlice, hangovers, smoking addiction, obesity, arthritis, depression, stress and all childhood disorders and serious diseases etc.

- references to treatment or alleviation of adverse conditions such as decongests, relieves pain, reduces inflammation, calms, stops itching, cures insomnia etc.

- references to interference with the normal operation of a physiological function such as burns fat, increases metabolism, reduces blood pressure, lowers cholesterol levels, prevents jet-lag etc.

Marketing

You should note that the following forms of marketing are unacceptable in products that are unlicensed:

- References to medical conditions.
- Comparison with licensed medicines.
- References to interference with the normal operation of a physiological function.
- Product names which refer to adverse medical conditions.
- References to medical and / or clinical research and testing.
- References to the health risks of not taking a particular product.
- Editorial medicinal claims.
- Testimonials that include/imply medicinal claims.
- Graphics that imply medicinal uses.
- References to, or reproduction of "generic" information.
- Juxtaposing with any examples of the above.

Food law

The Food Labelling Regulations contain detailed provisions for both the labelling and advertising of food. In particular, any claim that a food has the property of preventing, treating or curing human disease is prohibited. This prohibition covers any implication that a foodstuff is capable of protecting against disease, infection or other adverse condition or relieving symptoms. Food safety law is

administered and enforced locally on behalf of the Food Standards Agency by the Trading Standards Service.

In addition, health claims must now be approved under The Nutrition & Health Claims (England) Regulations 2007

Your Product

Phytin Mineral Salt

The MHRA takes the view that, based on the information provided, Phytin Mineral Salt falls outside of the definition of a medicinal product.

However, please be aware that the opinion expressed in this message does not provide authority to place the product on the market as a Food. Food law is administered by the Trading Standards Service and we would recommend that you contact your local service for guidance, if you have not already done so.

The Agency reserves the right to change its view in the event of any information or evidence which has a bearing on the status of the product, including the way in which it is packaged, promoted or presented. The Agency can give no assurance that any particular product, including products under development, will not subsequently be classified as a medicinal product. It is the responsibility of those marketing a product to ensure that it is marketed in accordance with the relevant legislation.

Yours Sincerely

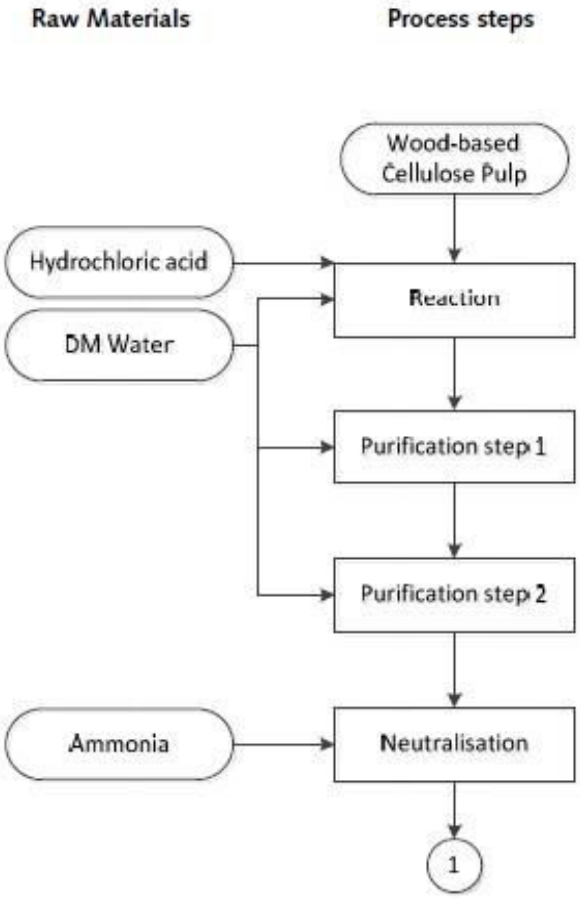
William Whitfield
Classifier, Medicines Borderline Section

T +44 (0)20 3080 6163
E william.whitfield@mhra.gov.uk

DFE pharma

Process Flow Sheet

Product group: MCC
Brand name: Pharmacel® 112
Product code: 13115-5355
Product description: Microcrystalline Cellulose
Document No.: PD-0597 Page 1 of 2



DMV-Fonterra Excipients GmbH & Co. KG

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BLZ 502 304 00
Account 1809898005
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Amtsgericht Kleve HRB 8945

Edition No.: 1 Issue date: 09 Jul 2015 Valid until: 30 Apr 2021

DFE pharma

Process Flow Sheet

Product group: MCC
Brand name: Pharmacel ® 112
Product code: 13115-5355
Product description: Microcrystalline Cellulose
Document No.: PD-0597 Page 2 of 2

Process Steps	Process parameters
1	
Spray-drying	
Magnet	Magnet strength min. 6000 Gauss
Sieving	400 µm mesh size
Packaging	A white multi-layered polyethylene-aluminum bag with a polyethylene inner liner, 25Kg net

This statement substitutes all previous versions issued for the brand names mentioned above.
We trust this information, which is made up to the best of our knowledge, will be helpful to you.

Name : Wouter H. Noordman, PhD
Job title : Global Technology & Innovation Director
Signature :

This document is controlled by a validated, electronic system and is valid without signature.
The above facsimile signature is only for display.

Edition No.: 1 Issue date: 09 Jul 2015 Valid until: 30 Apr 2021

DFE pharma

Product Specification

Product group: MCC
Brand name: Pharmacel®112
Product code: SAP 743842; Navision 13115-535
Product descr: Microcrystalline Cellulose
Document No.: PD-0601

Production site: DFE Pharma India LLP, Cuddalore India
Product name: Pharmacel® 112
Conforms to USP-NF, Ph. Eur., JP, IP Specifications on date of manufacture
Product description: A white or almost white fine, crystalline or granular, slightly hygroscopic powder having fluidity, odorless
Solubility: Practically insoluble in water, acetone, anhydrous ethanol, toluene, diethyl ether dilute acid and swells with 50 g/L sodium hydroxide solution.

Test	Specification
Identification A (USP-NF & Ph.Eur.)	Pass
Identification 2 (JP)	Pass
Identification C (IP)	Pass
Degree of polymerisation	Max. 350
Solubility in ammoniacal copper tetrammine	Pass
pH	5.5 – 7.0
Conductivity	Max. 75µS/cm
Ether soluble substances	Max. 0.05%
Water soluble substances	Max. 0.20%
Heavy metals	Pass
Assay (IP)	97.0-102.0%
Arsenic	Pass
Starch and Dextrins (IP)	Pass
Organic impurities (IP)	Pass
Loss on Drying	Max. 1.5%
Residue on Ignition	Max. 0.05%
Particle size %< 32µm (Airjet)	Max. 30.0%
Particle size %< 75µm (Airjet)	30.0-55.0%
Particle size %< 250µm (Airjet)	Min. 92.0%
Particle size D10 (Malvern)	Report value
Particle size D50 (Malvern)	Report value
Particle size D90 (Malvern)	Report value
Bulk density	0.28-0.34 g/mL
Total Aerobic Microbial Count	Max. 100 cfu/g
Total Yeast and Mould Count	Max. 20 cfu/g
Escherichia coli in 10g	Negative
Pseudomonas aeruginosa in 10g	Negative
Staphylococcus aureus in 10g	Negative

DMV-Fonterra Excipients GmbH & Co. KG

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General partner: DMV-Fonterra Excipients Verwaltungs-GmbH
Directors: Jan Jongsma
Stephen Gajzago
pharma@dfepharma.com
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Stat. seat: Goch
Amtsgericht Kleve HRB 8945

Edition No.: 4 Issue date: 01 Jan 2017 Valid until: 31 Dec 2019

DFE pharma

Product Specification

Product group: MCC
Brand name: Pharmacel®112
Product code: SAP 743842; Navision 13115-535
Product description: Microcrystalline Cellulose
Document No.: PD-0601

Salmonella species in 10g	Negative

Packaging:
A white multi-layered polyethylene-aluminum bag with a polyethylene inner liner, 25Kg net.

Storage:
Keep in original, unopened packing in ambient conditions, protected from humidity and away from strongly odorous materials. The storage of Pharmacel® 112 in original special packaging ensures complete protection from moisture absorption. If the packaging is opened, we strongly recommend closing the bag by heat-sealing, when material is not fully used.

Shelf life:
For storage periods exceeding 48 months, we recommend re-testing.

This document substitutes all previous versions issued for the brand names mentioned above.
We trust this information, which is made up to the best of our knowledge, will be helpful to you.

Name : Wouter H. Noordman, PhD
Job title : Global Technology & Innovation Director
Signature :

This document is controlled by a validated, electronic system and is valid without signature.
The above facsimile signature is only for display.

DMV-Fonterra Excipients GmbH & Co. KG

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Account 1809898005
BIC: ABNADEFFFRA
IBAN: DE85502304001809898005
VAT DE 246736318

General partner: DMV-Fonterra Excipients Verwaltungs-GmbH
Directors: Jan Jongsma
Stephen Gajzago
pharma@dfepharma.com
www.dfepharma.com
Stat. seat: Goch
Amtsgericht Kleve HRB 8965

DFE pharma

Safety Information

Product group: MCC
Brand name: Pharmacel® 101, 102, 112
Article codes: 743678, 743679, 743842
733326, 73317
Product description: Microcrystalline Cellulose
Document No.: PD-0282

Safety Information

- This product is not a dangerous product according to the applicable United Nations rules;
- The Safety Information is provided as a service to our customers and there is no legal obligation in the European Union to provide it in your national language and in the format of the Regulation (EC) 1907/2006 (consolidated);
- This safety information provides hazard information according to 29 CFR 1910.1200(a)(2);
- This document should be read in conjunction with the **Ingredient Declaration** and **Certificate of Standards/Product Specification** for the relevant product or product category

1. Product and company identification

Product name: Microcrystaline Cellulose

DFE Pharma Brand names; Pharmacel® 101, Pharmacel® 102, Pharmacel® 112

Manufacturer/supplier identification: See Footer

Identified uses; Excipient used in the manufacturing of pharmaceutical products

2. Hazards Identification

No hazardous product as specified in the current EU legislation and in UN-rules.

GHS-US classification; combustible dust.

Warning! May form combustible dust concentrations in air (during processing)

DFE pharma

Safety Information

Product group: MCC
Brand name: Pharmacel® 101, 102, 112
Article codes: 743678, 743679, 743842
733326, 73317
Product description: Microcrystalline Cellulose

3. Composition/information on ingredients

Product contains 100% (Microcrystalline) Cellulose

EINECS/ELINCS No.: 232-674-9
CAS No.: 9004-34-6
RTECS No.: FJ5691460

4. First aid measures

After inhalation: fresh air
After skin contact: wash off with plenty of water.
After eye contact: rinse out with water.
After swallowing (large amounts): get medical attention.

5. Fire-fighting measures

Explosion; Avoid generating dust; fine dust dispersed in air in sufficient concentrations, and in the presence of an ignition source (e.g. static electricity) is a potential dust explosion hazard

This product is flammable. Suitable extinguishing media: water, powder, spray foam, CO_2
In adaptation to materials stored in the immediate neighborhood.

6. Accidental Release measures

Dust deposits should not be allowed to accumulate on surfaces, as these may form an explosive mixture if they are released into the atmosphere in sufficient concentration.

Avoid dispersal of dust in the air (i.e. clearing dust surfaces with compressed air)

No sparking tools should be used

DFE pharma

Safety Information

Product group: MCC
Brand name: Pharmacel® 101, 102, 112
Article codes: 743678, 743679, 743842
733326, 73317
Product description: Microcrystalline Cellulose

7. Handling and storage

For safety reasons, store in tightly closed packing protected from solvents.

Minimize dust generation and accumulation.

Routine housekeeping should be instituted to ensure that dusts do not accumulate on surfaces.

Dry powders can build static electricity charges when subjected to the friction of transfer and mixing operations. Provide adequate precautions, such as electrical grounding and bonding, or inert atmospheres.

8. Exposure controls/personal protection

Respiratory protection required when dusts are generated.
Eye protection is required.
The use of hand protection is recommended.
Wash hands after working with substance.

It is recommended that all dust control equipment such as local exhaust ventilation and material transport systems involved in handling of this product contain explosion relief vents or an explosion suppression system or an oxygen- deficient environment.

Ensure that dust-handling systems (such as exhaust ducts, dust collectors, vessels, and processing equipment) are designed in a manner to prevent the escape of dust into the work area (i.e., there is no leakage from the equipment).
Use only appropriately classified electrical equipment and powered industrial trucks

DFE pharma

Safety Information

Product group: MCC
Brand name: Pharmacel® 101, 102, 112
Article codes: 743678, 743679, 743842
733326, 73317
Product description: Microcrystalline Cellulose
Document No.: PD-0282 Page 4 of 5

9. Physical and chemical properties

For chemical and physico-chemical data see the Certificate of Standards (COS)

10. Stability and reactivity

Like any other powdered product, there is a risk of explosion in a confined cloud (combustible dust formation).

LEL g/m³	Pmax Bar	Kst bar.m/s	MIE mJ
60	8.8	97	≤ 50
MIT °C	Smoulder °C	Dust Explosion class	
400	330	1	

LEL= Lower explosion limit; Pmax= Maximum explosion pressure; Kst=Maximum rate of pressure rise; MIE= Minimum ignition energy; MIT= Determination of the minimum ignition temperature; Smoulder= Smoulder temperature

11. Toxicological information

No toxic effects are to be expected when the product is handled appropriately

12. Disposal considerations

Products and Packaging;

There are no uniform EC Regulations for the disposal of chemicals or residues. Chemical residues generally count as special waste. The disposal of the latter is regulated in the EC member countries through corresponding laws and regulations. We recommend that you contact either the authorities in charge or approved waste disposal companies which will advise you on how to dispose of special waste.

13. Transport information

Not subject to limitations due to transport regulations.

14. Regulatory information

Labeling according to applicable local legislation; Keep away from sources of ignition – No smoking

DFE pharma

Safety Information

Product group: MCC
Brand name: Pharmacel® 101, 102, 112
Article codes: 743678, 743679, 743842
733326, 73317
Product description: Microcrystalline Cellulose
Document No.: PD-0282

15. Other information

Please refer to;

NFPA 654, *Standard for the Prevention of Fire and Dust Explosions from the Manufacturing, Processing, and Handling of Combustible Particulate Solids*, for safe handling

OSHA information on combustible dusts;
https://www.osha.gov/pls/publications/publication.athruz?pType=Industry&pID=250

ATEX **Directive 2014/34/EU**

16. Ecological information

No ecological problems are to be expected when the product is handled and used with due care and attention.

DFE pharma

G M O (Modified Organisms)

Product group: MCC
Brand name: Pharmacel®
Product description: Microcrystalline Cellulose
Document No.: PD-0478 Page 1 of 1

Dear Customer,

Pharmacel® is pharmaceutical grade Microcrystalline Cellulose. To meet the high quality standards from the pharmaceutical industry with respect to colour and performance of the product, wooden pulps are used as starting material for Pharmacel®.
Herewith we confirm to you that we do not use genetically modified organisms (GMO's) or GMO derived products in the production of Pharmacel®.

Referring to the current legislation in the European Union* the product does not have to be labeled as GMO or GMO derived.

* Relevant EU legislation:
Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed

Regulation (EC) No 1830/2003 of the European Parliament and of the Council of 22 September 2003 concerning the traceability and labeling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC

This statement substitutes all previous versions issued for the brand names mentioned above.
We trust this information, which is made up to the best of our knowledge, will be helpful to you.

With kindest regards,

Name : Armand M. Janssen
Job title : Manager Regulatory Affairs
Signature :

This document is controlled by a validated, electronic system and is valid without signature.
The above facsimile signature is only for display.

DMV-Fonterra Excipients GmbH & Co. KG

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47568 Goch, Germany
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F. +49 2823 9288 7799
Stat. seat: Goch
Amtsgericht Kleve HRA 3212

Bank
The Royal Bank of Scotland
BLZ 502 304 00
Account 1809898005
BIC: ABNADEFFFNA
IBAN: DE65502304001809898005
VAT DE 246726318

General partner: DMV-Fonterra Excipients Verwaltungs GmbH
Directors: Bas van Driel
Richard Whiteman
pharma@dfepharma.com
www.dfepharma.com
Stat. seat: Goch
Amtsgericht Kleve HRB 8965

DFE pharma

EU Regulation (EC) 231/2012

Product group:	MCC
Brand name:	Pharmacel®101, Pharmacel®102, Pharmacel®112
Article code:	743678, 743679, 743842
Product description:	Microcrystalline Cellulose
Document No.:	PD-0564 Page 1 of 1

Dear Customer,

In the Regulation (EC) 231/2012 a detailed specification for microcrystalline cellulose is mentioned.
Our products Pharmacel®101, Pharmacel®102 and Pharmacel®112 fully comply with all requirements mentioned in this regulation.
Pharmacel®101, Pharmacel®102 and Pharmacel®112 can thus be used as food additives (E460(i)) in the European Union in the legally defined categories.

This statement substitutes all previous versions issued for the brand names mentioned above.
We trust this information, which is made up to the best of our knowledge, will be helpful to you.

With kindest regards,

Name : Armand M. Janssen
Job title : Manager Regulatory Affairs
Signature :

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BIC: ABNADEFFFRA
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General partner: DMV-Fonterra Excipients Verwaltungs-GmbH
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www.dfepharma.com
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Amtsgericht Kleve HRB 8945

DFE pharma

Ingredient Declaration

Product group: MCC
Brand name: Pharmacel®
Product description: Microcrystalline Cellulose
Document No.: PD-0476 Page 1 of 1

Dear Customer,

We, DMV-Fonterra Excipients GmbH & Co. KG (DFE Pharma) herewith declare that our Pharmacel® is a pharmaceutical grade filler-binder prepared from the following ingredients:

Microcrystalline Cellulose	Ph. Eur./ USP-NF	100%

This statement substitutes all previous versions issued for the brand names mentioned above.
We trust this information, which is made up to the best of our knowledge, will be helpful to you.

