Effects of oat phenolic acids and avenanthramides on gut microbiota

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DECLARATION

I confirm is my own work and use of all material from other sources has been properly and fully acknowledged

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ABSTRACT

High consumption of wholegrains is related to positive health effects, including reduced risk of cardiovascular disease. Oat β-glucans have been considered to be partly responsible for these effects; furthermore, dietary phenolic acids and avenanthramides, are natural phenolic components in oats, which may also contribute to health benefits, whilst mechanisms for these effects remain unclear. Dietary components are well known to impact on the gut microbiome (GM), this consortium of bacteria are becoming increasingly associated with health effects, as such some of the health effects attributed by oats could be driven by the microbiota. This thesis investigates the potential interaction between of the gut microbiota, β-glucans, polyphenols and oats, to explore if this is a potential route for positive health outcomes. *In vitro* batch culture fermentations identified oats as a food matrix that significantly increased the abundance of Proteobacteria and Bacteroidetes, the lower dose resulted a bifidogenic effect and influenced microbial production of short chain fatty acids (SCFA). This is mediated by the synergy of all oat compounds within the complex food matrix, rather than its main bioactive β-glucan or polyphenols. During in vitro microbial fermentation, bound phenolic fractions largely remained intact up to 4h, following a significant increase in total free phenolic metabolites at 24 h and reached the same levels as control, pure free phenolic acids of microbial metabolites. 28 healthy adults enrolled on a randomised cross-over trial with three 4-week dietary intervention periods, comprising of a high (68.1 mg of phenolic acids) or moderate (38.9 mg of phenolic acids) avenanthramide and phenolic acid rich-wholegrain oat diet, or fibre matched control diet. The high avenanthramides and phenolic acids rich-wholegrain oat diet significantly increased the relative abundance of Proteobacteria phyla and *Sutterella* genus, without the alteration of the serum inflammatory markers. These data suggest that daily consumption of oats may beneficially modificate of the microbiota and metabolic activity.

LIST OF PUBLICATIONS

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CHAPTER 1

General introduction: The gut microbiota and cardiovascular health benefits: A focus on wholegrain oats

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Abstract

Existing scientific data suggests that a high intake of wholegrain foods contributes to improved gut health and a reduced risk of cardiovascular disease. Wholegrain oats are rich in dietary fibre and an important source of many bioactive components including minerals, vitamins, and phenolic compounds. The oat β-glucans have been reported to lower low-density lipoprotein cholesterol through their ability to increase the viscosity of intestinal chime, change the gut microbiota composition and increase the production of short-chain fatty acids, which may contribute to inhibition of hepatic cholesterol synthesis. Oats are also a rich source of phenolic acids, which are predominantly bound to cell wall polysaccharides through ester bonds. This bound state within oats means that phenolic acid bioavailability will largely be determined by interactions with the colonic microbiota in the large intestine. However, results from *in vitro*, animal and human studies have been inconsistent in relation to the impact of oats on the gut microbiota. This review will focus on the interaction of oat β-glucans and phenolic acids with gut microbiota, and the subsequent link to cardiovascular health.

1.1. Introduction

A high intake of wholegrains is related to improved gut health [1] and reduced risk of cardiovascular disease (CVD) [2, 3]. The health benefits of wholegrains might be due to effects on inflammation [4], fasting blood glucose [5, 6] and markers of lipid metabolism [2, 7-11]. A recent meta-analysis of observational studies indicates that diets rich in wholegrains are associated with a 21% reduction [Relative Risk (RR) = 0.79 (95% CI: 0.74, 0.85)] in CVD risk [12]. However, the median daily wholegrain intake in the UK, estimated from secondary analysis of data from the *National Diet and Nutrition Survey* rolling programme 2008/2009–2010/2011, is 20 g/day for adults and 13 g/day for children which is higher than in 2000/2001 but remains low and well below the US wholegrain recommendation of 48 g/day (no UK wholegrain dietary reference values exist) [13].

It has been suggested that the cardiovascular protective effects of wholegrain oats are mainly due to their dietary fibre content, in particular, soluble oat β glucans, with established blood cholesterol and glucose lowering properties [14-17]. However, oats are also a rich dietary source of phenolic acids and avenanthramides (a class unique to oats) and several randomised, controlled, crossover studies have suggested that a range of phenolic compounds may be responsible for some of the health effects [18-20]. However, the phenolic acids in oats are linked to cell wall polysaccharides by ester linkages and this means their absorption will be limited until they reach the large intestine where they may undergo extensive bioconversion by colonic microbiota to yield various bioactive metabolites that express local intestinal effects in the gut, and also systemic effects following absorption [21, 22]. Previous data have suggested that, in addition to fibre and non-digestible carbohydrate, phenolics may beneficially modulate the gut microbiota composition and activity [23, 24], which may play role in the prevention of chronic diseases [25]. For example, the secreting metabolites, such as secondary bile acids (BAs), short chain fatty acids (SCFA) and trimethylamine-N-oxide (TMAO), act as hormone-like factors

and are sensed by dedicated receptor systems in the human host to play a role in the pathogenesis of CVD [26]. In this review we provide a concise introduction into human gut health and the contribution that the microbiota play in influencing markers of CVD pathophysiology, before outlining the cardiovascular benefits of oat intake and how these are mediated, in part, by the gut microbiome.

1.2. Gut health and the host gut microbiota

The human gastrointestinal tract is a diverse and dynamic microbial ecosystem, comprising approximately 10^{14} bacterial cells and over 1000 different species [27]. Firmicutes and Bacteroidetes are the two dominant phyla, representing more than 90% of all the phylotypes, followed by lower relative abundances of Actinobacteria, Proteobacteria, Fusobacteria and Verrucomicrobia [28-30]. Commonly considered as the most beneficial bacterial genera are bifidobacteria, lactobacilli and butyrate producers, such as *Eubacterium rectale*, *Roseburia* species [28]. An appropriate balance between harmful and health promoting gut microbiota can support human health by: 1) maintaining host immune homeostasis; 2) increasing the efficiency of energy production in the gut through fermentation of non-digestible dietary compounds; 3) synthesising vitamins, such as B_{12} and K; 4) controlling intestinal epithelial cell proliferation; 5) stimulating immunological defence; 6) creating a protective barrier; and 7) inhibiting the growth of potential pathogens [31-35].

Conversely, an unfavourable gut microbiota composition and function (*i.e*. dysbiosis) can trigger the development of diseases through intestinal-derived endogenous endotoxins, such as lipopolysaccharides, indoxyl sulphate and L-carnitine. These metabolites may potentiate the development of acute diseases, such as diarrhoea and chronic diseases including obesity, metabolic diseases, cancer and CVD [36-38].

There has been a long understanding of the interaction between environmental factors and gut microbiota, including that of pH, peristalsis, redox potential and nutrient availability [39]. Dietary changes are thought to be responsible for around 20% of the variation in the gut microbiota, compared with only 1.9% for genetics [40, 41]. For example, a study showed that the microbiota of mice fed a low-fat, polysaccharide-rich diet markedly increased in populations of Firmicutes, and decreased in levels of *Clostridium, Eubacterium*, *Enterococcus* and *Bacteroides* spp when the mice were switched to a high sugar/fat diet [42, 43]. On the other hand, a high fibre diet can limit the growth of potentially pathogenic *E. coli.* [44], which has been observed in humans within 24 hours following a change from a high fat/low fibre diet to one which is low fat/high fibre [45].