Name : Wouter H. Noordman, PhD
Job title : Global Technology & Innovation Director
Signature :

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General partner: DMV-Fonterra Excipients Verwaltungs-GmbH
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www.dfepharma.com
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Edition No.: 2 Issue date: 15 Dec 2015 Valid until: 15 Dec 2018

DFE pharma

ALBA – Statement

Product group: MCC
Brand name: Pharmacel®
Product description: Microcrystalline Cellulose
Document No.: PD-0043 Page 1 of 2

Dear Customer,

Herewith we certify the presence or absence of allergens in the above mentioned products. The format is based on the former Alba database (now LeDA database) as maintained by the The Netherlands Nutrition Centre/National Institute for Public Health and the Environment.

LeDa code	GS1 code	Allergen	Recipe without (Z)	Recipe contains (M)	May contain (and recipe without) (K)	Unknown (0)
1.1	UW	Wheat	X			
1.2	NR	Rye	X			
1.3	GB	Barley	X			
1.4	GO	Oats	X			
1.5	GS	Spelt	X			
1.6	GK	Kamut	X			
1	AW	Gluten	X			
2.0	AC	Crustaceans	X			
3.0	AE	Egg	X			
4.0	AF	Fish	X			
5.0	AP	Peanuts	X			
6.0	AY	Soy	X			
7.0	AM	Cow's milk	X			
8.1	SA	Almonds	X			
8.2	SH	Hazelnuts	X			
8.3	SW	Walnuts	X			
8.4	SC	Cashews	X			
8.5	SP	Pecan nuts	X			
8.6	SR	Brazil nuts	X			
8.7	ST	Pistachio nuts	X			
8.8	SM	Macadamia/ Queensland nuts	X			
8	AN	Nuts	X			
9.0	BC	Celery	X			
10.0	BM	Mustard	X			
11.0	AS	Sesame	X			

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Edition No. 4 Issue date: 16 Jun 2017 Valid until: 16 Jun 2020

DFE pharma

ALBA – Statement

Product group: MCC
Brand name: Pharmacel®
Product description: Microcrystalline Cellulose
Document No.: PD-0043 Page 2 of 2

LeDa code	GS1 code	Allergen	Recipe without (Z)	Recipe contains (M)	May contain (and recipe without) (K)	Unknown (0)
12.0	AU	Sulpher dioxide and sulphites (E 220;E 228) at concentrations of more than 10 mg/kg or 10 mg/l, expressed as SO2	X			
13.0	NL	Lupin	X			
14.0	UM	Molluscs	X			
		Additional allergens	X			
20.0	ML	Lactose	X			
21.0	NC	Cocoa	X			
22.0	MG	Glutamate (E 620-E 625)	X			
23.0	MK	Chicken meat	X			
24.0	NK	Coriander	X			
25.0	NM	Corn/maize	X			
26.0	NP	Legumes	X			
27.0	MC	Beef	X			
28.0	MP	Pork	X			
29.0	NW	Carrot	X			

In addition we can confirm the absence of:
BHA/BHT (E 329,E 321), Gallates (E 310-E 312), Benzoic acid (E 210-E 213), Parabens (E 214-E 219), Azo colors, Tartrazine (E 102), Sunset yellow (E 110), Carmoisine (E 122), Amaranth (E 123), Poncean 4R (E 124), Sorbic acid (E 200-E 203), Cinnamon, Vanillin, Seafood and groundnuts/-oil.

This statement substitutes all previous versions issued for the brand names mentioned above.
We trust this information, which is made up to the best of our knowledge, will be helpful to you.

With kindest regards,

Name : Armand M. Janssen
Job title : Manager Regulatory Affairs
Signature :

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Edition No.: 4 Issue date: 16 Jun 2017 Valid until: 16 Jun 2020

DFE pharma

Residual Solvents Statement

Product group: MCC
Brand name: Pharmacel® 101, 102 & 112
Document No.: PD-0540
Page 1 of 1

Dear Customer,

Herewith we certify the following:
For the manufacturing of our microcrystalline cellulose, Pharmacel® 101, 102 and 112, product code 743678, 743679, 743842, 733317 and 733326 no other solvents than water are used.
This is certified with reference to the solvents listed in the "Q3C ICH guidelines on residual solvents (EMA/CHMP/ICH/82260/2006)" and USP general chapter <467>: no class 1, 2, 3 solvents are used in the raw materials, manufacturing process and product. General test 5.4 of the European Pharmacopoeia is also applicable. In addition, we do not use other organic solvents that are not listed in the ICH Q3C or the USP<467> either.
Since only water is used during the raw material and end product production, the final product is only tested on presence of free water by the pharmacopoeia test 'Loss on Drying'.
Our pharmaceutical grade microcrystalline cellulose complies with the current Ph. Eur. Requirements for Residual Solvents and USP requirements for Organic Volatile Impurities / Residual Solvents.

This statement substitutes all previous versions issued for the brand names mentioned above.
We trust this information, which is made up to the best of our knowledge, will be helpful to you.

Name : Wouter H. Noordman, PhD
Job title : Global Technology & Innovation Director
Signature :

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Edition No.: 2 Issue date: 22 Nov 2017 Valid until: 22 Nov 2020

SAFETY DATA SHEET
MAIZE STARCH AMYLO N-400

SECTION 1: Identification of the substance/mixture and of the company/undertaking

1.1 Product identifier:

Product name: MAIZE STARCH AMYLO N-400
Chemical name: Amylose
REACH Registration No.: Exempted
CAS-No.: 9005-82-7 **EC No.:** 232-685-9 **INCI Name:**

1.2 Relevant identified uses of the substance or mixture and uses advised against:

Identified uses:	Uses advised against:
Industrial. , Food. , Animal Feed. , Pharmaceuticals.	No data available.

1.3 Details of the supplier of the safety data sheet:

Supplier:
ROQUETTE FRERES
1 Rue de la Haute Loge
62136 LESTREM - France

Telephone: +33 3 21 63 36 00
Fax: +33 3 21 63 38 50
E-mail: sds@roquette.com

1.4 Emergency telephone number: NPIS (24/24) : 844 892 0111

SECTION 2: Hazards identification

2.1 Classification of the substance or mixture:

The product has not been classified as dangerous according to the legislation in force : CLP Regulation (EC) No 1272/2008.

2.2 Label elements: Not applicable

2.3 Other hazards: Dust may form an explosive mixture in the atmosphere.
Not fulfilling PBT (persistent/bioaccumulative/toxic) criteria
Not fulfilling vPvB (very persistent/very bioaccummulative) criteria

SECTION 3: Composition/information on ingredients

ROQUETTE

3.1 Substance:

Chemical name	Concentration	CAS-No.	EC No.	REACH Registration No.
Amylose	>=88%	9005-82-7	232-685-9	Exempted

SECTION 4: First aid measures

4.1 Description of first aid measures:

Inhalation: Move the exposed person to fresh air at once. Get medical attention if any discomfort continues.

Eye contact: Flush thoroughly with water. If irritation occurs, get medical assistance.

Skin contact: Wash with soap and water.

Ingestion: Get medical attention if symptoms occur.

4.2 Most important symptoms and effects, both acute and delayed:

Dust may irritate the eyes and the respiratory system.

4.3 Indication of any immediate medical attention and special treatment needed:

Treatment: Treat symptomatically.

SECTION 5: Firefighting measures

5.1 Extinguishing media:

Suitable extinguishing media:

Water spray.

Unsuitable extinguishing media:

Dry chemicals or foams.

5.2 Special hazards arising from the substance or mixture:

Fire or excessive heat may produce hazardous decomposition products. Dust may form an explosive mixture in the atmosphere. See Section 10.

5.3 Advice for firefighters:

Special Fire Fighting Procedures:

Prevent dust cloud.

Special protective equipment for fire-fighters:

Firefighters must use standard protective equipment including flame retardant coat, helmet with face shield, gloves, rubber boots, and in enclosed spaces, SCBA.

SECTION 6: Accidental release measures

See Section 8 of the SDS for Personal Protective Equipment.

6.1 Personal precautions, protective equipment and emergency procedures:

Avoid discharge to the aquatic environment.

6.2 Environmental precautions:

6.3 Methods and material for containment and cleaning up: Remove material, as much as possible, using mechanical equipment. Prevent dust cloud. Collect and dispose of spillage as indicated in section 13 of the SDS.

6.4 Reference to other sections: For waste disposal, see section 13 of the SDS.

SECTION 7: Handling and storage

7.1 Precautions for safe handling: Avoid generation and spreading of dust.

7.2 Conditions for safe storage, including any incompatibilities: Keep containers tightly closed. Store in original container.

7.3 Specific end use(s): Industrial., Food., Animal Feed., Pharmaceuticals.,

SECTION 8: Exposure controls/personal protection

8.1 Control parameters:

Occupational exposure limits:

This product does not contain any component with occupational exposure limits

Chemical name	Type	Exposure Limit Values	Source
Dust - Inhalable dust.	TWA	10 mg/m3	UK. EH40 Workplace Exposure Limits (WELs) (2007)
Dust - Respirable dust.	TWA	4 mg/m3	UK. EH40 Workplace Exposure Limits (WELs) (2007)

8.2 Exposure controls:

Appropriate engineering controls: Ventilate as needed to control airborne dust. Use explosion-proof ventilation equipment if airborne dust levels are high.

Individual protection measures, such as personal protective equipment:

Eye/face protection: Wear dust-resistant safety goggles where there is danger of eye contact.

Skin protection:

Hand Protection: No special precautions.

Other:	No special precautions.
Respiratory Protection:	In case of inadequate ventilation or risk of inhalation of dust, use suitable respiratory equipment with particle filter (type P1).
Hygiene measures:	Handle the product in accordance with the good hygiene practices and safety instructions.
Environmental exposure controls:	Avoid discharge to the aquatic environment.

SECTION 9: Physical and chemical properties

9.1 Information on basic physical and chemical properties:

Physical State:	solid
Form:	Powder
Color:	Off-white
Odor:	Odorless
pH:	~ 5.6 at 20 %
Melting Point:	No data available.
Boiling Point:	Not Applicable
Flash Point:	Not Applicable
Vapor pressure:	Not Applicable
Vapor density (air=1):	Not Applicable
Relative density:	~ 0.5

Solubility in Water:	Insoluble in water at 20 °C ~ 150 g/l at 90 °C

Explosive properties: - INERIS -Data from similar product.

Ignition Temperature:	~ 480 °C (Godbert-Greenwald) MIT in Cloud.
MIE (Minimum Ignition Energy):	~ 225 mJ (EN 13821 (Without Inductance)) Sensitive to ignition by an electrostatic phenomenon.
dP/dtmax (Maximum Rate of explosion Pressure rise):	~ 460 bar/s (EN 14034-2)
Pmax (Maximum Explosion OverPressure) ±10%:	~ 8.5 bar (EN 14034-1)
Kst value (±20%):	~ 124 barm/s (EN 14034-2)
Dust Explosion Class:	st 1 (VDI 3673)
Volume resistivity:	7,5x10^13 Ω.cm (IEC 61241-2-2 / Group IIIB nonconductive dust.)
Moisture:	~ 12.38 % (ISO 589)
Mv (Median value):	~ 18 µm (NFX 11-666)
Other Data:	LEL (Lower explosion limit) : 30-60 g/m3 BZ (Combustion class) : 3 (VDI 2263-1)

9.2 Other information:

SECTION 10: Stability and reactivity

Oxidizing agents.

10.1 Reactivity:

10.2 Chemical stability:

Material is stable under normal conditions.

10.3 Possibility of hazardous reactions:

No hazardous reactions under ordinary conditions of use and storage.

10.4 Conditions to avoid:

Prevent dust cloud. Dust clouds may be explosive under certain conditions. Avoid dust close to ignition sources.

10.5 Incompatible materials:

Strong oxidizing substances.

10.6 Hazardous decomposition products:

Carbon Dioxide. Carbon Monoxide.

SECTION 11: Toxicological information

11.1 Information on toxicological effects:

No data available.

Remarks:

The ingredients of this product are not classified as carcinogenic by the ACGIH, the CIRC, the OSHA or the NTP. No data on possible toxicity effects have been found.

SECTION 12: Ecological information

There are no data on the ecotoxicity of this product.

12.1 Toxicity: No data available.

12.2 Persistence and degradability: No data available.

12.3 Bioaccumulative potential: No data available.

12.4 Mobility in soil: No data available.

12.5 Results of PBT and vPvB assessment: Exempted

12.6 Other adverse effects: None known.

SECTION 13: Disposal considerations

13.1 Waste treatment methods:

Product: Dispose of waste in an appropriate authorised treatment facility in accordance with regulations in force and product characteristics at time of disposal. (for example, energy recovery).

Packaging material: Single use packaging. Collect for salvage or disposal.

SECTION 14: Transport information

The product is not covered by international regulation on the transport of dangerous goods (IMDG, IATA, ADR/RID).

14.5 Environmental hazards: Not regulated.

14.6 Special precautions for user: No special precautions.

14.7 Transport in bulk according to Annex II of MARPOL73/78 and the IBC Code: Not applicable.

SECTION 15: Regulatory information

15.1 Safety, health and environmental regulations/legislation specific for the substance or mixture:

This Safety Data Sheet is not mandatory according to the requirements of regulation (EC) N°1907/2006 (REACH) article 31 and is provided for information.

15.2 Chemical safety assessment: Exempted

SECTION 16: Other information

Revision Information: Not relevant.

Key literature references and sources for data: No data available.

Abbreviations and acronyms used in the SDS.:

CAS: Chemical Abstracts Service (division of the American Chemical Society) CLP : Classification, Labelling and Packaging.
PBT: Persistent, Bioaccumulative and Toxic
REACH : Registration, Evaluation, Authorisation and Restriction of Chemicals.
vPvB: very persistent and very bioaccumulative substance.

Disclaimer:

The information provided in this Safety Data Sheet (SDS) relates only to the specific product designated and may not be applicable when such product is used in combination with other materials or in any process. It is the responsibility of the user to be aware of and to follow the regulations applying to our product for its possession, handling and use.
The information given is designed only as a guidance and is not to be considered a warranty or quality specification.
All information and instructions provided in this SDS are based on the current state of our knowledge at the latest revision date indicated.

SAFETY DATA SHEET (EC 1907/2006)

EUDRAGIT S 100 / 20 KG

Version: **1.1 / GB** VA-No.
Revision date: **30.11.2017**
Issue date: 19.05.2016
replaces version: 1.0
Page: **1** / **9**

SECTION 1: Identification of the substance/mixture and of the company/undertaking

1.1. Product identifier

Trade name : EUDRAGIT S 100 / 20 KG

Chemical Name : Acrylic polymer

1.2. Relevant identified uses of the substance or mixture and uses advised against

Relevant applications : pharmaceutical and cosmetic excipient for oral and dermal use identified

Applications which are not advised : None known.

1.3. Details of the supplier of the safety data sheet

Company : Evonik Nutrition & Care GmbH
Health Care
Kirschenallee
D-64293 Darmstadt

Telephone : +49 (0)201 173-01

Telefax : +49 (0)201 173-3000

E-mail : productsafety-cs@evonik.com

1.4. Emergency telephone number

+49 (0)2365 49-2232 (TUIS - Interpreting service available)

+49 (0)2365 49-4423 (TUIS - Fax)

SECTION 2: Hazards identification

2.1. Classification of the substance or mixture

Classification according to Regulation (EC) No. 1272/2008 [CLP]

Not a hazardous substance or mixture.

2.2. Label elements

The product does not require a hazard warning label in accordance with GHS. The normal safety precautions for the handling of chemicals must be observed.

2.3. Other hazards

None known.

SECTION 3: Composition/information on ingredients

Acrylic polymer

3.1. Substances

No hazardous ingredients

3.2. Mixtures

-

SECTION 4: First aid measures

4.1. Description of first aid measures

General advice	: Remove soiled or soaked clothing immediately
Inhalation	: Ensure supply of fresh air. In the event of symptoms seek medical advice.
Skin contact	: In case of contact with skin wash off immediately with soap and water In the event of symptoms seek medical advice.
Eye contact	: In case of contact with eyes rinse thoroughly with water. In the event of symptoms seek medical advice.
Ingestion	: Thoroughly clean the mouth with water In the event of symptoms seek medical advice.

4.2. Most important symptoms and effects, both acute and delayed

Symptoms : Up to now no symptoms are known.

4.3. Indication of any immediate medical attention and special treatment needed

Treat symptomatically.

SECTION 5: Firefighting measures

5.1. Extinguishing media

Suitable extinguishing media : foam, carbon dioxide, dry powder, water spray.

Unsuitable extinguishing : Full water jet media

5.2. Special hazards arising from the substance or mixture

In the event of fire the following can be released:

carbon dioxide, carbon monoxide
Under certain conditions of combustion traces of other toxic substances cannot be excluded

5.3. Advice for firefighters

Do not inhale explosion and/or combustion gases Use self-contained breathing apparatus

SECTION 6: Accidental release measures

6.1. Personal precautions, protective equipment and emergency procedures

High risk of slipping due to leakage/spillage of product.
Use personal protective equipment.
Avoid dust formation.

6.2. Environmental precautions

Do not allow to enter drains or waterways Do not discharge into the subsoil/soil.

6.3. Methods and material for containment and cleaning up

Pick up mechanically
Dispose of absorbed material in accordance with the regulations.

6.4. Reference to other sections

For further information on exposure monitoring and disposal see sections 8 and 13.