1.3. The role of the gut microbiota in the pathophysiology of cardiovascular disease

1.3.1. Prebiotic effects

Shifts in the gut microbiota composition have been associated with a wide variety of diseases, including CVD [46]. The promotion of the growth of specific beneficial gut microbiota is believed to have preventative effects on CVD due to the influence of these bacteria on human physiology/metabolism, including an ability to reduce total serum cholesterol, low-density lipoprotein (LDL) cholesterol, and inflammation [47, 48]. Dietary substrates which induce such changes in the growth of favourable probiotic bacteria are referred to as ‘prebiotics’, which pass largely unmetabolised in the upper gastrointestinal tract and are selectively utilised by host microorganisms conferring a health benefit [49]. Established prebiotics are carbohydrate-based but other substances, such as polyphenols [50, 51] and polyunsaturated fatty acids [52, 53], might also fit the updated definition as they can affect the gut microbiome, although more studies are needed to show subsequent health effects.

Prebiotics may reduce risk factors of CVD through the stimulation of growth of *Bifidobacterium* and *Lactobacillus*, and the subsequent production of SCFA [54], or possibly through the reduction of plasma cholesterol [55] and/or fasting plasma glucose and insulin [56-58]. Furthermore, consumption of prebiotics has been linked to improved intestinal function, such as reduced gastrointestinal inflammation [59, 60] and mineral absorption, and modulation of energy metabolism, satiety and immune function [32, 33, 39, 61].

1.3.2. Structural effects

The large gut's enormous surface area helps to absorb nutrients, water, and electrolytes from food but at the same time it needs to provide a tight barrier against harmful substances and pathogens. One way in which the gut microbiota may confer health effects is via their potential to maintain large gut integrity [62]. Gut hyper-permeability (leaky gut) results from structural changes induced by Gram negative bacteria, which allows bacterial cell wall products, such as lipopolysaccharide and peptidoglycans, to enter into the bloodstream and activate macrophages. Also, gut microbiota derived lipopolysaccharide can induce foam cell formation and this can reduce reverse cholesterol transport and increase insulin resistance, hyper lipidemia, vascular inflammation [63] and thus increase CVD risk.

1.3.3. Bile acid synthesis/clearance/metabolism

BAs have indirect (through cholesterol metabolic pathways) and direct effects (through interaction with myocytes) on blood cholesterol levels, atherosclerotic plaque formation and myocardial function, and thus are hypothesised to reduce CVD risk [64].

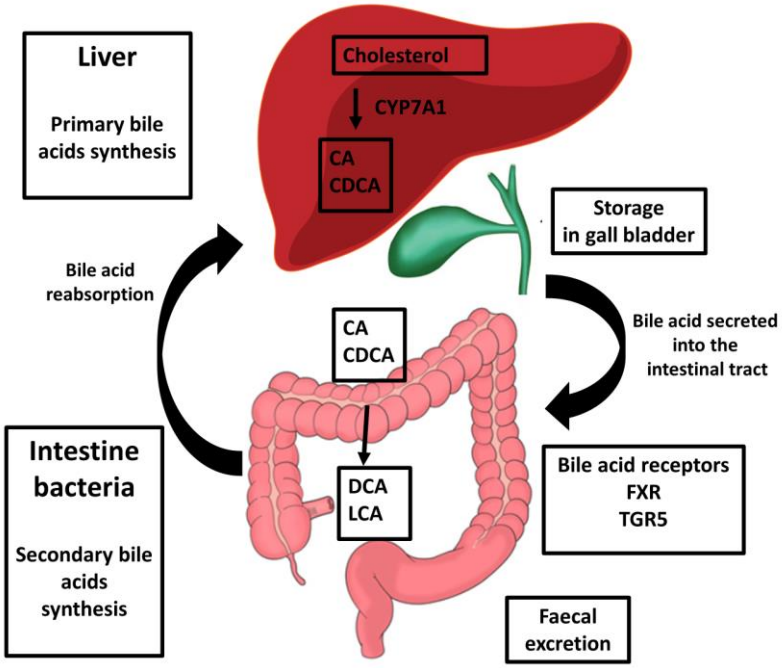


Figure 1. *Bile acid biosynthesis. Primary bile acids synthesis in the liver: cholesterol is synthesised to cholic acid (CA) and chenodeoxycholic acid (CDCA) by the Cholesterol 7 alpha-hydroxylase (CYP7A1) enzyme. In the intestinum, the bacterial 7 alpha-dehydroxylase converts the CA and CDCA to deoxycholic acid (CDA) and lithocholic acid (LCA), respectively. The bile acid receptors FXR farnesoid x receptor (FXR) and G protein-coupled bile acid receptor (TGR5) regulate bile acid synthesis, glucose homeostasis, lipid metabolism*

Figure 1 illustrates the pathways of BA synthesis and cholesterol biotransformation in the liver [65]. Primary BAs are further metabolised via conjugation to glycine or taurine in the liver, synthesised to bile salts and transported to the gallbladder. Following a meal and the release of cholecystokinin from the duodenum, the gallbladder contracts, resulting in bile salts flowing into the duodenum, which in turn promotes the absorption of dietary lipids [66].

The bacteria in the lower intestine are capable of converting primary BAs, by deconjugation and hydroxyl group oxidation at C-3, C-7, and C-12, and 7a/b-dehydroxylation, to secondary BAs, which predominate in human faeces [66, 67]. Even though some are lost in faeces, most BAs are absorbed in the ileum and return to the liver where they are deconjugated and released into the small intestine [65]. The genera of the gut microbiota involved in BA metabolism are *Bacteroides, Bifidobacterium, Clostridium, Lactobacillus* and *Eubacterium* [68]. BA metabolites resulting from microbial transformation may act as signalling molecules and regulate cardiovascular function through the TGR5 (G-coupled protein receptor) and FXR (farnesoid x receptor), thus potentially inhibiting inflammation and maintaining epithelial cell integrity [69], and modifying vascular tone [66, 70]. Furthermore, *Bacteroides fragilis*, *B. vulgatus*, *Clostridium perfringens*, *Listeria monocytogenes* and several species of *Lactobacillus* and *Bifidobacterium* interfere with cholesterol absorption from the gut by deconjugating bile salts, via bile salt hydrolases [71], which means cholesterol is less easily absorbed and more likely to be excreted in faeces, resulting in lowered blood cholesterol [36, 39, 65, 72].

1.3.4. Short chain fatty acids

SCFA are the major end product of both carbohydrate and amino acid bacterial fermentation in the human large intestine. The main SCFA are acetate, propionate and butyrate, less common are formate, valerate, caproate and branched-chain fatty acids [39, 73]. Butyrate can be metabolised by colonocytes or absorbed and metabolised further in the liver, where they are metabolised [74]. SCFA can act as an energy source for gut epithelial cells, improve intestinal defence against pathogens, modulate inflammation and possibly influence satiety [75]. Butyrate may play a key role in regulating gene expression, inflammation, and maintaining homeostasis of colonic mucosa through stimulating the production and release of the gut hormone glucagon-like peptide-2 (GLP-2) in enteroendocrine L cells [76-78].