SECTION 7: Handling and storage

7.1. Precautions for safe handling

Advice on safe handling	: Provide good ventilation of working area (local exhaust ventilation if necessary). Avoid the formation and deposition of dust.
Hygiene measures	: Wash hands before breaks and after work. Do not eat, drink or smoke when working. Remove soiled or soaked clothing immediately.
General protective measures	: Do not inhale dust/fumes/aerosols. Avoid contact with eyes and skin

7.2. Conditions for safe storage, including any incompatibilities Prevention of fire and explosion

Information	: Keep away from sources of ignition Take precautionary measures against electrostatic loading. Dust can form an explosive mixture with air. Cool endangered containers by water spray
Dust explosion class	: St2

Storage

Information	: Avoid contamination during sampling.
Further information on storage conditions	: Keep container tightly closed in a cool, well-ventilated place Protect from atmospheric moisture and water Keep away from direct sunlight. Keep in a dry place. Keep away from heat.

7.3. Specific end use(s)

No further recommendations.

SECTION 8: Exposure controls/personal protection

8.1. Control parameters

Contains no substances with occupational exposure limit values.

DNEL	: No DNEL/DMEL values on file.
PNEC	: No PNEC values on file.

8.2. Exposure controls

Eye protection	: safety glasses
Hand protection	: Protective gloves
Body Protection	: protective clothing
Respiratory protection	: in case of formation of vapours/dusts: Short term: filter apparatus, Filter P3

SECTION 9: Physical and chemical properties

9.1. Information on basic physical and chemical properties

Physical state	: solid
Form	: Powder
Colour	: white
Odour	: slight, typical
Odour Threshold	: not measured
pH	: not measured

Melting point	: Melting point/range > 200 °C
Boiling point	: not measured
Flash point	: > 250 °C Method: ASTM D 1929-68
Evaporation rate	: not measured
Flammability	: not measured
Upper Explosion/Ignition Limit	: not measured
Lower explosion limit	: not measured
Vapour pressure	: not measured
Relative vapour density	: not measured
Relative density	: not measured
Solubility(ies)	: Medium: Acetone Remarks: soluble Medium: lower alcohols Remarks: soluble Medium: alkalines Remarks: soluble
Water solubility	: virtually insoluble
Partition coefficient: noctanol/water	: not measured
Autoignition temperature	: not measured
Thermal decomposition	: not measured
Viscosity, kinematic	: not measured
Viscosity, dynamic	: not measured
Explosive properties	: not measured
Oxidising properties	: not measured
Density	: not measured

9.2. Other information

Bulk density : 500 kg/m3
(20 °C)

Metal corrosion : not measured

Ignition temperature : > 400 °C
Method: ASTM D 1929-68

volatile organic compounds
:

SECTION 10: Stability and reactivity

10.1. Reactivity

see section "Possibility of hazardous reactions"

10.2. Chemical stability

The product is stable under normal conditions.

Depolymerization begins at 200 °C

10.3. Possibility of hazardous reactions

No hazardous reactions with proper storage and handling.

10.4. Conditions to avoid

Direct sunlight
humidity Heat

10.5. Incompatible materials

Unknown

10.6. Hazardous decomposition products

None with proper storage and handling.

SECTION 11: Toxicological information

11.1. Information on toxicological effects

Acute toxicity (oral) : LD50
Species: Rat
Dose: > 5.000 mg/kg

LD50
Species: dog
Dose: > 5.000 mg/kg

Acute toxicity (inhalation) : no data available

Acute toxicity (dermal) : LD50
Species: Rat
Dose: > 2.000 mg/kg
Method: OECD 402

Irritation/corrosion of the skin : Species: Rabbit
Result: non-irritant
Method: OECD 404

Serious eye damage/ eye irritation : Species: Rabbit
Result: non-irritant
Method: OECD 405

Respiratory/skin sensitization : Buehler Test
Species: Guinea pig
Result: non-sensitizing
Method: OECD 406

Repeated dose toxicity : Species: dog
Application Route: Oral
Exposure duration: 6 weeks
NOAEL: 2.000 mg/kg
GLP: Yes

Species: Rat
Application Route: Oral
Exposure duration: 6 months
NOAEL: 100 mg/kg
Remarks: This information is derived from evaluation of or a test result for a similar compound (conclusion based on analogy).

Genotoxicity in vitro : Ames test
Salmonella typhimurium
Result: negative

Carcinogenicity : no specific test data available no evidence for hazardous properties (structure-activity-relationships) (analogy)

Reprotoxicity / Fertility : no specific test data available no evidence for hazardous properties (structure-activity-relationships) (analogy)

Specific Target Organ Toxicity - Single exposure : no evidence for hazardous properties

Specific Target Organ Toxicity - Repeated exposure	: no evidence for hazardous properties
Aspiration hazard	: No aspiration toxicity classification
Other information	: The properties of this product which are hazardous to health have been calculated as per regulation (EC) No. 1272/2008. See section 2 "Hazards Identification".

SECTION 12: Ecological information

Ecotoxicology Assessment

Acute aquatic toxicity	: Based on available data, the classification criteria are not met.
Chronic aquatic toxicity	: Based on available data, the classification criteria are not met.

12.1. Toxicity

Aquatoxicity, fish	: Species: Poecilia reticulata (guppy) Exposure duration: 96 h LC50: > 100 mg/l Method: OECD 203 Remarks: The data are derived from the evaluations or test results achieved with similar products (conclusion by analogy).
Aquatoxicity, invertebrates	: no data available
Aquatoxicity, algae / aquatic plants	: no data available
Toxicity in microorganisms	: no data available
chronic toxicity in fish	: no data available
Chronic toxicity in aquatic Invertebrates	: no data available

12.2. Persistence and degradability

Photodegradation	: no evidence for hazardous properties (structure-activity-relationships) (analogy)

Biological degradability : Biological degradability: 8 %
Exposure duration: 28 d
Result: Slightly biodegradable
Method: OECD 302 B
Remarks: The data are derived from the evaluations or test results achieved with similar products (conclusion by analogy).

12.3. Bioaccumulative potential

Bioaccumulation : no evidence for hazardous properties
(structure-activity-relationships) (analogy)

12.4. Mobility in soil

Environmental distribution : no evidence for hazardous properties
(structure-activity-relationships)
(analogy)

12.5. Results of PBT and vPvB assessment

PBT and vPvB assessment : This substance/mixture contains no components considered to be either persistent, bioaccumulative and toxic (PBT), or very persistent and very bioaccumulative (vPvB) at levels of 0.1% or higher.

12.6. Other adverse effects

General Information : The product is considered to be a weak water pollutant (German law).
Do not allow to enter soil, waterways or waste water canal.

SECTION 13: Disposal considerations

13.1. Waste treatment methods

Product : In accordance with local authority regulations, take to special waste incineration plant

Contaminated packaging : If empty contaminated containers are recycled or disposed of, the receiver must be informed about possible hazards.

SECTION 14: Transport information

Not dangerous according to transport regulations.

14.1. UN number: **--**
14.2. UN proper shipping name: **--**

14.3.	Transport hazard class(es):	--
14.4.	Packing group:	--
14.5.	Environmental hazards:	--
14.6	Special precautions for user:	No

SECTION 15: Regulatory information

regulations/legislation specific for the substance or mixture

5.2.1

15.1. Safety, health and environment

National legislation **al**

Technical instructions on Air Quality	: not applicable
Major Accident Hazard Legislation	: slightly water endangering Classification acc. to German law
Water contaminating class (Germany)	
Other regulations	: none

15.2. Chemical safety assessment

Chemical safety assessment	: No chemical safety assessment was carried out for this product.

SECTION 16: Other information

List of references

Other information : Comply with national laws regulating employee instruction.

Changes since the last version are highlighted in the margin. This version replaces all previous versions.

This information and all further technical advice is based on our present knowledge and experience. However, it implies no liability or other legal responsibility on our part, including with regard to existing third party intellectual property rights, especially patent rights. In particular, no warranty, whether express or implied, or guarantee of product properties in the legal sense is intended or implied. We reserve the right to make any changes according to technological progress or further developments. The customer is not released from the obligation to conduct careful inspection and testing of incoming goods. Performance of the product described herein should be verified by testing, which should be carried out only by qualified experts in the sole responsibility of a customer. Reference to trade names used by other companies is neither a recommendation, nor does it imply that similar products could not be used.

Legend

ADR	European Agreement concerning the International Carriage of Dangerous Goods by Road
ADN	European Agreement concerning the International Carriage of Dangerous Goods by Inland Waterways
ADNR	European agreement concerning the international carriage of dangerous goods by inland waterways (ADN)
ASTM	American Society for Testing and Materials
ATP	Adaptation to Technical Progress

BCF	Bioconcentration factor
BetrSichV	German Ordinance on Industrial Safety and Health
c.c.	closed cup
CAS	Chemical Abstract Services
CESIO	European Committee of Organic Surfactants and their Intermediates
ChemG	German Chemicals Act
CMR	carcinogenic-mutagenic-toxic for reproduction
DIN	German Institute for Standardization
DMEL	Derived minimum effect level
DNEL	Derived no effect level
EINECS	European Inventory of Existing Commercial Chemical Substances
EC50	half maximal effective concentration
GefStoffV	German Ordinance on Hazardous Substances
GGVSEB	German ordinance for road, rail and inland waterway transportation of dangerous goods
GGVSee	German ordinance for sea transportation of dangerous goods
GLP	Good Laboratory Practice
GMO	Genetic Modified Organism
IATA	International Air Transport Association
ICAO	International Civil Aviation Organization
IMDG	International Maritime Dangerous Goods
ISO	International Organization For Standardization
LOAEL	Lowest observed adverse effect level
LOEL	Lowest observed effect level
NOAEL	No observed adverse effect level
NOEC	no observed effect concentration
NOEL	no observed effect level
o. c.	open cup
OECD	Organisation for Economic Cooperation and Development
OEL	Occupational Exposure Limit
PBT	Persistent, bioaccumulative, toxic
PEC	Predicted effect concentration
PNEC	Predicted no effect concentration
REACH	REACH registration
RID	Convention concerning International Carriage by Rail
STOT	Specific Target Organ Toxicity
SVHC	Substances of Very High Concern
TA	Technical Instructions
TPR	Third Party Representative (Art. 4)
TRGS	Technical Rules for Hazardous Substances
VCI	German chemical industry association
vPvB	very persistent, very bioaccumulative
VOC	volatile organic compounds
VwVwS	German Administrative Regulation on the Classification of Substances Hazardous to Waters into Water Hazard Classes
WGK	Water Hazard Class
WHO	World Health Organization

SECTION 1: Identification of the substance/mixture and of the company/undertaking

1.1. Product identifier

Trade name : PLASACRYL T20 / 5 KG

Chemical Name : Aqueous emulsion with fatty acid glycerides and plasticizer

1.2. Relevant identified uses of the substance or mixture and uses advised against

Relevant applications : pharmaceutical and cosmetic excipient for oral and dermal use identified

Applications which are not advised : None known.

1.3. Details of the supplier of the safety data sheet

Company : Evonik Nutrition & Care GmbH
Health Care
Kirschenallee
D-64293 Darmstadt

Telephone : +49 (0)201 173-01

Telefax : +49 (0)201 173-3000

E-mail : productsafety-cs@evonik.com

1.4. Emergency telephone number

+49 (0)2365 49-2232 (TUIS - Interpreting service available)

+49 (0)2365 49-4423 (TUIS - Fax)

SECTION 2: Hazards identification

2.1. Classification of the substance or mixture Classification according to Regulation (EC) No. 1272/2008 [CLP]

Not a hazardous substance or mixture.

2.2. Label elements

The product does not require a hazard warning label in accordance with GHS. The normal safety precautions for the handling of chemicals must be observed.

2.3. Other hazards

None known.

SECTION 3: Composition/information on ingredients

Aqueous emulsion with fatty acid glycerides and plasticizer

3.1. Substances

-

3.2. Mixtures

No hazardous ingredients

SECTION 4: First aid measures

4.1. Description of first aid measures

General advice	: Remove soiled or soaked clothing immediately
Inhalation	: Ensure supply of fresh air. In the event of symptoms seek medical advice.
Skin contact	: In case of contact with skin wash off immediately with soap and water In the event of symptoms seek medical advice.
Eye contact	: In case of contact with eyes rinse thoroughly with water. In the event of symptoms seek medical advice.
Ingestion	: Thoroughly clean the mouth with water In the event of symptoms seek medical advice.

4.2. Most important symptoms and effects, both acute and delayed

Symptoms : Up to now no symptoms are known.

4.3. Indication of any immediate medical attention and special treatment needed

Treat symptomatically.

SECTION 5: Firefighting measures

5.1. Extinguishing media

Suitable extinguishing media : foam, carbon dioxide, dry powder, water spray.

Unsuitable extinguishing : Full water jet media

5.2. Special hazards arising from the substance or mixture

In the event of fire the following can be released:

- carbon dioxide, carbon monoxide

Under certain conditions of combustion traces of other toxic substances cannot be excluded

5.3. Advice for firefighters

Do not inhale explosion and/or combustion gases Use self-contained breathing apparatus

SECTION 6: Accidental release measures

6.1. Personal precautions, protective equipment and emergency procedures

High risk of slipping due to leakage/spillage of product.

Use personal protective equipment.
Ensure adequate ventilation.

6.2. Environmental precautions

Do not allow to enter drains or waterways Do not discharge into the subsoil/soil.

6.3. Methods and material for containment and cleaning up

Take up with absorbent material (eg sand, kieselguhr, universal binder) Dispose of absorbed material in accordance with the regulations.

6.4. Reference to other sections

For further information on exposure monitoring and disposal see sections 8 and 13.

SECTION 7: Handling and storage

7.1. Precautions for safe handling

Advice on safe handling	: Provide good ventilation of working area (local exhaust ventilation if necessary).
Hygiene measures	: Wash hands before breaks and after work. Do not eat, drink or smoke when working. Remove soiled or soaked clothing immediately.
General protective measures	: Do not inhale gases/vapours/aerosols. Avoid contact with eyes and skin

7.2. Conditions for safe storage, including any incompatibilities Prevention of fire and explosion

Information	: No special measures required.

Storage

Information	: Avoid contamination during sampling.

Further information on storage : Keep container tightly closed in a cool, well-ventilated place conditions
Protect from frost.

7.3. Specific end use(s)

No further recommendations.

SECTION 8: Exposure controls/personal protection

8.1. Control parameters

Contains no substances with occupational exposure limit values.

DNEL : No DNEL/DMEL values on file.

PNEC : No PNEC values on file.

8.2. Exposure controls

Eye protection : safety glasses

Hand protection : The protective gloves to be worn must satisfy the specifications of EC Guideline 89/686/EEC and the resulting Standard EN374.
Specific workplace situations must be considered separately.

Glove material: Nitrile rubber
Break through time: 480 min
Glove thickness: 0,33 mm

Body Protection : Wear suitable protective clothing.

Respiratory protection : in case of formation of vapours/aerosols:
Short term: filter apparatus, combination filter A-P2

SECTION 9: Physical and chemical properties

9.1. Information on basic physical and chemical properties

Physical state : liquid

Form : Liquid

Colour : white

Odour : slight, typical

Odour Threshold : not measured

pH : 2,1 - 4,5

Melting point	: Freezing point approx. 0 °C
Boiling point	: Boiling point/range approx. 100 °C
Flash point	: not measured
Evaporation rate	: not measured
Flammability	: not measured
Upper Explosion/Ignition Limit	: not measured
Lower explosion limit	: not measured
Vapour pressure	: not measured
Relative vapour density	: not measured
Relative density	: not measured
Solubility(ies)	: not measured
Water solubility	: dispersible
Partition coefficient: noctanol/water	: not measured
Autoignition temperature	: not measured
Thermal decomposition	: not measured
Viscosity, kinematic	: not measured
Viscosity, dynamic	: not measured
Explosive properties	: not measured
Oxidising properties	: not measured
Density	: not measured

9.2. Other information

Metal corrosion	: not measured
Ignition temperature	: not measured

SECTION 10: Stability and reactivity

10.1. Reactivity see section "Possibility of hazardous reactions"

10.2. Chemical stability

The product is stable under normal conditions.

10.3. Possibility of hazardous reactions

No hazardous reactions with proper storage and handling.

10.4. Conditions to avoid

freezing.

10.5. Incompatible materials

Unknown

10.6. Hazardous decomposition products

None with proper storage and handling.

SECTION 11: Toxicological information

11.1. Information on toxicological effects

Acute toxicity (oral)	: no data available
Acute toxicity (inhalation)	: no data available
Acute toxicity (dermal)	: no data available
Irritation/corrosion of the skin	: no data available
Serious eye damage/ eye irritation	: no data available
Respiratory/skin sensitization	: no data available
Repeated dose toxicity	: no data available

CMR assessment

Carcinogenicity	: no data available
Mutagenicity	: no data available
Teratogenicity	: no data available
Toxicity to reproduction	: no data available
Specific Target Organ Toxicity - Single exposure	: no data available
Specific Target Organ Toxicity - Repeated exposure	: no data available
Aspiration hazard	: No aspiration toxicity classification
Other information	: The properties of this product which are hazardous to health have been calculated as per regulation (EC) No. 1272/2008. See section 2 "Hazards Identification".

SECTION 12: Ecological information

Ecotoxicology Assessment

Acute aquatic toxicity	: Based on available data, the classification criteria are not met.
Chronic aquatic toxicity	: Based on available data, the classification criteria are not met.

12.1. Toxicity

Aquatoxicity, fish	: no data available
Aquatoxicity, invertebrates	: no data available
Aquatoxicity, algae / aquatic plants	: no data available
Toxicity in microorganisms	: no data available
chronic toxicity in fish	: no data available
Chronic toxicity in aquatic Invertebrates	: no data available

12.2. Persistence and degradability

Photodegradation : no data available

Biological degradability : no data available

12.3. Bioaccumulative potential

Bioaccumulation : no data available

12.4. Mobility in soil

Environmental distribution : no data available

12.5. Results of PBT and vPvB assessment

PBT and vPvB assessment : This substance/mixture contains no components considered to be either persistent, bioaccumulative and toxic (PBT), or very persistent and very bioaccumulative (vPvB) at levels of 0.1% or higher.

12.6. Other adverse effects

General Information : The product is considered to be a weak water pollutant (German law).
Do not allow to enter soil, waterways or waste water canal.

SECTION 13: Disposal considerations

13.1. Waste treatment methods

Product : In accordance with local authority regulations, take to special waste incineration plant

Contaminated packaging : If empty contaminated containers are recycled or disposed of, the receiver must be informed about possible hazards.

SECTION 14: Transport information

Not dangerous according to transport regulations.

14.1. UN number: **--**
14.2. UN proper shipping name: **--**
14.3. Transport hazard class(es): **--**
14.4. Packing group: **--**
14.5. Environmental hazards: **--** 14.6 Special precautions for user: No

SECTION 15: Regulatory information

15.1. Safety, health and environmental regulations/legislation specific for the substance or mixture National legislation

Technical instructions on Air Quality	: 5.2.5 (no class)
Major Accident Hazard Legislation	: not applicable
Water contaminating class (Germany)	: slightly water endangering Classification acc. to German law
Other regulations	: none

15.2. Chemical safety assessment

Chemical safety assessment	: No chemical safety assessment was carried out for this product.

SECTION 16: Other information

List of references

Other information	: Comply with national laws regulating employee instruction.

Classification and applied procedure to derive the classification of mixtures according to EU Regulation (EC) No. 1272/2008 (CLP)

Changes since the last version are highlighted in the margin. This version replaces all previous versions.

This information and all further technical advice is based on our present knowledge and experience. However, it implies no liability or other legal responsibility on our part, including with regard to existing third party intellectual property rights, especially patent rights. In particular, no warranty, whether express or implied, or guarantee of product properties in the legal sense is intended or implied. We reserve the right to make any changes according to technological progress or further developments. The customer is not released from the obligation to conduct careful inspection and testing of incoming goods. Performance of the product described herein should be verified by testing, which should be carried out only by qualified experts in the sole responsibility of a customer. Reference to trade names used by other companies is neither a recommendation, nor does it imply that similar products could not be used.

Legend

ADR	European Agreement concerning the International Carriage of Dangerous Goods by Road
ADN	European Agreement concerning the International Carriage of Dangerous Goods by Inland Waterways
ADNR	European agreement concerning the international carriage of dangerous goods by inland waterways (ADN)
ASTM	American Society for Testing and Materials
ATP	Adaptation to Technical Progress

BCF	Bioconcentration factor
BetrSichV	German Ordinance on Industrial Safety and Health
c.c.	closed cup
CAS	Chemical Abstract Services
CESIO	European Committee of Organic Surfactants and their Intermediates
ChemG	German Chemicals Act
CMR	carcinogenic-mutagenic-toxic for reproduction
DIN	German Institute for Standardization
DMEL	Derived minimum effect level
DNEL	Derived no effect level
EINECS	European Inventory of Existing Commercial Chemical Substances
EC50	half maximal effective concentration
GefStoffV	German Ordinance on Hazardous Substances
GGVSEB	German ordinance for road, rail and inland waterway transportation of dangerous goods
GGVSee	German ordinance for sea transportation of dangerous goods
GLP	Good Laboratory Practice
GMO	Genetic Modified Organism
IATA	International Air Transport Association
ICAO	International Civil Aviation Organization
IMDG	International Maritime Dangerous Goods
ISO	International Organization For Standardization
LOAEL	Lowest observed adverse effect level
LOEL	Lowest observed effect level
NOAEL	No observed adverse effect level
NOEC	no observed effect concentration
NOEL	no observed effect level
o. c.	open cup
OECD	Organisation for Economic Cooperation and Development
OEL	Occupational Exposure Limit
PBT	Persistent, bioaccumulative, toxic
PEC	Predicted effect concentration
PNEC	Predicted no effect concentration
REACH	REACH registration
RID	Convention concerning International Carriage by Rail
STOT	Specific Target Organ Toxicity
SVHC	Substances of Very High Concern
TA	Technical Instructions
TPR	Third Party Representative (Art. 4)
TRGS	Technical Rules for Hazardous Substances
VCI	German chemical industry association
vPvB	very persistent, very bioaccumulative
VOC	volatile organic compounds
VwVwS	German Administrative Regulation on the Classification of Substances Hazardous to Waters into Water Hazard Classes
WGK	Water Hazard Class
WHO	World Health Organization

SAFETY DATA SHEET

Preparation Date: 9/12/2013 **Revision Date:** 8/20/2018 **Revision Number:** G7

1. IDENTIFICATION

Product identifier

Product code: ET108 **Product Name:** ALCOHOL, 190 PROOF, USP

Other means of identification

Synonyms:	Alcool ethylique 190 proof (French) Alcohol etílico 190 proof (Spanish) Ethanol 190 proof Ethanol, undenatured 190 proof
CAS #:	64-17-5
RTECS #	KQ6300000
CI#:	Not available

Recommended use of the chemical and restrictions on use

Recommended use:	Solvent. Perfuming agent. In pharmaceuticals. Inks. In organic synthesis. In beverages.
Uses advised against	No information available

Supplier: Spectrum Chemical Mfg. Corp
14422 South San Pedro St.
Gardena, CA 90248
(310) 516-8000

Order Online At: https://www.spectrumchemical.com
Emergency telephone number Chemtrec 1-800-424-9300 **Contact Person:** Martin LaBenz (West Coast)
Contact Person: Ibad Tirmiz (East Coast)

2. HAZARDS IDENTIFICATION

Classification

This chemical is considered hazardous according to the 2012 OSHA Hazard Communication Standard (29 CFR 1910.1200)

Considered a dangerous substance or mixture according to the Globally Harmonized System (GHS)

Serious eye damage/eye irritation	Category 2
Reproductive toxicity	Category 1A
Specific target organ toxicity (single exposure)	Category 3
Specific target organ toxicity (repeated exposure)	Category 1

Flammable liquids	Category 2

Label elements

Danger

Hazard statements
Causes serious eye irritation
May damage fertility or the unborn child
May cause respiratory irritation. May cause drowsiness or dizziness
Causes damage to organs through prolonged or repeated exposure
Highly flammable liquid and vapor

Hazards not otherwise classified (HNOC)
Not Applicable

Other hazards
Causes mild skin irritation
Can burn with an invisible flame

Precautionary Statements - Prevention
Obtain special instructions before use
Do not handle until all safety precautions have been read and understood
Wash face, hands and any exposed skin thoroughly after handling
Wear protective gloves/protective clothing/eye protection/face protection
Do not breathe dust/fume/gas/mist/vapors/spray
Do not eat, drink or smoke when using this product
Use only outdoors or in a well-ventilated area
Keep away from heat/sparks/open flames/hot surfaces. — No smoking
Keep container tightly closed
Ground/bond container and receiving equipment
Use explosion-proof electrical/ventilating/lighting/.../equipment
Use only non-sparking tools
Take precautionary measures against static discharge
Keep cool

Precautionary Statements - Response
IF exposed or concerned: Get medical advice/attention
In case of fire: Use CO2, dry chemical, or foam to extinguish.
IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. If eye irritation persists: Get medical advice/attention. If skin irritation occurs: Get medical advice/attention
IF INHALED: Remove person to fresh air and keep comfortable for breathing. Call a POISON CENTER or doctor/physician if you feel unwell.

Precautionary Statements - Storage
Store locked up
Store in a well-ventilated place. Keep container tightly closed

Precautionary Statements - Disposal
Dispose of contents/container to an approved waste disposal plant

3. COMPOSITION/INFORMATION ON INGREDIENTS

Components	CAS-No.	Weight %
Ethyl Alcohol 200 proof	64-17-5	95
Water	7732-18-5	5

4. FIRST AID MEASURES

First aid measures

General Advice: National Capital Poison Center in the United States can provide assistance if you have a poison emergency and need to talk to a poison specialist. Call 1-800-222-1222.

Skin Contact: Wash off immediately with soap and plenty of water removing all contaminated clothing and shoes. Get medical attention. If skin irritation persists, call a physician.

Eye Contact: Flush eyes with water for 15 minutes. Get medical attention.

Inhalation: Move to fresh air. If breathing is difficult, give oxygen. If not breathing, give artificial respiration. Get medical attention.

Ingestion: Do not induce vomiting without medical advice. Never give anything by mouth to an unconscious person. Consult a physician if necessary.

Most important symptoms and effects, both acute and delayed

Symptoms

Causes eye irritation
May cause skin irritation
May cause irritation of respiratory tract
Dyspnea (Difficulty breathing and shortness of breath)
Central nervous system effects
Dizziness
Drowsiness
Headache
Ataxia
Staggering gait
Nausea
Vomiting
May cause cardiovascular effects

Indication of any immediate medical attention and special treatment needed

Notes to Physician: Treat symptomatically.

Protection of first-aiders

First-Aid Providers: Avoid exposure to blood or body fluids. Wear gloves and other necessary protective clothing. Dispose of contaminated clothing and equipment as bio-hazardous waste.

5. FIRE-FIGHTING MEASURES

Extinguishing Media

Suitable Extinguishing Media: Carbon dioxide (CO2). Dry chemical. Alcohol-resistant foam. Water spray.

Unsuitable Extinguishing Media: Do not use a solid (straight) water stream as it may scatter and spread fire.

Specific hazards arising from the chemical

Hazardous Combustion Products: Carbon Monoxide, Carbon Dioxide.

Specific hazards: Flammable. May be ignited by heat, sparks or flames. Material can burn with invisible flame. Vapor may travel considerable distance to source of ignition and flash back. Vapors may form explosive mixtures with air. Most vapors are heavier than air. They will spread along the ground and collect in low or confined areas (sewers, basements, tanks). Container explosion may occur under fire conditions or when heated. Fire may produce irritating, corrosive and/or toxic gases.

Special Protective Actions for Firefighters

Specific Methods: Water mist may be used to cool closed containers. For larger fires, use water spray or fog. Cool containers with flooding quantities of water until well after fire is out.

Special Protective Equipment for Firefighters: As in any fire, wear self-contained breathing apparatus pressure-demand, MSHA/NIOSH (approved or equivalent) and full protective gear

6. ACCIDENTAL RELEASE MEASURES

Personal precautions, protective equipment and emergency procedures

Personal Precautions: Ensure adequate ventilation. Keep people away from and upwind of spill/leak. Avoid contact with skin, eyes and clothing. Use personal protective equipment. Remove all sources of ignition. Pay attention to flashback. Take precautionary measures against static discharges. All equipment used when handling the product must be grounded. Use spark-proof tools and explosion-proof equipment. In case of large spill, water spray or vapor suppressing foam may be used to reduce vapors, but may not prevent ignition in closed spaces.

Environmental precautions Prevent further leakage or spillage if safe to do so. Prevent product from entering drains. Prevent entry into waterways, sewers, basements or confined areas.

Methods and material for containment and cleaning up

Methods for containment Stop leak if you can do it without risk. Absorb spill with inert material (e.g. vermiculite, dry sand or earth), then place in a suitable chemical waste container. In case of large spill, dike if needed. Dike far ahead of liquid spill for later disposal.

Methods for cleaning up Use appropriate tools to put the spilled material in a suitable chemical waste disposal container. Use only non-sparking tools. Clean contaminated surface thoroughly.

7. HANDLING AND STORAGE

Precautions for safe handling

Technical Measures/Precautions:

Provide sufficient air exchange and/or exhaust in work rooms. Remove all sources of ignition. To avoid ignition of vapors by static electricity discharge, all metal parts of the equipment must be grounded. Keep away from incompatible materials.

Safe Handling Advice

Wear personal protective equipment. Use only in well-ventilated areas. Avoid contact with skin, eyes and clothing. Keep away from heat and sources of ignition. Do not breathe vapors or spray mist. Do not ingest. When using do not smoke. Handle in accordance with good industrial hygiene and safety practice.

Conditions for safe storage, including any incompatibilities

Technical Measures/Storage Conditions:

Hygroscopic. Keep container tightly closed in a dry and well-ventilated place. Store at room temperature in the original container. Sensitive to light. Store in light-resistant containers. Keep away from heat and sources of ignition. Store in a segregated and approved area. Store away from incompatible materials.

Incompatible Materials:

Oxidizing agents
Acids
Alkali Metals
Halogens
Caustics
isocyanates
Metals
Bases
Acid anhydrides
Acid chlorides

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

Control parameters

National occupational exposure limits

United States

Components	CAS-No.	OSHA	NIOSH	ACGIH	AIHA WEEL
Ethyl Alcohol 200 proof	64-17-5	1000 ppm TWA 1900 mg/m^3 TWA	1000 ppm TWA 1900 mg/m^3 TWA	1000 ppm STEL	None
Water	7732-18-5	None	None	None	None

Canada

Components	CAS-No.	Canada - Alberta	Canada - British Columbia	Canada - Ontario	Canada - Quebec
Ethyl Alcohol 200 proof	64-17-5	1000 ppm TWA 1880 mg/m^3 TWA	1000 ppm STEL	1000 ppm STEL	None
Water	7732-18-5	None	None	None	None

Australia and Mexico

Components	CAS-No.	Australia	Mexico
Ethyl Alcohol 200 proof	64-17-5	1000 ppm TWA 1880 mg/m^3 TWA	1000 ppm TWA 1900 mg/m^3 TWA
Water	7732-18-5	None	None

Appropriate engineering controls

Engineering measures to reduce exposure: Ensure adequate ventilation. Provide exhaust ventilation or other engineering controls to keep the airborne concentrations of vapors and mist below their respective threshold limit value.

Individual protection measures, such as personal protective equipment

Personal Protective Equipment

Eye protection: Goggles or Safety glasses with side-shields
Skin and body protection: Chemical resistant apron
Long sleeved clothing
Gloves

Respiratory protection: Vapor respirator. Be sure to use an approved/certified respirator or equivalent.

Hygiene measures: Avoid contact with skin, eyes and clothing. When using, do not eat, drink or smoke. Wash hands before breaks and immediately after handling the product.

9. PHYSICAL AND CHEMICAL PROPERTIES

Physical state:
Liquid

Appearance:
No information available.

Color:
Clear. Colorless.

Odor:
Pleasant. Alcoholic. Mild. Ethereal. Like wine or whiskey.

Taste
Pungent. Burning.

Formula:
C2-H5-OH

Molecular/Formula weight (g/mole):
46.07

Flammability:
No information available

Flash point (°C):
17

Flashpoint (°C/°F):
17 °C/63 °F

Flash Point Tested according to:
Closed cup

Autoignition Temperature (°C/°F):
363 °C/685.4 °F

Lower Explosion Limit (%):
3.3%

Upper Explosion Limit (%):
19%

Melting point/range(°C/°F): -114.1-117.3 °C/-173.38-179.14 °F

Decomposition temperature(°C/°F):
No information available

Boiling point/range(°C/°F):
79 °C/174.2 °F

Bulk density:
No information available

Density (g/cm3):
No information available

Specific gravity:
0.8 @ 20 °C

pH:
No information available

Vapor pressure @ 20°C (kPa):
5.93

Evaporation rate: No information available

Vapor density:
1.59

VOC content (g/L):
789

Odor threshold (ppm):
5-10 (recognition)
84 (tolerance)

Partition coefficient (n-octanol/water): No information available

Viscosity:
No information available

Miscibility:
Miscible with water
Miscible with Acetone
Miscible with Ether
Miscible with Benzene
Miscible with glacial Acetic Acid
Miscible with many organic solvents

Solubility:
Very soluble in water

10. STABILITY AND REACTIVITY

Reactivity
When Ethanol comes in contact with Sodium, it liberates flammable hydrogen gas

It can react vigorously or explosively with acid hydrides or acid chlorides
It reacts with alkali metals to liberate flammable hydrogen gas
It reacts with ammonia + silver nitrate to form silver nitride and silver fulminate
It reacts with acetyl bromide to evolve hydrogen bromide
Ethyl alcohol can react with freshly cut/etched/scratched aluminum with the evolution of heat and release of hydrogen gas. The Ethyl alcohol has to be on the aluminum surface as it is being cut/scratched/etched Ethyl Alcohol reacts vigorously with acetyl chloride.
Ethyl alcohol reacts with silver (I) oxide + ammonia or hydrazine to form silver nitride and silver fulminate
Ethanol ignites and then explodes on contact with the following compounds: acetic anhydride + sodium hydrosulfate, disulfuric acid + nitric acid, phosphorus (III) oxide, platinum, potassium tert-butoxide + acids
Ethanol rapidly absorbs moisture from the air. Can react vigorously/explosively with oxidizers. Ethanol can react vigorously/explosively with the following: ammonium hydroxide & silver oxide, chlorine or chlorine oxides, perchlorates (barium perchlorate, chloryl perchlorate, magnesium perchlorate (forms ethyl perchlorate), nitrosyl perchlorate , potassium perchlorate, silver perchlorate, uranyl perchlorate), acetic anhydride, acetyl bromide (evolves hydrogen bromide), acetyl chloride, aluminum sesquibromide ethylate, bromine pentafluoride, calcium hypochlorite, chromic anhydride, , chromium trioxide, chromyl chloride, cyanuric acid + water, dichloromethane + sulfuric acid + nitrate (or) nitrite, manganese perchlorate + 2,2-dimethoxy propane, dioxygen difluoride, disulfuryl difluoride, fluorine nitrate, hydrogen peroxide, iodine heptafluoride, manganese heptoxide, iodine + methanol + mercuric oxide, iodine + Phosphorus (forms ethane iodide), mercuric nitrate, nitric acid, perchloric acid, permanganic acid, peroxodisulfuric acid, platinum black, potassium dioxide, potassium permanganate, potassium superoxide, potassium tert-butoxide, ruthenium(VIII) oxide, silver +nitric acid (forms silver fulminate), silver nitrate (forms ethyl nitrate), silver peroxide, sodium hydrazide, hydrogen peroxide + sulfuric acid, sulfuric acid + permanganates, uranium hexafluoride, sulfuric acid + sodium dichromate, tetrachlorisilane + water, silver & nitric acid, tetraphosphorus hexaoxide

Chemical stability

Stability: Stable under recommended storage conditions.