In addition, SCFA decrease systemic levels of blood lipids by inhibiting hepatic cholesterol synthesis and/or redistributing cholesterol from plasma to the liver [61]. SCFAs are also thought to bind to specific G protein-coupled receptors, leading to the favourable regulation of lipid and glucose metabolism in the context of CVD [79-81]. Another gut microbiota metabolism dependent effect is the microbial conversion of choline and L-carnitine to trimethylamine (TMA) to TMAO. TMAO is thought to increase atherosclerotic CVD by altering cholesterol transport, potentially increasing macrophage activation [82].

1.4. Oat components

Oats (*Avena sativa*) are unique among cereals, due to their multifunctional characteristics and nutritional profile (Table 1).

Table 1. *Nutritional composition of raw oat bran. Data from the US Department of Agriculture National Nutrient Database [83]*

Nutrient	Value per 100 g
Energy (kcal)	246
Protein (g)	17.30
Total fat (g)	7.03
Fatty acids, total saturated (g)	1.6
Fatty acids, total monounsaturated (g)	2.38
Fatty acids, total polyunsaturated (g)	2.77
Total carbohydrate(g)	66.22
Sugar (g)	1.45
[&] β-glucans (g)	4.5-5.6
[&]Arabinoxylans (g)	3.83-13.20
Non-starch polysaccharide (g)	7.1
Calcium (mg)	58
Iron (mg)	5.41
Potassium (mg)	566
[&]Sodium (mg)	4
Magnesium (mg)	235
Zinc (mg)	3.11
Thiamine(mg)	1.17
Riboflavin (mg)	0.22
Niacin (mg)	0.93
Vitamin B (mg)	0.16
Folate (µg)	52
Vitamin E (mg)	1.01
Vitamin K (µg)	3.2
[*]Total phenolic (mg)	35.1-87.4

[*]*Data from Shewry et al. (2008)*

They supply protein, carbohydrate (primary starch content), crude fat, dietary fibre (non-starch), unique antioxidants and vitamins and minerals [84-86]. Oats have been grown for thousands of years, mainly as an animal feed crop, but during the 19th century oats gained recognition as a ‘healthy’ food [85].

Numerous reviews suggest that diets rich in oats may reduce inflammatory bowel disease and coeliac disease [87, 88], attenuate CVD progression [3, 89], and regulate glucose control in type 2 diabetes [24, 90].

The nutrient profile and quality of oats depends on several factors, such as growth environment, genotype and the interaction between environment and genotype [91]. The main constituent (60%) of oat grains is starch (rapidly digestible, slowly digestible and resistant), located in the endosperm. Resistant starch is recognised as a fermentable fibre source for gut microbiota, which results in the production of SCFA in the colon [92-94]. Moreover, oats are a source of high-quality protein, lipids (especially unsaturated fatty acid such as oleic, linoleic and palmitic acid), traces of minerals (mainly calcium and iron), B and E vitamins [85, 93, 94], and phenolic compounds.

1.4.1. Oat phytochemicals

Dietary phenolics, such as hydroxycinnamic, hydrobenzoic acids and avenanthramides, are natural phenolic components in oats [95]. They are found in three different forms within the oat food matrix: as soluble free acids; as soluble conjugates esterified to low molecular weight components such as sugars; and as insoluble bound acids esterified to high molecular weight components including lignin, cell wall polysaccharides such as arabinoxylan [96] and storage proteins in the aleurone layer and the pericarp [95].

The main hydroxycinnamic acids present in oats include, ferulic acid, caffeic, p-coumaric (Figure 2), o-coumaric and sinapic acids. Hydroxybenzoic acid derivatives are protocatechuic, syringic, vanillic, *p*-hydroxybenzoic and gallic acids [97-100].

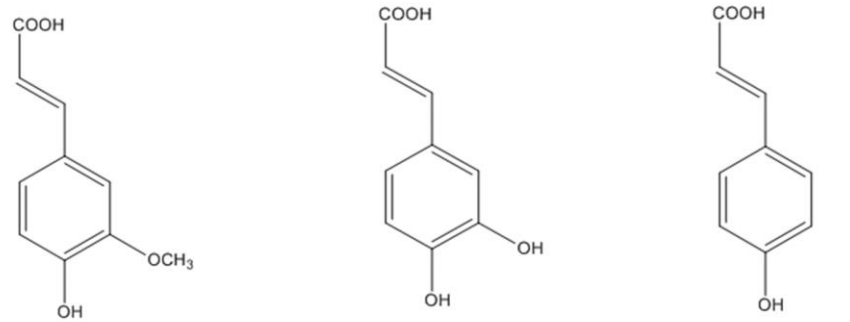
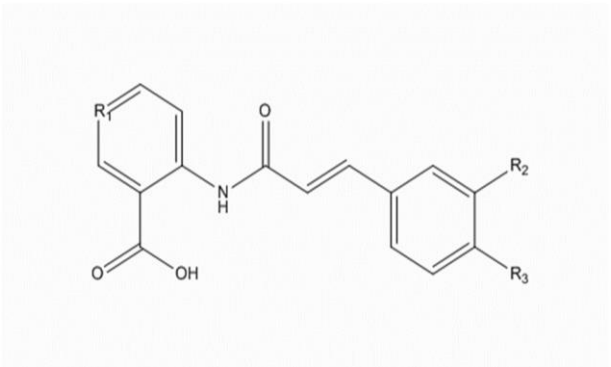


Figure 2. *Chemical structure of the main hydroxycinnamates in oats*

Hydroxycinnamic acids all have a C_6C_3 carbon skeleton with a double bond in the side chain that may have a cis or trans configuration. By far the most abundant hydroxycinnamic acids in oats is the trans-ferulic acid, with contents ranging from 95-386 µg/g [95]. Analytical data indicate that, on average, 19% of these phenolic acids (range: 50-110 µg/g) are present in the soluble state, 34% as conjugates (range: 111-314 µg/g) and 47 % bound (range: 131- 640 µg/g) [95].



Avenanthramides

AV A – R_1= OH, R_2=H

AV B - R_1= R_3=OH, R_2=OCH_3

AV C - R_1= R_2 = R_3=OH

Figure 3. *Chemical structure of the avenanthramide in oats.*

Avenanthramide - A (AVA), Avenanthramide – B (AVB) and Avenanthramide – C (AVC)

Another phenolic group, which is specific to oats are the avenanthramides (Figure 3) consisting of an amide conjugate of anthranilic acid and hydroxycinnamic acids. The three major subgroups are avenanthramide - A (AVA), avenanthramide – B (AVB) and avenanthramide – C (AVC), which occur in the bran or outer layers of the kernel [101]. Total contents of avenanthramides in oats range from 42-91 µg/g [101-104].

1.4.2. Fibre

Wholegrain oats are typically 10%-12% fibre, of which roughly 40% is soluble and 60% insoluble (cellulose) [85]. Mixed - linkage (1-3)(1-4)- β-D-glucans (β-glucans) (82% water - soluble fraction) together with arabinoxylans are important sources of soluble as well as insoluble dietary fibre [95, 105]. The soluble β-glucans located throughout the subaleurone cell walls are one of the most commonly studied components of oats [106-109]. 100 g of oats contains 3 – 6 g β-glucans [95].