Possibility of Hazardous Reactions: Hazardous polymerization does not occur

Conditions to avoid: Heat. Ignition sources. Incompatible materials.

Incompatible Materials:

Oxidizing agents
Acids
Alkali Metals
Halogens
Caustics
isocyanates
Metals
Bases
Acid anhydrides
Acid chlorides

Hazardous decomposition products: Carbon monoxide. Carbon dioxide. When heated to decomposition it emits acrid smoke and irritating fumes.

Other Information

Corrosivity: No information available

Special Remarks on Corrosivity: No information available

11. TOXICOLOGICAL INFORMATION

Information on likely routes of exposure

Principal Routes of Exposure:
Ingestion. Skin. Eyes. Inhalation.

Acute Toxicity

The following values are calculated based on chapter 3.1 of the GHS document

ATEmix (oral) 7432 mg/kg

ATEmix (inhalation-vapor) 131.26 mg/l

Component Information

Ethyl Alcohol 200 proof	
CAS-No.	64-17-5

LD50/oral/rat = 7060 mg/kg Oral LD50 Rat
LD50/oral/mouse = 3450 mg/kg Oral LD50 Mouse
LD50/dermal/rabbit = No information available
LD50/dermal/rat = No information available
LC50/inhalation/rat = 124.7 mg/L Inhalation LC50 Rat 4 h
LC50/inhalation/mouse = 39000 mg/m^3 4 h
Other LD50 or LC50information = >60000 ppm Inhalation LC50 Mouse 1 h
5900 mg/m^3 Inhalation LC50 Rat 6 h
20000 ppm Inhalation LC50 Rat 10 h
5560 mg/kg Oral LD50 Guinea Pig
6300 mg/kg Oral LD50 Rabbit

Water	
CAS-No.	7732-18-5

LD50/oral/rat = > 90 mL/kg Oral LD50 Rat
LD50/oral/mouse = No information available
LD50/dermal/rabbit = No information available
LD50/dermal/rat = No information available
LC50/inhalation/rat = No information available
LC50/inhalation/mouse = No information available
Other LD50 or LC50information = No information available

Product Information

LD50/oral/rat =
VALUE- Acute Tox Oral = No information available

LD50/oral/mouse =
Value - Acute Tox Oral = No information available

LD50/dermal/rabbit
VALUE-Acute Tox Dermal = No information available

LD50/dermal/rat
VALUE -Acute Tox Dermal = No information available

LC50/inhalation/rat
VALUE-Vapor = No information available
VALUE-Gas = No information available
VALUE-Dust/Mist = No information available

LC50/Inhalation/mouse
VALUE-Vapor = No information available
VALUE - Gas = No information available
VALUE - Dust/Mist = No information available

Symptoms

Skin Contact: Mildly to moderately irritating to the skin.

Eye Contact: Causes serious eye irritation.

Inhalation May cause irritation of respiratory tract. Symptoms may include coughing and shortness of breath. May cause nausea and headache. It may affect behavior/central nervous system (ataxia, general anesthetic, drowsiness). May affect respiration (respiratory depression). Inhalation of high concentrations of vapor may cause anesthetic effects. Inhalation of high concentrations of vapors may cause dizziness or suffocation. May affect the brain.

Ingestion Ingestion may cause gastrointestinal irritation, nausea, vomiting and diarrhea. May cause gastritis. May cause loss of appetite. May cause flushed skin. May affect the cardiovascular system (change in heart rate). May affect the cardiovascular system (hypotension or hypertension, tachycardia, dysrhythmias). It may affect behavior/central nervous system (excitation, mild euphoria, excessive talking, fatigue, headache, dizziness, drowsiness, staggaring gait, ataxia, hallucinations, slurred speech, amnesia, confusion, release of inhibitions, agressive behavior, convulsions, coma). May affect respiration (dyspnea, respiratory depression). It may affect the brain. May affect liver. May affect the blood. May affect the endocrine system. It may affect the spleen. May affect urinary system (kidneys).

Aspiration hazard No information available.

Delayed and immediate effects as well as chronic effects from short and long-term exposure

Chronic Toxicity Prolonged or repeated skin contact may cause dermatitis, and dryness and cracking of the skin. Prolonged or repeated ingestion may affect behavior/central nervous system. Prolonged or repeated ingestion may affect metabolism (cause anorexia, weight loss). Prolonged or repeated ingestion may affect the liver (fatty liver degeneration, cirrhosis of the liver. Prolonged or repeated ingestion may affect the cardiovascular system.

Sensitization: No information available.

Mutagenic Effects:

For Ethyl alcohol:
May affect genetic material
Experiments with bacteria and/or yeast have shown mutagenic effects
Cytogenic analysis - hamster ovary
Cytogenic Analysis (Hamster embryo)
Cytogenic analysis - human leukocyte
Cytogenic Analysis: human lymphocyte
Sister Chromatid Exchange - Hamster ovary
Sister Chromatid Exchange (human lymphocyte)

Carcinogenic effects: May cause cancer based on animal test data. Equivocal tumorigenic agent by RTECS criteria.

Components	CAS-No.	IARC	ACGIH - Carcinogens	NTP	OSHA HCS - Carcinogens	Australia - Notifiable Carcinogenic Substances	Australia - Prohibited Carcinogenic Substances

Ethyl Alcohol 200 proof	64-17-5	Group 1 Monograph 100E [2012] in alcoholic beverages Monograph 96 [2010] in alcoholic beverages	A3 Confirmed Animal Carcinogen with Unknown Relevance to Humans	Not listed	Present	Not listed	Not listed
Water	7732-18-5	Not listed	Not listed	Not listed	Not listed	Not listed	Not listed

ACGIH (American Conference of Governmental Industrial Hygienists)

IARC (International Agency for Research on Cancer)

NTP (National Toxicology Program)

OSHA (Occupational Safety and Health Administration of the US Department of Labor)

Reproductive toxicity May damage fertility or the unborn child
Reproductive Effects: Causes adverse reproductive effects
Developmental Effects: May cause adverse developmental effects May cause harm to the unborn child
Teratogenic Effects: Causes birth defects (teratogenic effects)
Specific Target Organ Toxicity

STOT - single exposure Respiratory system. central nervous system.
STOT - repeated exposure Causes damage to organs through prolonged or repeated exposure. **Target Organs:** Skin. Liver. Central nervous system. Nervous system. Heart.

12. ECOLOGICAL INFORMATION

Ecotoxicity

Ecotoxicity effects: Aquatic environment.
Ethyl Alcohol 200 proof - 64-17-5
Freshwater Fish Species Data: 12.0 - 16.0 mL/L LC50 Oncorhynchus mykiss 96 h static 1 100 mg/L LC50 Pimephales promelas 96 h static 1 13400 - 15100 mg/L LC50 Pimephales promelas 96 h flow-through 1
Water Flea Data: 9268 - 14221 mg/L LC50 Daphnia magna 48 h 2 mg/L EC50 Daphnia magna 48 h
10800 mg/L EC50 Daphnia magna 24 h
Persistence and degradability: No information available
Bioaccumulative potential: No information available.
Mobility: No information available.

13. DISPOSAL CONSIDERATIONS

Disposal Methods

Waste from residues / unused products:
Waste must be disposed of in accordance with Federal, State and Local regulation.

Contaminated packaging:

Empty containers should be taken for local recycling, recovery or waste disposal

Components	CAS-No.	RCRA - F Series Wastes	RCRA - K Series Wastes	RCRA - P Series Wastes	RCRA - U Series Wastes
Ethyl Alcohol 200 proof	64-17-5	None	None	None	None
Water	7732-18-5	None	None	None	None

14. TRANSPORT INFORMATION

DOT

UN-No: UN1170
Proper Shipping Name: Ethanol solution
Hazard Class: 3
Subsidiary Class No information available
Packing group: II
Emergency Response Guide Number 127

Marine Pollutant No data available
DOT RQ (lbs): No information available
Special Provisions 24, IB2, T4, TP1
Symbol(s): No information available
Description: UN1170, ETHANOL SOLUTION, 3, II

TDG (Canada)

UN-No: UN1170
Proper Shipping Name: Ethanol solution
Hazard Class: 3
Subsidiary Risk: No information available
Packing Group: II
Marine Pollutant No Information available
Description: UN1170, ETHANOL SOLUTION, 3, II

ADR

UN-No: UN1170
Proper Shipping Name: Ethanol solution
Hazard Class: 3
Packing Group: II
Subsidiary Risk: No information available
Special Provisions 144, 601
Description: UN1170, ETHANOL SOLUTION, 3, II

IMO / IMDG

UN-No: UN1170
Proper Shipping Name: Ethanol solution
Hazard Class: 3
Subsidiary Risk: No information available
Packing Group: II
Marine Pollutant No information available
EMS: F-E
Special Provisions 144
Description UN1170, ETHANOL SOLUTION, 3, II

RID

UN-No: UN1170
Proper Shipping Name: Ethanol solution
Hazard Class: 3
Subsidiary Risk: 3
Packing Group: II
Special Provisions 144, 601
Description: UN1170, ETHANOL SOLUTION, 3, II

ICAO

UN-No: UN1170
Proper Shipping Name: Ethanol solution
Hazard Class: 3
Subsidiary Risk: No information available
Packing Group: II
Description: UN1170, ETHANOL SOLUTION, 3, II
Special Provisions A58, A180, A3

IATA

UN-No: UN1170
Proper Shipping Name: Ethanol solution
Hazard Class: 3
Subsidiary Risk: No information available
Packing Group: II
ERG Code: 3L
Special Provisions No information available
Description: UN1170, ETHANOL SOLUTION, 3, II

15. REGULATORY INFORMATION

International Inventories

Components	CAS-No.	U.S. TSCA	KOREA KECL	Philippines (PICCS)	Japan ENCS	CHINA	Australia (AICS)	EINECS-No.
Ethyl Alcohol 200 proof	64-17-5	Present(ACTIVE)	KE-13217	Present	(2)-202	Present	Present	Present 200-578-6
Water	7732-18-5	PresentACTIVE	Present KE-35400	Present	Not present	Present	Present	Present 231-791-2

U.S. Regulations

Ethyl Alcohol 200 proof

Massachusetts RTK: Present
New Jersey RTK Hazardous Substance List: 0844
Pennsylvania RTK: Present
Minnesota - Hazardous Substance List: Present
Louisana Reportable Quantity List for Pollutants: Present (listed as Volatile Organic Compounds)
California Directors List of Hazardous Substances: Present
FDA - Food Additives Generally Recognized as Safe (GRAS): 21 CFR 184.1293

FDA - 21 CFR - Total Food Additives 169.175, 169.176, 169.177, 169.181, 172.340, 172.560, 172.580, 175.105, 176.180, 176.200, 177.1200, 177.1650, 178.1010, 184.1293, 73.30, 73.345, 73.615

California Prop. 65: Safe Drinking Water and Toxic Enforcement Act of 1986.

Chemicals Known to the State of California to Cause Cancer:

⚠WARNING: This product can expose you to chemicals including (see table below) which is (are) known to the State of California to cause cancer. For more information go to www.p65warnings.ca.gov.

Chemicals Known to the State of California to Cause Reproductive Toxicity:

⚠WARNING: This product can expose you to chemicals including (see table below) which is (are) known to the State of California to cause birth defects or other reproductive harm. For more information go to www.p65warnings.ca.gov.

Components	CAS-No.	Carcinogen	Developmental Toxicity	Male Reproductive Toxicity	Female Reproductive Toxicity:
Ethyl Alcohol 200 proof	64-17-5	carcinogen (Ethanol in alcoholic beverages)	developmental toxicity (Ethyl alcohol in alcoholic beverages)	Not Listed	Not Listed
Water	7732-18-5	Not Listed	Not Listed	Not Listed	Not Listed

CERCLA/SARA

Components	CAS-No.	CERCLA - Hazardous Substances and their Reportable Quantities	Section 302 Extremely Hazardous Substances and TPQs	Section 302 Extremely Hazardous Substances and RQs	Section 313 - Chemical Category	Section 313 - Reporting de minimis
Ethyl Alcohol 200 proof	64-17-5	None	None	None	None	None
Water	7732-18-5	None	None	None	None	None

U.S. TSCA

Components	CAS-No.	TSCA Section 5(a)2 - Chemicals With Significant New Use Rules (SNURS)	TSCA 8(d) -Health and Safety Reporting
Ethyl Alcohol 200 proof	64-17-5	Not Applicable	Not Applicable
Water	7732-18-5	Not Applicable	Not Applicable

Canada

WHIMIS 2015 - GHS Classifications

WHMIS 2015 Hazard Classification Information: .

Component	WHMIS 2015 Hazard Classification
Ethyl Alcohol 200 proof 64-17-5 (95)	Flammable liquids - Category 2: H225 Highly flammable liquid and vapour.; Serious Eye Damage/Eye Irritation - Category 2B: H320 Causes eye irritation.
Water 7732-18-5 (5)	Not a dangerous product according to HPR classification criteria

Canada Hazardous Products Regulation This product has been classified according to the hazard criteria of the HPR (Hazardous Products Regulation) and the SDS contains all of the information required by the HPR

WHMIS 1988 Hazard Class
B2 Flammable liquid
D2B Toxic materials

Components	WHMIS 1988
Ethyl Alcohol 200 proof	B2,D2B
Water	Uncontrolled product according to WHMIS classification criteria

Canada Controlled Products Regulation:
This product has been classified according to the hazard criteria of the CPR (Controlled Products Regulation) and the MSDS contains all of the information required by the CPR.

Components	WHMIS Ingredient Disclosure List -
Ethyl Alcohol 200 proof	0.1 %

Inventory

Components	CAS-No.	Canada (DSL)	Canada (NDSL)
Ethyl Alcohol 200 proof	64-17-5	Present	Not Listed
Water	7732-18-5	Present	Not Listed

Components	CAS-No.	CEPA Schedule I - Toxic Substances

Ethyl Alcohol 200 proof	64-17-5	Not listed
Water	7732-18-5	Not listed
Components	CAS-No.	CEPA - 2010 Greenhouse Gases Subject to Mandatory Reporting
Ethyl Alcohol 200 proof	64-17-5	Not listed
Water	7732-18-5	Not listed

EU Classification

EU GHS - SV - CLP 1272/2008

Components	CAS-No.	EU GHS - SV - CLP (1272/2008)
Ethyl Alcohol 200 proof	64-17-5	Flammable liquids - Flam. Liq. 2: H225 Highly flammable liquid and vapour.603-002-00-5
Water	7732-18-5	

EU - CLP (1272/2008)

R-phrase(s)

R11 - Highly flammable.

S -phrase(s)

S 7 - Keep container tightly closed.

S16 - Keep away from sources of ignition - No smoking.

Components	CAS-No.	**Classification**	**Concentration Limits:**	**Safety Phrases**
Ethyl Alcohol 200 proof	64-17-5	F; R11	No information	S(2) S7 S16
Water	7732-18-5		No information	

The product is classified in accordance with Annex VI to Directive 67/548/EEC

Indication of danger:

F - Highly flammable.

F

16. OTHER INFORMATION

Preparation Date: 9/12/2013
Revision Date: 8/20/2018
Prepared by: Sonia Owen

Disclaimer:

All chemicals may pose unknown hazards and should be used with caution. This Safety Data Sheet (SDS) applies only to the material as packaged. If this product is combined with other materials, deteriorates, or becomes contaminated, it may pose hazards not mentioned in this SDS. The physical properties reported in this SDS are obtained from the literature and do not constitute product specifications. Information contained herein does not constitute a warranty, whether expressed or implied, as to the safety, merchantability or fitness of the goods for a particular purpose. Spectrum Chemicals & Laboratory Products, Inc. assumes no responsibility for results obtained or for incidental or consequential damages, including lost profits, arising from the use of these data. No warranty against infringement of any patent, copyright or trademark is made or implied. It shall be the user's responsibility to develop proper methods of handling and personal protection based on the actual conditions of use. While this SDS is based on technical data judged to be reliable, Spectrum assumes no responsibility for the completeness or accuracy of the information contained herein.

End of Safety Data Sheet

SIGMA-ALDRICH® sigma-aldrich.com

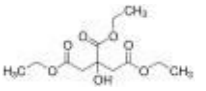
3050 Spruce Street, Saint Louis, MO 63103, USA
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

Product Specification

Product Name:

Triethyl citrate – natural, ≥99%, FG

Product Number: W308302 CAS Number: 77-93-0



Formula: C12H20O7
Formula Weight: 276.29 g/mol

TEST	Specification
Appearance (Color)	Colorless
Appearance (Form)	Liquid
Refractive index at 20 ° C	1.440 - 1.444
Infrared Spectrum	Conforms to Structure
Purity (GC)	>_ 99.0 %
Arsenic (As)	<_ 3 ppm
Cadmium (Cd)	<_ 1 ppm
Mercury (Hg)	<_ 1 ppm
Lead (Pb)	<_ 10 ppm
Expiration Date Period	----------
5 Years	

Specification: PRD.1.ZQ5.100000

SIGMA-ALDRICH®

Sigma-Aldrich
Flavors & Fragrances
6000 N. Teutonia Avenue
Milwaukee, WI 53209 USA
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Fax 414-438-4216
Visit us at www.sigma-aldrich.com

Print this page

September 12, 2018

GMO Statement

To the best of our knowledge, the following Sigma Aldrich Flavors and Fragrances material:

W308302 **Triethyl citrate-natural, ≥99%, FG**

It has not been derived from or produced using genetically modified organisms or their derivatives. This material does not contain DNA and/or protein from genetic modification.

Based on this information, the product stated above, will not on their own, require labeling of the foodstuffs and food ingredients as indicated in EC 1829/2003 and EC 1830/2003

Best Regards,

Sigma-Aldrich Flavors & Fragrances

SIGMA-ALDRICH®

Sigma-Aldrich
Flavors & Fragrances
6000 N. Teutonia Avenue
Milwaukee, WI 53209 USA
Tel. 800-227-4563 / 414-438-2608
Fax 414-438-4216
Visit us at www.sigma-aldrich.com

Print this page

September 12, 2018

FOOD GRADE STATEMENT

W308302 Triethyl citrate-natural, ≥99%, FG

To the best of our knowledge, this product is manufactured under current Good Manufacturing Practices (cGMP) for food facilities and conforms to Hazard Analysis and Critical Control Points (HACCP) principles. The product is intended for use in manufacturing food and/or designated Generally Recognized As Safe (GRAS) by:

FEMA # 3083
Flavis # 09.512
EU Regulation 1223/2009
EU Regulation 1334/2008 & 178/2002
FDA 21 CFR (117)

To the best of our knowledge, this product was not adulterated or misbranded as defined by the Federal Food, Drug, and Cosmetic Act, state, or municipal ordinances in which the definition of adulteration and misbranding is substantially the same as defined by the Act. This product is allowed under the provision of Section 404 or 505 of the Act, to be introduced into interstate commerce.

Sigma-Aldrich warrants that its products conform to the information contained in this and other Sigma-Aldrich publications. Purchaser must determine the suitability of the product for their particular use.

Best Regards,

Sigma-Aldrich Flavors & Fragrances

SIGMA-ALDRICH®

Intract Pharma

Prof. Arjan Narbad,
Quadram Institute Bioscience,
Norwich Research Park,
NR4 7UA

Phloral® use in Human Clinical Trials

Dear Professor Narbad,

Phloral® is the world's most advanced oral technology for delivering drugs to the human colon. Phloral coated tablets and capsules have been evaluated for both efficacy and safety in multiple human clinical trials. These studies include administering the formulation to healthy volunteers[1], as well as disease states such as ulcerative colitis[2] and *Clostrium difficile* infection (CDI)[3].

D'Haens et al (2017) evaluated a Phloral®-coated mesalazine formulation in 409 patients with ulcerative colitis as part of a global Phase III randomised non-inferiority study. As a result of successfully completing this pivotal trial, the formulation is expected to be available to patients in 2019, marketed by Tilllotts Pharma.

Allegretti et al. (2018) conducted an open label dose finding study with Phloral® formulations containing faecal microbiota transplants (FMT). Twenty-six patients with recurrent CDI were administered a Phloral®-coated FMT formulation. The Phloral® formulations even at a low dose, significantly improved the clinical cure rate and microbial diversity when compared to gastric-release formations.

In all human clinical trials using Phloral® to date, the formulation has been deemed to be both safe and efficacious.

Bill Lindsay
CEO

[1] Ibekwe VC et al. Aliment Pharmacol Ther. 2008;(28):911-916
[2] D'Haens GR et al. Aliment Pharmacol Ther. 2017;46(3):292–302
[3] Allegretti J et al. Microbial-Based Therapy. 2018

Annex 14, Catalogue B Uncoated Capsules

B1. QualiCaps

B1.1 QualiCaps Certificate of Analysis

B1.2 QualiCaps Technical Brochure

B1.3 QualiCaps Technical Information

Qualicaps Engineered to perform

Qualicaps Europe, S.A.U.
Avda. Monte Valdelatas, 4
28108 Alcobendas, Madrid, Spain
T +34 91 663 08 00
F +34 91 663 08 30

Quali-V®

QUALI-V®
HPMC CAPSULE

CERTIFICATE OF ANALYSIS

This is to certify that the information below has been approved by QA as conforming to Qualicaps Europe specifications, as described in the current technical manual and applicable regulatory requirements.

PRODUCT DESCRIPTION: EMPTY HYPROMELLOSE CAPSULES
CAP/BODY COLOR DESCRIPTION: ZAB/ZAB
MANUFACTURING DATE : 07/11/2017
LOT NUMBER : E1704902
EXPIRY DATE: 5 YEARS
SIZE : 00
LOT QUANTITY APPROVED : 867000

HPMC: Complies with the requirements of the European and the United States Pharmacopeiae.

COLORANT: Comply with the EC directives 2009/35 & 231/2012 and where applicable with the requirements of the EP and USP/NF.

FINISHED PRODUCT TESTS

CAPSULES: Are manufactured without preservatives and no Ethylene Oxyde treatment.

PARAMETERS	LIMITS	RESULT
Total Aerobic Microbial Count (TAMC)	10^3 CFU/G	10 (Average)
E. Coli	Negative / 1 G	Meets test
Total Yeast Mould Count (TYMC)	10^2 CFU/G	10

VISUAL QUALITY

All the capsules are controlled statistically to ensure conformance to specifications. These values are derived from the MIL.STD.105E.
The quality specifications are as follow :

VISUAL DEFECTS	Major A	Major B	Minor
AQL (%)	0,015	0,065	1,0

DIMENSIONAL TEST

All the capsules are controlled statistically to ensure conformance to the following specifications:

PARAMETERS		LIMITS	REFERENCE	RESULT			
DIAMETER	CAP (mm)	Meets spec	TS*	8,55	-	8,57	(min-max)
	BODY (mm)	Meets spec	TS*	8,21	-	8,24	(min-max)
LENGTH	CAP (mm)	Meets spec	TS*	11,73	-	11,86	(min-max)
	BODY (mm)	Meets spec	TS*	20,07	-	20,32	(min-max)
END THICKNESS	CAP (µ)	Meets spec	QS*	154	-	230	(min-max)
	BODY (µ)	Meets spec	QS*	106	-	164	(min-max)
WEIGHT (g/100 caps		Meets spec	TS*	11,60		(Average)	
MOISTURE (%)		Meets spec	TS*	5,4		(Average)	

*TS = Qualicaps Technical Sheet
*QS = Qualicaps internal specification
"Meets test" means conforms to specification

FEDERICO GARCIA
QUALITY ASSURANCE MANAGEMENT

 DATE:28/11/2017

Quali-V®

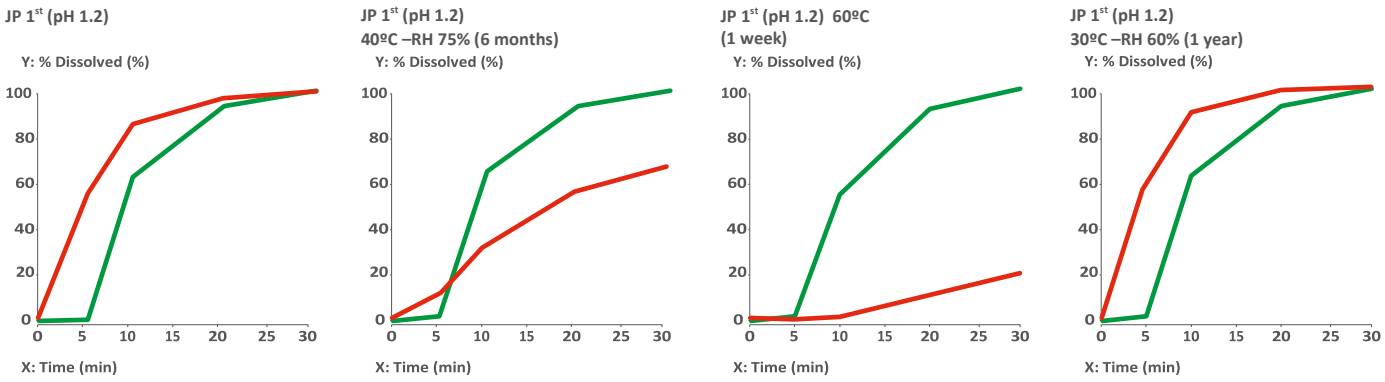
HE TREND IN MARKET PREFERENCE FOR PHARMACEUTICAL APPLICATIONS

QUALI-V®
THE PREFERRED CHOICE FOR PHARMACEUTICAL SOLID ORAL DOSAGE FORMS

QUALI-V® HPMC CAPSULES, THE BEST ALTERNATIVE TO GELATIN

- Quali-V® HPMC capsules are **equivalent in their dissolution profile** to traditional hard gelatin capsules, with a similar disintegration time[1] and release properties[2], proving **identical in-vivo performance behavior**.

- While gelatin capsules undergo cross-linking at **high temperature** or **high relative humidity**[3], such conditions **do not affect the Quali-V® HPMC dissolution profile,** as these capsules are chemically stable.



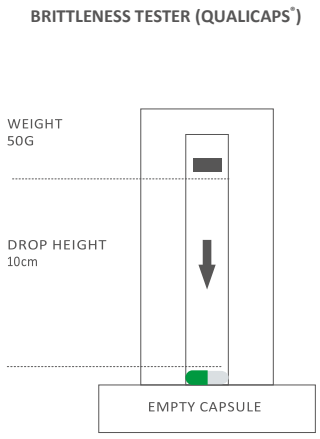
***Capsule fill formulation**: Acetaminophen 35 mg, Lactose 280 mg, Croscarmellose 35 mg*

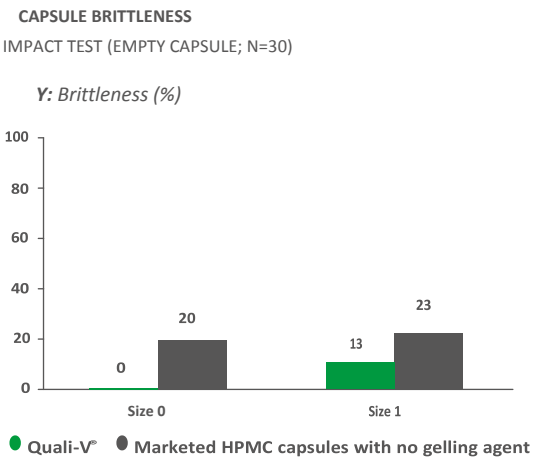
***Fill weight**: 350 mg (Size 1 capsule)*
***Dissolution test method**: Paddle at 50 rpm*

- In addition to having a lower moisture content (4.0%-6.0%), Quali-V® HPMC capsules demonstrate **better performance than gelatin capsules in terms of brittleness**. The moisture content of Quali-V® can be reduced minimizing significantly the occurrence of brittleness[4] that takes place when drying gelatin capsules below a certain threshold.
- Moisture has more influence on static electricity in gelatin capsules than in Quali-V® HPMC capsules[5].

QUALI-V® CAPSULES, SUPERIOR PROPERTIES FOR HYGROSCOPIC DRUGS

- Quali-V® capsules **maintain their physical stability** when filled with hygroscopic materials or are exposed to low relative humidity conditions. They also hold many types of formulations: powders, pellets, tablets, semi-solids and non-aqueous liquids.
- Quali-V® capsules are proven to be **more resistant to breaking and fracturing** at very low relative humidity (12% RH), as they are **more elastic**[6].





	Capsule	Tensile strength [N]	Elongation al break (%)
RH 45 - 50% (ambient humidity)	**Quali-V®** HPMC with no gelling agent	165 ± 16 153 ± 15	14.6 ± 2.4 9.7 ± 0.8
RH 12% (low humidity)	**Quali-V®** HPMC with no gelling agent	172 ± 21 119 ± 40	11.1 ± 2.0 8.5 ± 1.2

(size 1, N=10, Average ± SD)

- Superior resistance to breaking and superior de-blistering performance[4,6] make Quali-V® capsules the **ideal option to safeguard encapsulated products during long-term storage,** even in less than ideal conditions.

QUALI-V® CAPSULES, THE PERFECT CHOICE FOR RAPID DISSOLVING DRUG PRODUCTS

- Quali-V® capsules demonstrate a very **rapid and predictable dissolving drug product profile** where 85% of the API is released in the first 15 minutes at different pH levels.

- Quali-V® capsules show **high reproducibility of invitro performance** between manufactured lots.

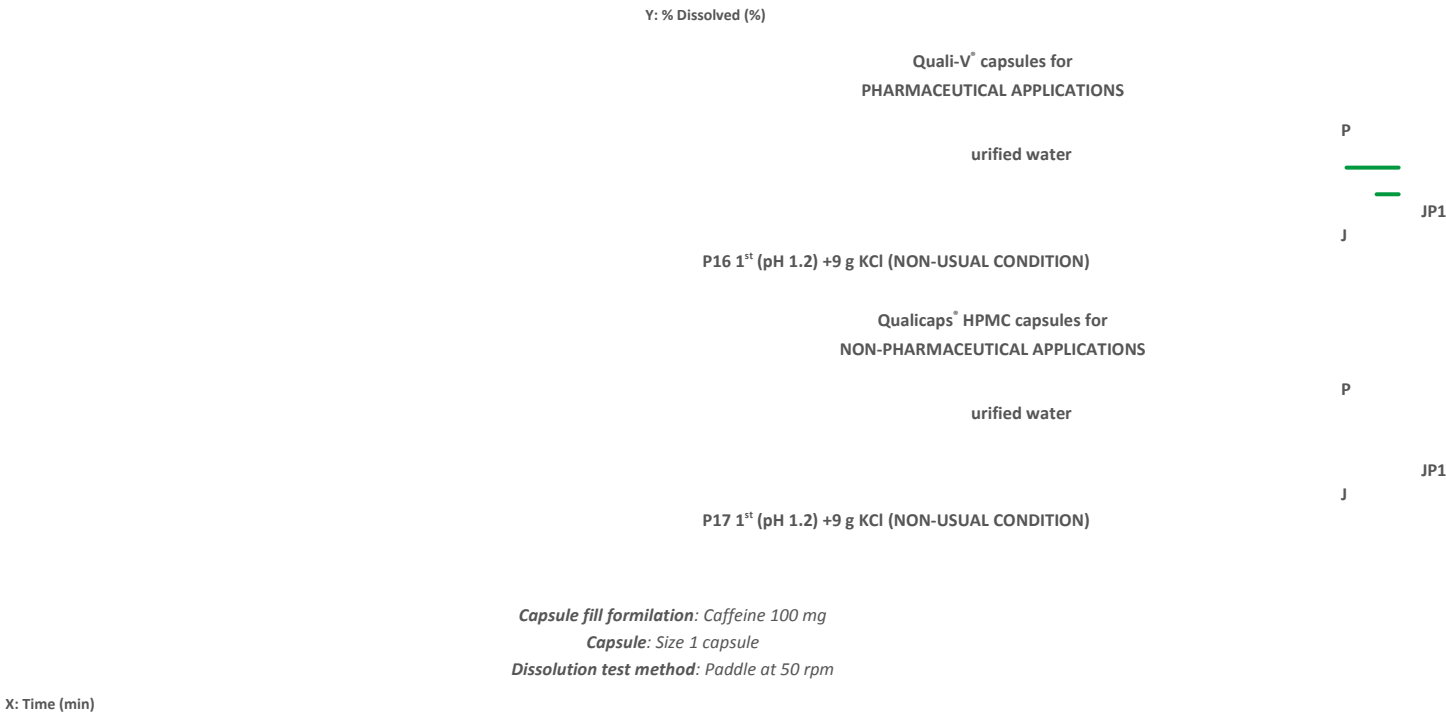
DISSOLUTION PROFILE FOR THREE QUALI-V® BATCHES (pH 1.2)

Studies of Universitat de València 2012-2013

E1101855 Mu-1 E1102536 C-9 E1102536 C-3

- **Quali-V® capsules have a faster release profile,** with a shorter timeframe until first rupture[7], 3-4 minutes less than other HPMC capsules on the market. Quali-V® also shows quicker dissolution rates with less variation[6].

- Quali-V® capsules are specifically designed for oral **pharmaceutical applications** where dissolution properties are prioritized over mechanical properties to ensure a **consistent dissolution profile in standard conditions**.



QUALI-V® CAPSULES, DESIGNED TO MEET THE DEMANDING REQUIREMENTS OF PHARMACEUTICAL INDUSTRY

- 100% plant-based and preservative-free, Quali-V® is acceptable for consumption within certain dietary and religious limitations. Quali-V® also responds to the clean label movement among today's consumers.

- Pharmaceutical-grade quality. The manufacturing process is carried out following strict pharmaceutical criteria and certified according to ISO 9001 and ISO 14001. Drug Master Files for the US and Canada have been registered.

- Quali-V® capsules do not undergo any changes in physical and chemical performance throughout their 5-year shelf-life; all parameters meet the specifications during stability studies.

Our scientific business development team can support R&D in capsule-forms

Our technical service engineers can assist in achieving productivity based dosage yields in capsule filling

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References

1 Tuleu, C., Khela, M., Evans, D., Jones, B., Nagata, S. and Basit. A., 2004. A comparative scintigraphic assessment of the disintegration of HPMC and gelatin capsules in fasting subjects. Poster, AAPS Meeting, Baltimore, 2004. **2** Honkanen, O., Eerikäinen, S., Tuominen, R. and Marvola, M., 2001. Bioavailability of ibuprofen from orally administered hydroxypropyl methylcellulose capsules compared to corresponding gelatine capsules. S. T. P. Pharma Sci., 11, 181-185. **3** Nagata, S, Tochio, S., Sakuma, S. and Suzuki, Y., 2001. Dissolution profiles of drugs filled into HPMC and gelatin capsules. Poster AAPS Meeting, Denver, 2001 **4** Nagata, S., 2002. Advantages to HPMC capsules: a new generation's capsules. Drug Del. Technol., 2(2), 34-39. **5** Satoshi Sakuma, Shinji Tochio, Shunji Nagata. Shionogi Qualicaps CO., LTD. Investigation of the Static Electrical Charging of HPMC and Gelatin Capsules. Poster AAPS Meeting, Salt Lake City, 2003 **6** Evaluation of the properties of HPMC capsules manufactured using different methods. Tomo Uyama, Asami Inui, Tohru Kokubo. Qualicaps Co. Ltd., 321-5, Ikezawa-cho, Yamato-Koriyama, Nara 639-1032, Japan. Poster AAPS Meeting, New Orleans, 2010. **7** Ku, M.S., Lu, Q., Li, W., Chen, Y., Performance qualification of a new hypromellose capsule: Part II. Disintegration and dissolution comparison between two types of hypromellose capsules. Internationa Journal of Pharmaceutics, 386 (2010) 30-41.