1.4.3. Bioavailability of phytochemicals and physiological effects of β-glucans

It has been estimated that only 5% -10 % of dietary polyphenols are absorbed in the small intestine, with the remaining fraction reaching the large intestine where they are metabolised by the gut microbial community [110, 111]. The absorption and metabolism of ferulic acid can be affected by the form of food matrix ingested [97, 112]. Counterintuitively, fibre bound ferulic acid in wheat bran has been observed to be more bioavailable than free ferulic acid [113]. The differences observed in their absorption may relate to the rapid cleavage of the fibre-phenolic ester bond by the intestinal microbiota, which generated a higher amount of free phenolic acids in the large gut, increases their time in the plasma and decreases the level of urinary excretion [113, 114]. Our group reported intake of 60 g oat bran (2.5 mg avenanthramides, 28.6 mg phenolic acids) resulted in elevated urinary excretion of 30 phenolic acids/metabolites, amounting to total

recovery of 22.9% ± 5.0%, mainly between 0-2 and 4-8 hours [115]. The predominant metabolites included vanillic acid, 4 - and 3-hydroxyhippuric acids and sulphate conjugates of benzoic and ferulic acid (accounting for two thirds of total phenolic excretion). The results suggest that bound phenolic acids present in oats are rapidly released by the microbiota. Similarly, another human study [116] showed peak plasma concentrations of avenanthramides between 2 and 3 hours after high (229.6 mg/kg) AVA intake and 1 and 2 hours for low AVA intake (32.7 mg kg). AVB demonstrated a longer half-life and slower elimination rate than AVA and AVC. The bioactive properties of polyphenols are greatly dependent on their bioavailability [101, 103, 111, 117, 118]. These polyphenols have been observed to inhibit vascular smooth muscle cell proliferation and enhance nitric oxide production [119].

The physiological activity of β-glucans is in part related to their effects on bile reabsorption and through their ability to increase intestinal chyme viscosity, effects that are dependent on both the concentration and molecular weight of the β-glucans consumed [85, 95]. The molecular weight of β-glucans varies between 100 000 and 1 200 000 g/mol, and those of a small molecular size (370 000 g/mol) have been reported to be less effective at reducing cholesterol levels [106] compared with those of high molecular weight [120]. The solubility of β-glucans is another important factor influencing their physiological activity [105]. Their ability to form highly viscous small intestinal chyme likely contributes to the health benefits of β-glucans, as it has been reported that there is an inverse linear relationship between measures of postprandial blood glucose and insulin responses (to an oral glucose load) and the viscosity of the chyme [121-123].

1.5. Cardiovascular benefits of the oat intake

Atherosclerosis is one of the main underlying pathophysiological processes in CVD development [124, 125], with several different actions, such as oxidation of LDL cholesterol, adhesion of monocytes across the endothelial surface, development of monocytes to macrophages, and the formation of foam cells [126]. The potential modulation of atherogenic pathways following oat intake has been suggested and may involve anti-inflammatory activity, the maintenance of endothelial function and the reduction of plasma cholesterol (Figure 4) [3, 10, 14, 127-129]. Additionally, oat fibre and phenolics are capable of interacting directly with the gut microbiota, leading to a shift in their profile and composition, secondary changes in cholesterol and bile metabolism, and the production of key metabolites such as SCFA and phenolic acids metabolites (Figure 4).

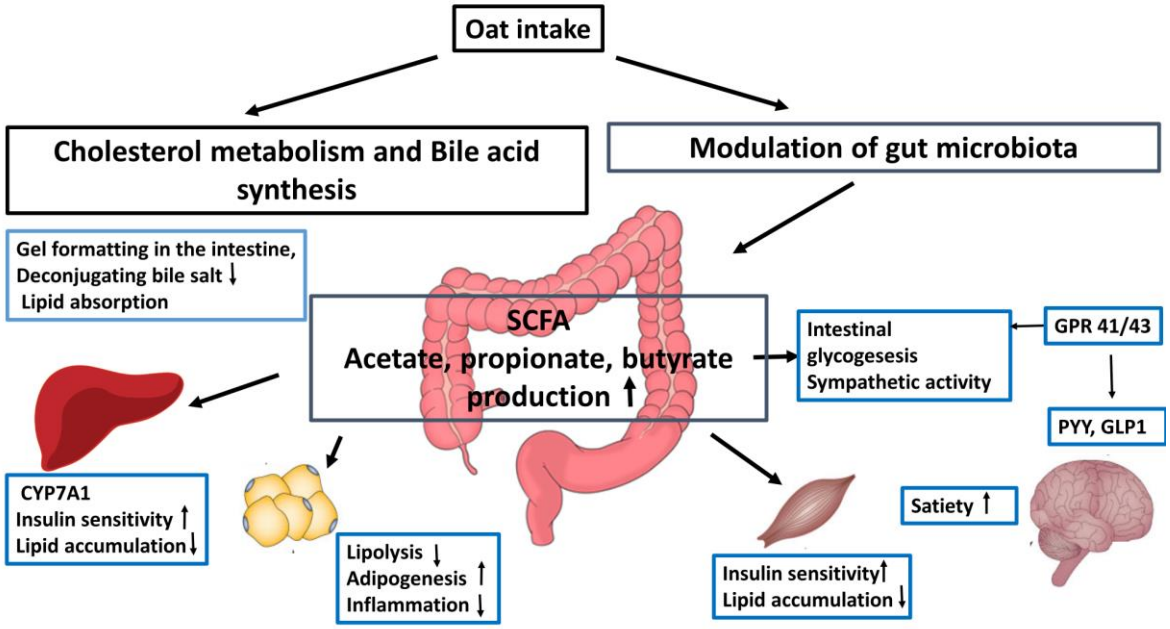


Figure 4. *Potential mechanisms in the digestion system of the cardiovascular benefits of oats. Oats may interact directly with the gut microbiota leading to a shift in their profile and composition, changes in cholesterol and bile metabolism, and the production of key metabolites such as short chain fatty acids. Abbreviations: SCFA (short-chain fatty acid), GPR41 and GPR43 (G-protein coupled receptor), PYY (peptide YY), GLP1 (glucagon-like peptide 1), CYP7A1 (cholesterol 7 alpha-hydroxylase)*

1.5.1. Effects of oat intake on microbiome composition- in vitro and animal data

Several *in vitro* fermentation (Table 2) and animal studies (Table 3) suggest that increased oat intake leads to gut microbiota alterations. *In vitro* fermentation of oat grains has been shown to increase *Bifidobacterium* and *Lactobacillus* populations [130] and *Bacteroides* and *Provotella* groups [131], whilst decreasing clostridia levels [132]. However, although oat-derived β-glucans extracts increased *Lactobacillus-Enterococcus* after 5 hours and *Bacteroides* after 24 hours in one *in vitro* study, they were found to not influence bifidobacterial growth [133]. In another *in vitro* study, it has been reported that the β-glucans are fermented by *Bacteroides* spp. but not by *Lactobacillus* and *Bifidobacterium* [134]. The fermentation of oats in anaerobic, pH controlled, faecal batch cultures has been shown to increase SCFA production, with significant increases in acetate, propionate and butyrate levels [130, 132, 133, 135, 136].