TECHNICAL DOSSIER

Nutra'V

Hard two-piece HPMC capsules produced with the consumer well-being in mind

Qualicaps Engineered to perform

1 Raw Material Specifications

1.1 Hypromellose

Nutra'V **capsules are made from hypromellose that complies with the principal Pharmacopoeiae: the United States Pharmacopoeia (USP/NF), the European Pharmacopoeia (EP) and the Japanese Pharmacopoeia (JP), as well as with the pu-rity criteria defined for E464 (HPMC) in Commission Regula-tion (EU) No 231/2012.**

1.2 Colourants

The colourants used are in compliance with the EU Directives and when required with the requirements of the EP, USP/NF.

1.3 Purified Water

The water used by Qualicaps® complies with the requirements of the EP, USP/NF and JP.

1.4 Additives

Nutra'V **capsules contain small amounts of carra-geenan as a gelling agent and potassium chloride as a gelling promoter. In addition, carnauba wax is applied as a surface lu-bricant on the capsules. These additives comply with the re-quirements of the following regulations: carrageenan - the EEC food regulations, USP/NF, and Japanese Pharmaceutical Excipients (JPE) regulations; potassium chloride - the EP, USP/NF and JP; carnauba wax and/or maize (corn) starch - the EP, USP/NF and JP.**

2 Dimensional Specifications

2.1 Weight

Capsule weight can vary by ± 10% from the target value. The values are determined by weighing a sample of 100 capsules at the standard moisture content of 3.0% to 7.0%. Customers should determine tare weights for filling by testing samples from in-house batches. These values are not applicable to individual capsules but rather to the average of the batch.

2.2 Length

Capsule lengths are controlled in the manufacturing process and audited for each batch.

2.3 Closed Joined Length

This value is given as a filling machine set-up recommendation and not as an approval/rejection criterion for empty capsules. The closed joined length has been calculated to ensure the correct location of the special positive locking features on the cap and body. If the filling machine is set so that the capsules are closed to a shorter length, then the cap or body may be damaged and the locking mechanism may fail; if longer, they may come apart. It is recommendable to provide this value to packaging equipment manufacturers prior to making a decision on blister pocket specifications.

2.4 Outside Diameter

The outside diameters, provided as a guideline for evaluating packaging material dimensions, are measured by passing the caps and bodies through calibrated bushes under specified conditions that simulate those of filling machines. This dimension should never be considered as an approval/rejection criterion.

Size		00	0E	0	1
Weight	Target weight (mg/100 capsules)	120	110	90 81-99	72 64.8-79.2
	Weight limits (mg/100 capsules)	108-132	99-121		
Capacity	Approximate body volume (ml)	0.93	0.76	0.67	0.48
Outside diameter	Cap diameter (mm)	8.57	7.69	7.68	6.92
	Body diameter (mm)	8.23	7.34	7.34	6.61
	Tolerance (mm)	± 0.06	± 0.06	± 0.06	± 0.06
Length	Cap length (mm)	11.84	11.99	10.72	9.78
	Body length (mm)	20.17 ± 0.5	20.98	18.44	16.61
	Tolerance (mm)		± 0.5	± 0.5	± 0.5
Closed joined length	Closed joined length (mm)	23.6	24.2	21.7	19.4
	Tolerance (mm)	± 0.3	± 0.3	± 0.3	± 0.3
Size		2	3	4	
Weight	Target weight (mg/100 capsules)	61	49	38	
	Weight limits (mg/100 capsules)	54.9-67.1	44.1-53.9	34.2-41.8	
Capacity	Approximate body volume (ml)	0.37	0.28	0.21	

Outside diameter	Cap diameter (mm)	6.36	5.83	5.33
	Body diameter (mm)	6.07	5.56	5.06
	Tolerance (mm)	± 0.06	± 0.06	± 0.06
Length	Cap length (mm)	8.94	8.08	7.21
	Body length (mm)	15.27	13.59	12.19
	Tolerance (mm)	± 0.5	± 0.5	± 0.5
Closed joined length	Closed joined length (mm)	18.0	15.9	14.3
	Tolerance (mm)	± 0.3	± 0.3	± 0.3

3 Visual Quality Specifications (AQLs)

The visual quality of a capsule batch is determined using sampling plans defined in ANSI/ASQ Z 1.4–2008 (normal inspection level, single sampling plan).

The specifications are derived from the ANSI/ASQ Z 1.4–2008 and assessed on a combined sample taken randomly throughout the batch from √N + 1 cartons (N is the total number of cartons in the controlled batch).

Qualicaps® capsules are controlled statistically to ensure conformance to the specifications found in the following section.

4 Visual Acceptable Quality Level (AQL)

AQL as defined in ANSI/ASQ Z 1.4-2008, is the maximum percent of defective units that for the purpose of sampling inspection can be considered satisfactory as a process average. A normal inspection level, single sampling plan is used.

Defect classification	AQL
Major A	0.015%
Major B	0.065%
Minor	1.0%

5 Classification and Descriptions of Visual Defects

Visual defects are classified according to the following definitions:

- **Major A: Affects the performance of a capsule as a package for the final product, or could contribute to a major subjective problem in filling.**
- **Major B: Would cause a problem on a capsule filling machine.**
- **Minor: Has no effect on the performance of a capsule as a package; it is a slight blemish that makes the capsule visually imperfect.**

MAJOR A	
Cracked	A cap or body with many splits
Double dip	Extra thick cap due to being dipped twice which makes the capsule not separate properly
Failure to separate	A joined cap and body that does not separate properly
Hole	An irregular opening in the cap or body
Joined in lock	A capsule in locked position
Long cap/body	Length of cap or body 1 mm more than specified length
Mashed	A mechanically damaged capsule that has been squashed flat
Pinched	Inward cap or body pinches > 3 mm
Short cap	Cap length 1 mm less than specified length
Short body	Body length 0.6 mm less than specified length
Split	A split in the film starting from the cap or body edge > 2 mm
Telescope	A closed capsule with a protruding piece of either cap or body produced by a double split
Thin spot (cap shoulder)	A thin area in the cap shoulder that may rupture when the capsule is filled
Trimming	A piece > 5 mm of, or the whole trimmed end of a cap or body inside a closed capsule
Uncut cap/body	An untrimmed cap or body

MAJOR B	
Damaged edge-large	Roughly trimmed cap edge. The imperfection at its greatest is > 1 mm into the specified length
Double cap	A capsule with an additional cap covering the body end
Different dye speck	A coloured spot different from the colour of cap or body

Grease	Mould release aid spots on the inside of capsule
Inverted end	A cap or body with the end pushed inwards > 3 mm in length
Long joined	A capsule not closed sufficiently to engage the prelock
Small pinched	Inward cap or body pinches < 3 mm
Thin spot	A thin area in the cap or body wall which may rupture when the capsule is filled
Turned edge	Folded-over edge > 2 mm on body cut line
Unjoined	A single cap or body

MINOR	
Black speck	A non-contaminant black spot > 2 mm
Bubble	An air bubble in the visible part of the capsule with a diameter > 0.4 mm (excluding overlapping area between cap and body)
Chips, tails	Small fragments of gelatin > 3 mm still attached or free within the capsule
Crimp	Cap or body has external surfaces crimped > 3 mm
Damaged edge-small	Roughly trimmed cap edge. The imperfection is V shaped and < 1 mm into the specified length
Dent	A depression formed in the end of cap or body. The dent is less than half of the diameter of the capsule part
Dye speck	A colour spot from the colour of the cap or body > 2 mm
Grease light	Small grease marks > 3 mm
Scrape	A scratch mark > 3 mm on the surface of a cap or body
Starred end	An individual imperfection of the tip of cap or body > 3 mm generated by turbidity or surface deformation
Strings	Strings between 3-4 mm at the cutting edge

6 Print Quality Specifications

(AQLs)

The print quality of a capsule batch is determined using statistical sampling plans defined in the ANSI/ASQ Z 1.4–2008 (normal inspection level, single sampling plan).

The specifications are derived from the ANSI/ASQ Z 1.4–2008 and assessed on a combined sample taken randomly throughout the batch from √N + 1 cartons (N is the total number of cartons in the controlled batch).

Qualicaps® printed capsules are controlled statistically to ensure compliance with the specifications found in the following section.

7 Print Acceptable Quality Level (AQL)

AQL as defined in ANSI/ASQ Z 1.4-2008, is the maximum percent of defective units that for the purpose of sampling inspection can be considered satisfactory as a process average.
A normal inspection level, single sampling plan is used.

Defect classification	AQL
Major A	0.010%
Major B	0.040%
Minor	1.0%

8 Classification and Descriptions

of Print Defects

Multiple Images (image is illegible)

MAJOR A

Unprinted

Incorrect Image

MAJOR B

Ink line/Spot > 5 mm

Misplaced Image
(off-register; not identifiable)

Partial Image
)

Smudged Image
(image is illegible)

MINOR

Ink Line/Spot (1-5 mm)

Misplaced Image
(off-register; still identifiable)

Multiple Images
(image is still legible)

Partial Image
(part of image is missing, but still legible)

Smudged Image
(image is still legible)

9 Chemical Specifications

Parameter	Specification
Moisture content/Loss on drying	3.0% - 7.0%

10 Microbiological Specifications

Parameter	Specification
Total Aerobic Microbial Count (TAMC)	10^3 cfu/g
Escherichia coli	Absence in 1 g
Total Yeats and Mould Count (TYMC)	10^2 cfu/g

11 Packaging

Qualicaps® capsules are supplied in a package that has two components:

- An inner liner made of a laminate of pharmaceutical-grade materials: polyester/polyethylene/aluminium foil. This is heat-sealed after inserting the capsules, creating a container with minimal moisture transfer properties.
- A cuboid cardboard carton of standard dimensions. This protects the inner liner during transportation.

Capsule size	00	0E	0	1	2	3	4
Capsules per carton in 000's*	75	75	100	135	175	225	300

Cartons size: 60 cm long x 40 cm wide x 75 cm high
* Tolerance: Capsule quantity variance is ± 5% per delivered carton box

12 Storage

Qualicaps® packaging is designed to maintain the quality of the empty capsule between manufacturing and filling. It is essential to read and understand the following information in order to ensure that Nutra'V capsules maintain their quality during this period.

12.1 Transportation

Nutra'V capsules are supplied in sturdy cardboard car-tons, each having heat-sealed, moisture-proof liners. These cartons may be grouped on a European size case pallet.

12.2 Warehousing conditions

The conditions in the areas in which capsules are stored or filled can affect the machinability of the Nutra'V **capsule. The ideal temperature for the storage of capsules should be between 15°C and 30°C (59°F and 86°F). The containers should be kept away from exposure to direct heat, sunlight and moisture. Maintaining the capsules within the liner bag (without perforations) safeguards them from both light degradation and loss of moisture, regardless of ambient humidity. Properly stored and sealed containers will provide optimum capsule performance in production.**

12.3 Capsule Shelf Life

Under the aforementioned storage conditions, Nutra'V **capsules will maintain their quality for five years from the date of manufacture.**

13 Filling Area Conditions

The moisture content of capsules is directly related to the relative humidity of the air to which they are exposed. When capsules are removed from their original packaging (sealed aluminium liner) and exposed during the filling process, their moisture content will equilibrate to filling room conditions.

The ideal conditions for a filling area are a temperature between 20°C and 25°C and a relative humidity between 35% and 55%, which will maintain the moisture content of the capsules within the desired range of 3.0% to 7.0% for Nutra'V**.**

An important consideration is to expose the minimum number of capsules required for the process at any one time. Some filling machines can generate significant heat during running, and this may affect capsules in use.

The capsule filling machine may be located in a controlled area but the climatization system may be operated only during the working day. Empty capsules should preferably be removed from the hopper on the filling and/or intermediate conveying equipment if climatic conditions vary from the ideal during idle hours.

For capsule handling, it is best to avoid the use of plastic utensils because this could result in static electrical charging that could cause feeding problems on the filling machine.

14 Regulatory Information

Nutra'V **capsules are made from hypromellose that conforms to current editions of EP/USP/JP monographs and with the purity criteria defined for E464 (HPMC) in Commis-sion Regulation (EU) No 231/2012.**

- **Hypromellose, used as the main raw material in the manufacturing process of empty** Nutra'V **capsules, is de-rived from pine trees.**
- **None of the ingredients of** Nutra'V**capsules are of bovine origin and therefore, there is no TSE/BSE problem associated to** Nutra'V **capsules.**

- **None of the ingredients of** Nutra'V**capsules are listed on Commission Regulation 881/2006, and therefore can be considered non-risk materials as far as contaminants mentioned in such regulation are concerned.**
- Nutra'V **capsules do not contain GMOs (genetically modified organisms).**
- Nutra'V **capsules do not contained preservatives and are not treated with either Ethylene Oxide nor gamma radiations.**
- **Neither gluten, sugar, nor lactose are used in the manufacturing process of** Nutra'V **capsules.**
- **The residual level of solvents in capsules fully complies with guideline CPMP/ICH/283/95**

Annex 14, Catalogue C Coated Capsules

C1. Microbial testing of trial capsules

eurofins

PO Number **QIB0117738A**

Bhavika Parmanand
Quadram Institute Bioscience
Norwich Research Park
Colney
Norfolk
NR4 7UA

AR-18-UD-247696-01

Reported on **03/07/2018**
Reported by **Narinder Ramewal, Head of Operational Excellence**

Certificate Of Analysis

Sample number	**400-2018-60163259**	Received on	**26/06/2018**
		Analysis Started on	**27/06/2018**
Your sample reference	**Capsules Containing inert powder**	Your sample code	**None supplied**

Test Code	Analyte	Result	SOP No.
Microbiology			
UMRXB	Clostridium Perfringens	< 10 cfu/g	EUMM3.07
UM02R	Coagulase positive staphylococcus	< 20 cfu/g	EUMM3.06
UMMKF	Listeria Species	Not Detected /25 g	EUMM3.19
UMX0I	Moulds 25°C	< 10 cfu/g	EUMM3.16
UMX0I	Yeast 25°C	< 10 cfu/g	EUMM3.16
UM8SQ	Presumptive Bacillus cereus 30°C	< 10 cfu/g	EUMM3.03
UMPEC	Presumptive Enterobacteriaceae 37°C	< 10 cfu/g	EUMM3.05
UMRJX	Salmonella	Not Detected /25 g	EUMM3.28

Unless stated, all results are expressed on a sample as received basis.
† Indicates that this test was subcontracted

Key: cfu colony forming units
< denotes less than
> denotes greater than

UKAS TESTING

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Regd Office: i54 Business Park
Valiant Way
Wolverhampton WV9 5GB
Regd in England No: 5009315

~ estimated value

* Indicates that this parameter is not included in the UKAS accreditation schedule for the laboratory.
Opinions and/or interpretations within this report are outside our accreditation scope.

Eurofins Food Testing UK Ltd i54
Business Park Valiant Way
Wolverhampton
WV9 5GB 0342

EPoM Food Frequency Questionnaire

This questionnaire asks for some background information about you, especially about what you eat.

Please answer every question. If you are uncertain about how to answer a question then do the best you can, but please do not leave a question blank.

1. On average, how often have you eaten these foods during the intervention period? Please answer all questions accurately.

Foods	Never	1-3 per week	4 or more per week
Tofu			
Wholemeal pasta			
Oat meal			
Beans inc. pinto, kidney, soybeans			
Brown rice			
Polished rice			
Chickpeas			
Lentils			
Potatoes			
Peas			
Egg (e.g. omelettes, flans, meringues, cakes, cookies, batter			
mixes, egg pasta, quorn, mayonnaise, quiches)			
Wheat (e.g. bread, cereals, pasta, pizza, cakes, pies, pastry)			
White fish (e.g. tuna, fish cakes, battered fish, fish fingers)			
Shellfish (e.g. crab, prawns, shrimps, lobster, crayfish)			
Oily fish (e.g. mackerel, salmon, sardines, pilchards,			
herring, kipper, white bait, trout, crab, FRESH tuna)			
Peanuts (e.g. Bombay mix, peanut butter, peanut brittle,			
peanut cookies, sate, some vegetarian meals)			
Tree nuts - almonds, brazil nuts, pecan nuts, hazel nuts, walnuts etc. (e.g. in chocolate, crunchy nut cornflakes,			
Citrus fruits (eg orange, tangerine, grapefruit, lemon, lime)			
Seeds e.g. sesame, poppy, sunflower			

2. How many portions of fruit and vegetables do you eat daily? (1 portion is 1 fruit, 1 bowl of salad, 2-3 tablespoons of vegetables, 1 bowl of fruit salad, a handful of dried fruit or a cupful of berries or grapes)

Less than 1 portion	
1 portion	
2 portions	
3 portions	
4 portions	
5 portions	
More than 5 portions	

3. How many cups of coffee/tea do you drink per day?

None	
1	
2	
3	
More than 3	

4. Please write any additional comments you may think will be of use to the study team:

Thank you for taking the time to complete this questionnaire. Please hand this questionnaire back to a study team member.

Participant Code:

Phase 1/2 *(please delete as appropriate)*

Treatment A/B *(please delete as appropriate)*

Date treatment started:

EPoM Study Participant Capsule Checklist

Below is a checklist that we require you to fill out as and when capsules have been consumed throughout the study. If you do forget to consume a capsule, please make a note of it in the space provided below.

Day	**Date**	**Meal 1** (2x capsules to be taken with meal 1)	**Meal 2** (2x capsules to be taken with meal 2)	**Meal 3** (2x capsules to be taken with meal 3)	**Reason for Omission**
Example	02.02.19	*x*	*x*	*x*	-
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					

Please mark the relevant box when you have consumed the required number of capsules for each day of the study in this phase. When completed, please tick the box and date the statement below:

I can confirm that I have consumed the capsules provided to me at the times indicated in the table above ☐

Date ……

Please use this space to provide us with any other information you may feel is relevant to the study:

PLEASE REMEMBER TO RETURN THE CAPSULE BOTTLE AT THE END OF THE PHASE

Effect of Phytin on Human Gut Microbiome (EPoM Study)

Faecal Collection Kit:

In order to collect your faecal sample, we have provided you with a faecal collection kit. The kit should include:

- An insulated container with a label on it
- A plastic pot
- A plastic bag
- A nappy sack
- A plastic clip to close the plastic bag
- When necessary, a urine sample collection bottle will be included, and we would advise you to put this somewhere separate from the faecal collection kit until it is required.

Please can you check your kit to ensure that you have all the items. Although we have checked the kit, if anything is missing please contact a member of the study team on the **study mobile (to be included when available)**, **Bhavika Parmanand on 01603 255021** or **Dr Lee Kellingray on 01603 255070** before you collect your sample.

The instructions as to how we would like you to collect your sample are on the other side of this piece of paper.

Please ensure you have read and understood the instructions in advance of trying to collect your sample. If you have any questions please get in touch with a member of the study team.

Instructions for faecal collection:

Day of the sample collection

1. Place the labelled plastic bag into the plastic pot as though you were lining a bin, roll the excess bag down over the outsides of the plastic pot (note pot will be white not black as in picture).

2. Collect your faecal sample directly into the labelled plastic bag in the pot. The pot is only used to make it easier to hold the bag. Please do not place any toilet paper into the pot and avoid collecting any urine in the pot if possible.

3. Once the faeces is inside the labelled plastic bag, roll the top of the bag back up and seal the bag, close to but not touching the sample, using the plastic clip provided. Remove the sealed bag from the plastic pot and throw the plastic pot away in your normal domestic waste.

4. Place the sealed bag inside the nappy sack and tie the nappy sack shut using the bag handles.

5. Place the tied nappy into the insulated container. Close the lid of the insulated container and clip it shut to secure it. Wash your hands.

6. Please write your volunteer number, and the date and time of collection on the label on the outside of the insulated container.

7. Please call the study mobile (**to be included when available**), or a study scientist Bhavika Parmanand on 01603 255021 or Dr Lee Kellingray on 01603 255070 before you deliver your sample so that we can be ready to receive it.

8. If for any reason a study scientist is unable to take your call, you can contact the Clinical Research Facility (CRF) (**to be included when available**). If you call the CRF and the answer phone is on it means there is no one at the CRF and you will need to contact one of the study scientists on the numbers above.

Thank you very much.

Effect of Phytin on Human Gut Microbiome (EPoM Study)

Stool chart

- This stool chart will allow us to monitor and record any changes to gut function during the study period.

- During the periods outlined below we would like you to keep a record of **each stool** using the Bristol stool chart on page 2 to identify the type of stool. During these periods, we would also like you to record whether you see any blood or mucus on/in your stool and rate any experience of abdominal discomfort/pain, abdominal bloating, straining when opening your bowels (constipation), and/or flatulence for **each day**, leaving a gap to separate each day (example on page 3).

- Ladies who are menstruating at the time entries are added to the stool chart should mark M in the box referring to stool type as this may help to interpret the data.

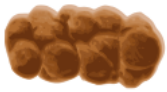
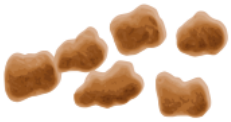
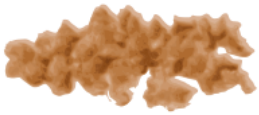
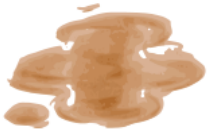
- We would like you to keep this record for 7 days whilst consuming each of the supplied capsules, during both Phases

- You can choose which days to complete, but **ideally it must be 7 consecutive days**. If this is not possible, then the 7 days should include one weekend.

- Key to gastrointestinal sensations:
  None – no discomfort.
  Mild – minimal discomfort but not interfering with everyday activities.
  Moderate - causes interference with everyday activities.
  Severe – prevents normal everyday activities.

Please call Bhavika Parmanand on 01603 255021
or Dr Lee Kellingray on 01603 255070
OR the study mobile (to be included when available)
during normal working hours for any help.

Bristol Stool Chart

Type 1		Separate hard lumps, like nuts (hard to pass)
Type 2		Sausage-shaped but lumpy
Type 3		Like a sausage but with cracks on the surface
Type 4		Like a sausage or snake, smooth and soft
Type 5		Soft blobs with clear-cut edges
Type 6		Fluffy pieces with ragged edges, a mushy stool
Type 7		Watery, no solid pieces. **Entirely Liquid**

Towards the bottom of the stool chart, which we would like you to complete, you will notice questions relating to any changes in medication. Should you have any changes to your medication, or start taking any medication of any kind, we would appreciate it if you could record what is being taken, any changes such as increased/decreased dose, date the medication was taken/changed, and the reason for taking/changing the medication.

The QI CRF Research Nurse may contact you by telephone to discuss information on the stool chart and might advise you to speak to your GP should it be deemed necessary, as well as advising on your continuation or exclusion from the study.

If you suffer from diarrhoea during the study, we would like you to contact a member of the study team and you will be asked to not collect a faecal sample for a minimum of 48 h after the last episode of diarrhoea. Should the diarrhoea persist for more than 72 h, you may be advised by the QI CRF Research Nurse to speak to your GP and will be excluded from the study.

NHS
Norfolk and Norwich
University Hospitals
NHS Foundation Trust

EPoM study stool chart example:

Date	Time	Type (refer to Bristol stool chart)	Abdominal discomfort (none, mild, moderate, severe)	Abdominal pain (none, mild, moderate, severe)	Type of abdominal pain (dull ache, sharp pain, cramping)	Bloating (none, mild, moderate, severe)	Constipation (none, mild, moderate, severe)	Flatulence (none, mild, moderate, severe)	Colour (pale cream, light brown, dark brown, black)	Blood Y/N If yes, what colour (bright red, dark red)	Mucus Y/N
01/06/19	09:05	3	none	none	none	none	none	none	Pale cream	N	Y
01/06/19	14:25	2	none	none	none	mild	none	moderate	Light brown	Y Bright red	N
02/06/19	10:50	4 M	none	none	none	none	none	none	Dark brown	N	N
02/06/19	17:15	2 M	mild	none	none	none	none	mild	Light brown	N	N
03/06/19	All day	none	moderate	moderate	dull ache	mild	severe	severe	n/a	n/a	n/a

Have there been any changes to your medication? (Y) N

What is the medication? **Senokot**……

How has your medication changed? **Took 2 tablets**……..

When did your medication change? **Tuesday 01st January 2019 at 10pm**……..

For what reason did your medication change? **I was constipated and experiencing abdominal pain** ……

……


NHS
Norfolk and Norwich
University Hospitals
NHS Foundation Trust

Quadram Institute Bioscience
Norwich Research Park
Colney
Norwich NR4 7UA
UK

www.quadram.ac.uk

[Insert Date]

[Participant Address]

Dear_____,

Re: Effects of Phytin on Human Gut Microbiome (EPoM Study)

I am writing to confirm your decision to withdraw from the EPoM study. I can confirm that your withdrawal from this research study will not affect your clinical care and participation in future studies at the Quadram Institute Bioscience or Norfolk and Norwich University Hospitals. No further data or sample will be collected from you, however, identifiable data or samples already collected with consent will be retained and used in this study if possible.

You will not be contacted again or be asked to provide any information in relation to the EPoM study, however, if you would like to discuss the withdrawal procedure in more detail, please do not hesitate to contact a member of the study team (contact information can be found in the participant information sheet and towards the end of this letter).

We would like to thank you for your participation in this study.

Yours sincerely,

Miss Bhavika Parmanand
Tel: 01603 255021
Email: bhavika.parmanand@quadram.ac.uk

Quadram Institute Bioscience
Norwich Research Park
Colney
Norwich NR4 7UA
UK

www.quadram.ac.uk

REPORT ON ADVERSE EVENT (AE)/REACTION (AR)/SERIOUS ADVERSE EVENT (SAE)
(SAE/SUSAR-internal use only as the official NRES form will be used and signed off by Chief Investigator)

Adverse Event / Adverse Reaction (circle as appropriate)

Study Title: EPoM

REC reference:……..

Participant Code Number:…….. Date of Birth: …….. Age: ……..

Male/Female (delete as appropriate)

Date/Time of occurrence: …….. Date/Time reported: ……..

Reported to (initially): …….. Place of occurrence: ……

Description of AE/AR/SAE/SUSAR:

Extent of Adverse Event (delete as appropriate)**:** Mild Moderate Severe

Treatment/Action:……

……

……

Outcome (delete as appropriate)**:** Recovered Not yet recovered Unknown

Description of Trial Material:

Drug Reaction (delete as appropriate)**:** Certain Probable Possible Unlikely Unclassified

Reporter details (print name):……**Title**:……

Signature of QI CRF Medical advisor …….. Date:……..

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Norwich Research Park
Colney
Norwich NR4 7UA
UK

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Chief Investigator **Professor Arjan Narbad**

Signature: **Date:**

Professional Address: QIB, Colney, Norwich, NR4 7UA **Tel. Number:** 01603 255131

Comments by QI CRF Medical advisor:

……

……

……

……

……

……

Participant's screening questionnaire attached (circle as appropriate): Yes No

If 'No' state reason:……

N.B. please ensure any relevant paperwork is attached

QI CRF medical advisor

Name:……

Quadram Institute Bioscience
Norwich Research Park
Colney
Norwich NR4 7UA
UK

www.quadram.ac.uk

Professional Address:

……

……

……

Telephone No.: ……

Signature:…… **Date:**……

University of East Anglia

Norfolk and Norwich University Hospitals NHS
NHS Foundation Trust

Joint Arrangements for Research

Serious Adverse Event report

1. What are you reporting?		
SAE / SAR ☐	SUSAR* ☐	
Note: If you are reporting a SUSAR the randomisation code for that participant will have to be unblinded		
Report Type:	Initial ☐ Follow-up Report ☐ Follow-up Report #	
2. Study information		
Study Title: (short)		
Sponsor:	**Chief Investigator Name:** **Email Address:**	
Eudract Number: *(for CTIMPs only)*	**R&D Reference Number / IRAS Number:**	
Protocol title and version number:		
Site Number: *(for multi-site studies only)*	**Site Name:**	
Principal Investigator	**Name:** **Email address:**	
Date of site becoming aware of the event *(dd/mm/yy)*:		

3. Participant information

Participant DOB: *(dd/mm/yy)*	**Participant initials:**	**Participant Gender:** ☐ Male ☐ Female
Participant Randomisation No:		

Evaluation of Event

4. Event/Reaction: *(keywords; e.g. body site, symptoms, severity, treatment)*

5. Date of onset: *(dd/mm/yy)*	**6. Date person completing form became aware of event:** *(dd/mm/yy)*

7. Criteria for definition as SAE *:

- ☐ Congenital abnormality/birth defect
- ☐ Resulted in death
- ☐ Life threatening
- ☐ In patient hospitalisation/prolongation of hospitalisation
- ☐ Persistent or significant disability

** If there is more than one criterion, choose the more/most significant one.*

8. Describe event: *(A summary of signs and symptoms, diagnosis, treatment of event, concurrent treatment, other relevant medical history, including re-challenge details if applicable. Please include the point in the study at which the event occurred.)*

9. In the investigators opinion was the event related to a research procedure?	☐ Definitely ☐ Likely ☐ Possibly ☐ Unlikely ☐ Not related
10. Please specify which procedure if applicable	
11. Is the study a Clinical Trial of Investigational Medicinal Product (CTIMP)? ☐ Yes *Please answer questions 12-17* ☐ No *Please go to question 18*	
12. In the investigators opinion was the event related to the Investigational Medicinal Product?	☐ Definitely ☐ Likely ☐ Possibly ☐ Unlikely ☐ Not related
13. Action taken with study drug:	☐ None ☐ Dose temporarily reduced ☐ Dose reduced ☐ Discontinued temporarily ☐ Discontinued
14. If related to IMP was this reaction unexpected (Suspected Unexpected Serious Adverse Reaction – SUSAR)?	

☐ Yes ☐ No ☐ Not applicable	
15. Did event/reaction abate after stopping drug?	☐ Yes ☐ No ☐ Not applicable
16. Did event/reaction reappear after reintroduction of drug?	☐ Yes ☐ No ☐ Not applicable
17. IMP & concomitant medication information	

(Please complete Appendix 1)	
18. Have urgent safety measures been implemented? ☐ Yes ☐ No ☐ Not applicable	**If yes, please detail below:**
Outcome of event	

19. What is the outcome of the SAE?	**20. Date event resolved:** *(dd/mm/yy)*	**21. Date patient died:** *(dd/mm/yy)*
☐Recovered ☐Recovered with sequalae ☐Continuing ☐Resulted in death ☐Unknown		

22. Cause of death obtained from:

- ☐ Coroner's inquest
- ☐ Death certificate
- ☐ Working diagnosis

Contact and signatures

Please supply contact details where further information may be obtained:

23. Person to contact:

24. Phone number:

25. Email address:

_____

Signature ***(person completing report)*** **Print name** **Date** *(dd/mm/yy)*

_____

PI Signature ***(if multicentre trial)*** **Print name** **Date** *(dd/mm/yy)*

_____

CI Signature ***(if not completing report)*** **Print name** **Date** *(dd/mm/yy)*

If the study is sponsored by NNUH please send the completed form to rdsae@nnuh.nhs.uk .

If the study is sponsored by the University of East Anglia and Hosted by NNUH, please scan and email the form to researchsponsor@uea.ac.uk and rdsae@nnuh.nhs.uk.

For R&D Office use only

Date form RECEIVED by R&D team: *(dd/mm/yy)* (___ /___/____)	Reviewed by: Date reviewed: *(dd/mm/yy)* (___ /___/____)
For SUSAR only:	Date reported to the REC: (___ /___/____) Date reported to MHRA: (___ /___/____)

Appendix 1

Section 17: IMP & concomitant medication information

Drug details (Daily dose and generic name)	Route of Administration (IV / Oral etc.)	Therapy Start Date (dd/mm/yy)	Therapy End Date (dd/mm/yy)	Date of last Administrered Dose prior to SAE onset (dd/mm/yy)	Indications for Use

Quadram Institute Bioscience
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UK
www.quadram.ac.uk

3 rd October 2018

To Whom It May Concern

Dear Sir or Madam,

The PHYTIN Study — Effect of ehytin on the Human Gut Microbiome (EPoM) IRAS Number 251932.

The Quadram Institute will maintain is current liability Insurance with the same insurer in 2018. The Institute has liability insurance in respect of research work involving human volunteers. The insurances comprise of:

1. No Fault Compensation for Clinical Trials/Human Studies
   000,000 for any one occurrence & in the annual aggregate

2. Legal Liability for Clinical Trials/Human Studies
   000,000 for any one occurrence & in the annual aggregate

3. Medical Malpractice 000,000 for any one occurrence & in the annual aggregate

No Fault Compensation for Clinical Trials/Human Studies

Compensation for bodily injury arising out of any Human Study or Healthy Volunteer Study conducted by QIB or on its behalf in connection with QIB business. Claims are covered only if they are made during the period of insurance.

Legal Liability for Clinical Trials/Human Studies

Legal liability to research subjects for bodily injury arising out of any Human Study conducted by QIB or on its behalf in connection with QIB business. Claims are covered only if they are made during the period of insurance.

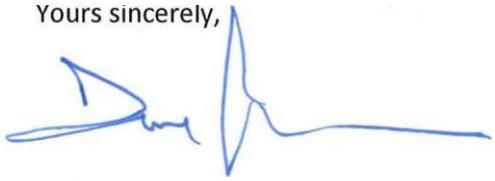
- The Insured shall also include any past employee who acted for the Insured and who agrees to be bound by the terms of the policy.

- Any sub-contractor, doctor, consultant physician, hospital or contract research organisation or nurse who will be performing work for the Insured in respect of Study/Trial are covered by this policy excluding liability which arises out of their own act error or omission outside the terms or instructions of the Study/Trial protocol.

Medical Malpractice
Legal liability to third parties arising from medical malpractice. Claims are covered only if they are made during the period of insurance, excluding any claim which is the subject of insurance indemnity or assistance provided by any Medical Defence Organisation or arising from products supplied.

The attached Schedule gives further details of all QIB liability insurances.

Yours sincerely,



Dave Foreman
Finance Director

Effect of Phytin on Human Gut Microbiome (EPoM Study)

Assessment day check list

Participant Code: _____ Date: ___ ___/ ___ ___/ ___ ___ Treatment: AB/BA (circle)

Is the participant willing to continue with the study?
(If no, do not continue study)

Allergies? Y _____N_____ If yes, please state _____

Weight kg

Has the participant developed any illnesses/medical conditions since the last study visit? YES / NO If YES:

Symptoms Description	**Onset Date**	**Severity:** 1= mild; 2=moderate; 3=severe; 4= life threatening	**AE / SAE**	**Comments/ Action taken**
	___ ___/___ ___/___ ___			
	___ ___/___ ___/___ ___			

Has the participant started medication or changed anything about his/her current medication/supplements since the last visit? YES / NO

If YES:

Medication / Supplements	**Dosage / Frequency change**	**Date of Change**	**Reason**	**Comments/Action taken**
		___ ___/___ ___/___ ___		
		___ ___/___ ___/___ ___		

Please use this space to provide us with any other information the participant may feel is relevant to the study:

Comments: Exclude / Postpone / Continue study day (circle)
Name of CRF Research Nurse/Study Scientist (circle)
(PRINT)…… Signature……Date ___ ___/___ ___/___ ___

Quadram Institute Bioscience
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www.quadram.ac.uk

25th July 2018

To whom it may concern

I hereby confirm that the Quadram Institute Bioscience is in receipt of £5,358,691.17 funding from the BBSRC, reference BB/R012512/1, for the Food Innovation and Health Institute Strategic Programme Grant starting 1st April 2018 and ending 31st March 2022.

Yours sincerely

Dr Mary Anderson
Head of Contracts