Table 2. *The impact of oats on the gut microbiota and short chain fatty acid (SCFA) production based on data from in vitro studies at 24 hours*

Reference	Intervention	Effects on bacterial composition	SCFA
Hughes *et al.* [133]	Oat derived β-glucans Low molecular weight (LMW) 150 kDa High molecular weight (HMW) 230kDa	*Atopobium* ↑ - LMW and HMV *Bacteroides – Provotella* ↑ - LMV *Lactobacillus/Enterococcus* ↓ - LMV and HMV *Clostridium histolyticum* ↑ - LMV and HMV	Acetate ↑ - LMV and HMV Propionate ↑ - LMV and HMV
Kedia *et al.* [132]	Oat bran fraction (OB) Whole oat flour (WOF)	*Bifidobacterium* ↑ - OB and WFO *Lactobacillus/ Enterococcus* ↑ - OB *Clostridium* ↓ - 24 hours, OB *Clostridium* ↑ - 24 hours, WOF	Acetate, butyrate, propionate ↑ - OB and WOF
Kim *et al.* [135]	Oat derived β-glucans Low β-glucans (LB) – 5.31% β-glucans High β-glucans (HB) – 7.70% β-glucans	*No data*	Acetate, butyrate, propionate ↑ - LB, HB No significant differences between LB vs. HB

Connolly *et al.* [130]	Oat grain flakes 0.53-0.63 mm (size 23) 0.85-1.0 mm (size 25-26)	*Bifidobacterium* genus↑ - size 25-26 *Eubacterium* ↑ - size 23 and 25-26	Acetate ↑ -size 23 Acetate, propionate, butyrate ↑ - size 25-26
Connolly *et al.* [24]	Wholegrain oat based cereals Jumbo porridge oat (JPO) 100% wholegrain aggregate (WGA) Granola (G) 70% whole grain loops (WGL) Instant porridge (IP)	JPO, IP, G, WGA *Bifidobacterium* ↑ JPO, G – *Atopobium* ↑ G, WGA, IP, WGL - *Bacteroides, Provotella* ↑ G ,WGA, WGL - *Lactobacillus/ Enterococcus* ↑ P, WGA -*Clostridium* ↓	Acetate, propionate ↑ - JPO, IP, WGA, WGL, IP Butyrate ↑ - IP, WGA
Connolly *et al.* [136]	Toasted (T), partially toasted (PT) and raw (R) wholegrain wheat flakes	*Bifidobacterium* genus ↑ – T, PT, R *C. hystolyticum* subgroup*, Lactobacillus* ↑ - R	Acetate, propionate ↑ - T, PT, R
Chappell *et al.* [131]	Belinda oats	Bacteroides ↑ Firmicutes ↓	Acetate, propionate, butyrate ↑

Consumption of oats by rodents has been reported to result in many specific changes in their microbiota, such as increases in the growth of *Prevotellaceae*, *Lactobacillaceae* and *Alcaligenaceae* families 175.5% (P = 0.03), 184.5% (P = 0.01), and 150.0% (P = 0.004) (Table 3). However, these results are not consistent with the findings from other animal studies that indicate oat bran intake increases only *Bifidobacterium* and *Lactobacillus* growth [137, 138]. The range of oat products used in these studies, including oat flour and bran [138], or insoluble fibre and soluble fibre combinations [139], might explain the reported variability in microbiota growth in rodents. Hence, further well-designed *in vitro* and human studies are required to examine which oat components may result in beneficial changes to the microbiota.

Table 3. *Animal studies examining the effects of oat intake on growth of gut microbiota and short chain fatty acid (SCFA) production*

Reference	Duration/ Intervention	Oat effects on bacterial composition and/or SCFA compared to control	Animals
Drzikova *et al.* [138]	Oat flour (F); oat bran (B); oat flour (F@) (autoclaved) *6 weeks*	*Bifidobacterium* genus↑ - F, B *Bacteroides* ↑ - F@	Rat
Immerstrand *et al.* [140]	Oat bran (OB; β-glucans – 1800 kDa or 2348 kDa) Processed oat bran (POB; β-glucans – 1311 kDa, 241 kDa, 56 kDa, 21 kDa, 10 kDa) *4 weeks*	Acetate, propionate, butyrate ↑ - OB, POB	Mice
Berger *et al.* [137]	High fat diet - barley husks, rye bran, fibre residue from oat milk *4 weeks*	*Lactobacillus* ↑ propionate, butyrate ↑	Mice
Zhou *et al.* [139]	Whole grain oat flour (WGO), Low bran oat flour (LBO) *8 weeks*	Prevotellaceae, Lactobacillaceae, and Alcaligenaceae families relative abundance– ↑ - WBO Clostridiaceae, Lachnospiraceae families – ↑ - LBO	Mice

Overall, these studies indicate inconsistent findings regarding the influence of oats on the growth of the microbiota and their diversity, which might be partly explained by the various different study and experimental designs. It is also important to note that these studies considered only the influence of the β-glucans fraction within oats and not other oat bioactives, such as polyphenols, which have also been observed to interact with the gut microbiota [110, 141, 142].

1.5.2. Effects of oat intake on microbiome composition- human trials

Whilst *in vitro* [130, 132, 136] and animal studies [137, 140] indicate that oat intake increases the production of SCFAs, these effects are difficult to quantify in humans as SCFAs are rapidly absorbed or utilised in the large intestine. For example, most of the butyrate is used by the colonocytes as their preferred energy substrate [143], propionate is primarily absorbed and removed by the liver [144], whilst acetate passes into the peripheral circulation [145]. The use of *in vitro* tools can help to investigate whether various substrates lead to increased SCFA generation, as faecal SCFA measurements are an uncertain estimate of colonic SCFA production [146].

Table 4. *The relationship between oats, the growth of gut microbiota and short chain fatty acid (SCFA) production based on data from human intervention trials*

Reference	Duration/ Intervention	Oat effects on bacterial composition and/or SCFA compared to control
Bridges *et al.* [147]	20 (38 – 73 years) hypercholesteraemic men Oat bran - 34 g total fibre and 13.4 g soluble fibre/day Control - 14 g total fibre and 3 g soluble fibre/day *3 weeks*	SCFA in peripheral serum Serum acetic acid ↑
Mårtensson *et al.* [148]	56 adults (20 - 70 years, 24 men/32 women) with moderately increased plasma cholesterol levels Fermented oat-based products (FO) (3-3.5 g – β-glucans/day) Fermented ropy, oat products (FRO) Fermented dairy-based product (control) *Control – 3 weeks, treatment - 5 weeks*	Total bacteria count ↑ *Bifidobacterium* ↑ - FO, FRO
Nilsson *et al.* [149]	25 healthy adults (20 - 47 years, 10 men/15 women) 40 g β-glucans enriched oat bran (40 g oat bran, 20 g dietary fibre, 10 g glucan in 4 slices of bread) Control – baseline samples, week 0 *12 weeks*	SCFA in faeces Acetic, propionic, butyric, isobutyric, isovaleric– ↑
Valeur *et al.* [146]	10 healthy adults (22-49 years, 2 men/8 women) 60 g oatmeal porridge (8.5 g fibre, 4.7 g glucans) Control – baseline samples, day 1 *1 week*	Intestinal gas production SCFA and NO changes - faeces β –galactosidase and urease - ↓ Rectal level of PGE_2 - no significant difference
Connolly *et al.* [23]	32 mild or hypercholesterolemia adults (23-64 years, 12 men/20 women) Wholegrain oat granola (WGO) - 45 g granola breakfast cereals (6.3 g fibre, 2.9 g glucans) Non-wholegrain (NWG) – 45 g non-wholegrain breakfast cereals (control) (3 g fibre, no glucans) *6 weeks*	SCFA no significance differences Total bacteria count ↑ *Bifidobacterium* ↑ *Lactobacilli* ↑

Findings from human trials on the effects of oats on the microbiota vary, possibly due to differences in study design (*i.e*. intervention dose, study duration, study population, the method of microbial enumeration) and because these studies assessed the effects of fibre but not the polyphenols in oats (Table 4). Two randomised controlled trials provide evidence that wholegrain wheat may exert effects on gut microbiota [150, 151]. A daily intake of 48 g of wholegrain wheat significantly increased the growth of *Bifidobacterium* (0.8 $\log_{10}$ cells per g faeces) and *Lactobacillus* (0.6 $\log_{10}$ cells per g faeces) and increased plasma ferulic acid levels [151]. In contrast, the intake of 70 g per day of wholegrain wheat was found not to increase levels of *Bifidobacterium*, although faecal ferulic acid levels were found to be associated with an increase in *Bacteroides*, Firmicutes and a reduction of *Clostridium* [150]. To date, no human trials have studied the direct effects of extracted or purified oat phenolic acids on the growth of the microbiota, or whether they contribute to the prebiotic effects of wholegrain intake, although data do exist on the impact of flavanols, which have been found to promote the growth of specific beneficial bacteria [50]. These data suggest that phenolic acids present in wholegrain cereals may potentiate gut microbiota diversity; however, further research is required to distinguish between the effects of fibre and polyphenols on gut health.

1.5.3. Impact of oat intake on cholesterol metabolism and bile acid synthesis

Oat β-glucans have been suggested to lower cholesterol by causing an increased viscosity of the intestinal chime [128]. The gel formed is thought to act as a physical barrier to lipid (triglycerides) absorption in the small intestine, in particular, that of cholesterol reabsorption [126, 152, 153]. β-glucans have also been shown to bind to luminal BAs, which increases the excretion of bile in the faeces and triggers an increase in hepatic conversion of cholesterol into BAs, thereby decreasing blood cholesterol levels [153, 154].

It has been reported that the intake of 75 g of extruded oat bran (11 g β-glucans) resulted in a near doubling of serum 7 alpha-hydroxy-4-cholesten-3-one concentration within 8 hours, which leads to increased BA synthesis [155]. BA excretion is well known to stimulate cholesterol uptake from the circulation, resulting in a decreased serum cholesterol concentration [154].

1.5.4. Oat intake and short chain fatty acid production

SCFA production is highly dependent on the donors gut microbiota composition and availability of substrates [156]. The ability of oats (and isolated β-glucans) to increase SCFA production is well established [130, 136, 137]. After 12 hours of *in vitro* fermentation, oat-bran (containing 22% oat β-glucan) induced significantly higher concentrations of propionate compared to other prebiotic dietary fibres, such as inulin and xylooligosaccharide [157]. The production of SCFAs in the large intestine following oat consumption has been reported and postulated to contribute to reductions in serum lipids and other CVD risk markers [126]. As such, SCFAs may act as mediators of the beneficial effects of wholegrain oat intake of human CVD. These effects might be dependent on SCFAs' ability to: 1) affect liver and muscle cellular metabolism via their potential to increase insulin sensitivity and decrease lipid accumulation in liver [158-162]; 2) suppress cholesterol synthesis, increase fatty acid oxidation and decrease *de novo* fatty acid synthesis in the liver [109, 155, 163, 164]; 3) increase adipogenesis and decrease lipolysis [165, 166]; 4) modulate satiety through their potential to bind to G-protein coupled receptors (GPR41, GPR43), leading to the increased production of the gut hormones glucagon-like peptide-1 and peptide YY [167-169] and regulate satiety centrally [81, 147, 170]; and 5) improve colon and liver glucose homeostasis via their induction of intestinal gluconeogenesis [79, 171-173] (Figure 4).

In addition, butyric acid has been reported to increase phenolic acid absorption in the colon [174], which subsequently may induce endothelium-dependent vasodilation [175-177]. Recent studies have also indicated that the ratio of acetate and propionate may be important for defining the precise effects of SCFAs on various physiological systems. For example, a high serum acetate:propionate ratio has been associated with reductions in total serum cholesterol in men [178-180].

1.6. Conclusions

Evidence suggests that wholegrain oats may reduce CVD risk due to their influence on lipid metabolism and plasma cholesterol levels. Oat β-glucans are likely to be partly responsible for any effects as they can change the gut microbiota composition, increase cholesterol excretion in the gut, and increase the levels of SCFA, which may inhibit hepatic cholesterol synthesis and affect glucose homeostasis in adipose tissues and muscle cells. However, inconsistent findings from *in vitro*, animal and human studies have been reported regarding the influence of oat intake on these outcomes, possibly due to differences in experimental techniques and the focus on β-glucan rather than other compounds present in oats, such as phenolic acids and avenanthramides, which may also contribute to beneficial changes in the gut microbiota and lipid/cholesterol metabolism. Clinical, observational and experimental studies to date have not explored the extent to which cardiovascular benefits are dependent on oat fibre or phenolic acid levels. Hence, further randomised controlled trials are required to examine the relative effects of oat phenolics on microbial pathways and cardiovascular risk markers.

Author Contributions

AK conducted the literature search and drafted the review. MYS, GS, SA, GGCK, GW and JPES critically revised the manuscript. All authors read and approved the final version of the paper. No conflict of interests has been declared.

1.7. Objectives and Hypothesis

The overall objectives of the thesis are to explore the effect of oat phenolic acids and avenanthramides on gut microbiota composition and metabolic activity using *in vitro* batch culture and a chronic human crossover randomised trial. The faecal microbiota compositions and dynamics were determined using tag-encoded 16S rRNA gene HiSeq high throughput sequencing and short chain fatty acid analysis, and UPLC-MS/MS profiles were used to identify the metabolomics activity of the microbiota.

The overall hypothesis of this thesis is that oat polyphenols frequent intake have relative contribution to the health benefits by improving gut microbiota composition and activity, inflammatory markers.

Study 1: Oat bran, but not its isolated bioactive β-glucans or polyphenols, have a bifidogenic effect in an *in vitro* fermentation model of the gut microbiota

Objectives: To examine if a physiologically relevant dose of oat polyphenol mix (avenanthramide, hydroxycinnamic acids and benzoic acid derivatives), β-glucan extract or digested oat bran as whole food matrix beneficially modulate the gut microbiota. Secondly, the study aimed to assess the impact of different doses of digested oat bran on the GM, notably using an oat bran that was well characterised in terms of physicochemical properties.

Hypothesis: Digested oat bran will beneficially, dose dependently, modulate gut microbiota composition by increasing beneficial genera. Moreover, short chain fatty acids will be produced by fibre fermentation. Oat polyphenol mix and β-glucan extract will exert less of a change in the gut microbiome.

Study 2: Bioconversion of oat phenolic acids and avenanthramides following i*n vitro* digestion: are fibre bound phenolic acids bioavailable?

Objectives: To determine the extent to which such oat phenolic acids, including bound phenolic acids, are released from the oat matrix during digestive processes in the GI tract and the fate of these once released.

Hypothesis: Some oat polyphenols will be absorbed before reaching the large intestine, but mainly bound forms of phenolic acid will persist to the large intestine and be broken down by gut microbiota.

Study 3: Effect of oat polyphenols on gut microbiota composition and inflammatory markers in healthy (above average blood pressure) adults

Objectives: To assess whether beta-glucan matched meals providing either a high dose or a moderate dose of oat avenanthramides and phenolic acids leads to dose-dependent chronic improvements in gut health and inflammatory markers relative to a fibre and energy matched control intervention

Hypothesis: The 4-week supplementation of high dose of oat avenanthramides and phenolic acids will results beneficial modulation of the gut microbiota composition by increasing beneficial genera and will improve the inflammatory markers.

CHAPTER 2

Oat bran, but not its isolated bioactive β-glucans or polyphenols, have a bifidogenic effect in an *in vitro* fermentation model of the gut microbiota

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Abstract

Wholegrain oats are known to modulate the human gut microbiota and have prebiotic properties (increase the growth of some health promoting bacterial genera within the colon). Research to date mainly attributes these effects to the fibre content; however, oat is also a rich dietary source of polyphenols, which may contribute to the positive modulation of gut microbiota. *In vitro* anaerobic batch-culture experiments were performed over 24 h to evaluate the impact of two different doses (1 and 3 % w/v) of oat bran, matched concentrations of β-glucan extract or polyphenol mix, on the human faecal microbiota composition using 16S RNA gene sequencing and short chain fatty acid analysis (SCFA).

Supplementation with oats increased the abundance of Proteobacteria ($p < 0.01$) at 10 h, Bacteroidetes ($p < 0.05$) at 24h and concentrations of acetic and propionic acid increased at 10 and 24 h compared to negative control. Fermentation of the 1 % w/v oat bran resulted in significant increase in SCFA production at 24 h (86 ($_{SD}$ 27) mM vs. 28 ($_{SD}$ 5) mM; $p < 0.05$) and a bifidogenic effect, increasing the relative abundance of *Bifidobacterium* unassigned at 10h and *Bifidobacterium adolescentis* ($p < 0.05$) at 10 and 24 h compared to negative control. Considering the β-glucan treatment induced an increase in the phylum Bacteroidetes at 24 h, it explains the Bacteroidetes effects of oats as a food matrix. The polyphenol mix induced an increase in Enterobacteriaceae family at 24 h. In conclusion, in this study, we found that oats increased bifidobacteria, acetic acid and propionic acid, and this is mediated by the synergy of all oat compounds within the complex food matrix, rather than its main bioactive β-glucan or polyphenols. Thus oats as a whole food led to the greatest impact on the microbiota.

2.1. Introduction

A large body of evidence from prospective and intervention studies suggests that a diet rich in oats could significantly reduce the risk of bowel disease [87, 88], cardiovascular disease [3, 12, 89] and lowers high blood cholesterol levels [3, 14, 89, 152]. Recent reports suggest that oats may act as a prebiotic, modulating the gut microbiota and impacting on metabolic disease risk [23, 136]. To date, it is believed that the protective effect of whole grain oats is mainly due to the presence of dietary fibre, in particular, soluble β-glucan [14-17], and resistant starch [138]. β-glucan is known to lower cholesterol and bile acid absorption through formation of viscous gels in the upper gut [107] and/or directly binding of cholesterol or bile acids. Resistant starch leads to the production of short chain fatty acids (SCFA) in the colon and may also increase the growth of some health promoting bacterial genera within, such as *Bifidobacterium* and *Lactobacillus* [92-94, 181]. Species of these genera influence the cholesterol metabolism through increasing bile-salt hydrolase enzyme activity and the deconjugation of bile acids [182].

Oats, however, are also rich dietary sources of polyphenols, including avenanthramides and phenolic acids [95]. These are likely to contribute to the health effects of a diet rich in oats [97, 117, 183] but have not yet been examined in detail. Phenolic acids are found in three different forms within the oat food matrix: as soluble free acids, as soluble conjugates esterified to low molecular weight components such as sugars, and as insoluble bound acids esterified to high molecular weight components including lignin, cell wall polysaccharides and storage proteins [95]. A large proportion of oat polyphenols are bound via an ester bond, and hence are poorly absorbed in the upper intestine and reach the colon [184], where they may beneficially modulate the microbiota [23]. There are no esterases in human tissues that break these ester links [185]; therefore, the main catalytic site is in the colon through cinnamoyl esterase activity of human faecal microbiota, such

as *Escherichia coli* (three isolates), *Bifidobacterium lactis* and *Lactobacillus gasseri* (two strains) [186, 187].

Gut microbiota (GM) alterations by whole grain oats have been observed in a number of *in vitro* fermentation models [24, 130-133, 136] and animal studies [137-140]. The fermentation of oats has led to increased *Bifidobacterium, Lactobacillus* [23, 24, 132, 136, 137, 139] and *Bacteroides* [131] populations and, at the same time decreased *Clostridium* [132, 139].

The soluble β-glucans have been one of the most commonly studied components of oats [109, 133, 188]. However, oat-derived isolated β-glucan only impacted on *Bacteroides* growth, not on *Bifidobacterium* in 24h pH-controlled anaerobic batch culture fermenters [133]. Furthermore, Crittenden *et al.* [134] reported that β-glucan was fermented by *Bacteroides* spp. but not by *Lactobacillus* or *Bifidobacterium.* The viscosity and molecular weight of β-glucan may strongly influence its ability to lead to SCFA production and act as a prebiotic [189]. Additionally, these studies have used the relatively high concentrations of 1% v/w of β-glucans dose [24, 190] which is much higher than the physiological β-glucans concentration delivered from eating oats. To date, none of the oat or β-glucan *in-vitro* fermentation studies assessed the polyphenol content of their treatment [131, 133, 136]. Thus, little information exists on the ability of oat-derived avenanthramides, hydroxycinnamic and hydroxybenzoic acids to influence the GM.

The main purpose of the present *in vitro* study was to examine if a physiologically relevant dose of polyphenol mix (avenanthramide, hydroxycinnamic acids and benzoic acid derivatives), β-glucan extract or digested oat bran as whole food matrix beneficially modulates the GM. Secondly, the study aimed to assess the impact of different doses of digested oat bran on the GM, notably using an oat bran that was well characterised in terms of physicochemical properties.

2.2. Materials and Methods

Contributions

The authors' contributions were as follows: AK, MYS and GW designed the study; OK analysed the β-glucan molecular weight, AK and PH conducted the *in vitro* fermentation experiments; MW and AK conducted the 16S rRNA gene sequencing, AK and MW conducted the statistical analyses. AK, MW, MYS, GW, GK, GS, SA and JS interpreted the data and AK drafted the manuscript, MW, MYS, GM, GGCK and JS critically revised the manuscript.

2.2.1. Reagents

All chemicals and reagents were purchased from Sigma-Aldrich Chemical Co Ltd. (Pool, Dorset, UK) or Fisher (Loughborough, Leics, UK) unless stated otherwise. Mixed-linkage β-glucan kit was supplied by Megazyme Co (Wicklow, Ireland). The anaerobic jar (AnaerojarTM 2,5L) and gas-generating kit (AnaeroGen TM) were obtained from Oxoid Ltd (UK), the dialysis tube from Spectrum (VWR International). Media and instruments were autoclaved at 121°C for 15 min. HPLC column and guard cartridges were obtained from Phenomenex (Cheshire, UK). PowerSoil®DNA Isolation Kit was purchased from Mo Bio Laboratories, Inc (USA), the primers for the 16S rRNA gene amplification from Integrated DNA Technologies, BVBA (Belgium), AccuPrimeTM SuperMix II from Life Technologies (CA, USA), AMPure XP beads from Beckman Coulter Genomic (CA, USA).

2.2.2. Oats and Controls

The oat bran was purchased from White's (Tandragee, Northern Ireland). The oat macronutrient composition was analysed by Campden BRI laboratories (Total carbohydrate, Available carbohydrate, Total dietary fibre- AOAC 991.43 method, Total fat – BS 4401, and protein – AOAC 981.10 method), whereas the detailed polyphenol content

was measured in our laboratory at the University of Reading based on a previous method [115]. The beta-glucan method used was employed specifically to quantify 1,3:1,4-β-D-glucan. Synergy1 the oligofructose-enriched inulin was supplied by Beneo (Belgium) and 94%-β-glucan extract from Megazyme Co (Wicklow, Ireland). Polyphenols were purchased from Sigma-Aldrich Chemical Co Ltd. (Pool, Dorset, UK).

2.2.3. In vitro digestion of oat bran (from mouth to small intestine)

The method employed was adapted from Mills et al. [191]. Oat bran was digested *in vitro* in three phases: the oral phase, the gastric phase and the small intestinal phase. 60 g of oat bran was mixed with 150 ml of sterile and distilled water and homogenised, transferred into a 500 ml Duran bottle, and microwaved for 1 minute. In the oral phase, 20 mg of α-amylase was dissolved in 6.25 ml $CaCl_2$ (1 mM, pH 7.0) and added to the solution, then incubated at 37°C for 30 minutes on a shaker set at 120 *xg*. After incubation, the pH was adjusted to 2.0 with 6 M HCl solution and the gastric phase introduced by adding 2.7 g pepsin in 25 ml HCl (0.1 M) and further incubated for 2 hours under the same conditions. In the small intestinal phase, 560 mg pancreatin and 3.5 g bile were mixed with 125 ml $NaHCO_3$ (0.5 M) and dispensed into the mix. The pH was adjusted to 7.0 then incubated for 3 hours at 37 °C with shaking. Finally, the sample solution was transferred to a seamless semi-permeable 100-500 Dalton molecular weight cut-off regenerated cellulose dialysis tubing and dialysed against NaCl (0.01 M at 5°C) to remove low molecular mass digestion products. After 15 hours, the dialysis fluid was changed and the process continued for an additional 2 hours. The digested oat bran mix was collected and transferred into several 250 ml clear plastic containers, frozen to -80° and freeze-dried for 5 days to remove all fluid content.

2.2.4. Extraction and analyses of polyphenols from undigested and digested oat bran

Polyphenols were extracted from undigested (raw) and digested (after *in vitro* digestion) oat bran in two separate fractions (i.e. free and conjugated or bound) using the method of Schar *et al.* [115]. The phenolic acids and avenanthramides in oat extracts were identified and quantified using a high-performance liquid chromatography (HPLC) Agilent 1100 series (Agilent Technologies Ltd) equipped with a quaternary pump, autosampler, column thermostat, sample thermostat and photo diode array detector. Compound separation was achieved by a Kinetex biphenyl column (100 A 250 x 4.6 mm length, 5 uM particle size; Phenomenex) and using a gradient elution. Mobile phase A consisted of 0.1 % (v/v) formic acid in HPLC water (A), while mobile phase B was 0.1 % (v/v) formic acid in methanol. The following optimised gradient protocol was run: 0 min, 95% A, 5 % B; 20 min, 75 % A, 25 % B; 25 min, 74 % A, 26 % B; 30 min, 65 % A, 35 % B; 40 min, 64 % A, 36 % B; 53 min, 30 % A, 70 % B; 56 min, 5 % A, 95 % B; 61 min, 5 % A, 95 % B; 62 min, 95 % A, 5 % B; 65 min, 95 % A, 5 % B. The flow rate of the mobile phase was 1.0 ml/min and the sample injection volume was 20 µl [192]. The absorbance was recorded at 254, 280 and 320 nm and quantification was based on 12 point linear calibration curves (mean $R^2 > 0.99$) and as a ratio to the internal standard (i.e. 3,5-dichloro-4-hydroxybenzoic acid) to account for losses during extraction ($R^2 \geq 0.99$).

2.2.5. Determination of the β-glucan content

The β-glucan content was analysed with the 1,3:1,4 mixed-linkage β-glucan kit. The assay uses lichenase and β-glucosidase to metabolize β-glucan to β-gluco-oligosaccharides and subsequently glucose. Glucose then reacts with GOPOD reagent and its absorbance was measured at 510 nm by UltroSpec 1100 photo spectrometer (Scinteck Instruments LLC, USA). The final β-glucan content was calculated by the Megazyme Mega-CalcTM tool [193].

The molecular weight of the β-glucan was determined by size-exclusion HPLC. The chromatography system consisted of three serially connected columns (Shodex SB-G, Shodex SB-806M, Shodex SB-804 HQ) and an UV-MALLS-Viscometer-dRI detector. The column temperature was set at 40°C, the mobile phase was MiliQ water (Millipore, Bedford, MA) containing 0.02 % sodium azide, and the flow rate set at 0.5 ml/min. Samples were prepared at a concentration of 10 mg/ml, heated at 60°C for 3 hours under constant shaking, syringe filtered (0.45 µm PVD; Whatman, NY) and diluted to a concentration of 1 mg/ml before injection.

2.2.6. pH controlled faecal batch culture fermentation

Substrate ability to modulate gut microbiota was determined using anaerobic, stirred, pH and temperature controlled faecal batch cultures. Batch culture fermentation vessels (300 ml volume: one vessel per treatment) were sterilised and filled with 135 ml of sterile basal medium (g/l: 2 g peptone, 2 g yeast extract, 0.1 g NaCl, 0.04 g K_2HPO_4, 0.04 g KH_2PO_4, 0.01 g MgSO4·7H_2O, 0.01 g CaCl2·6H_2O, 2 g $NaHCO_3$, 2 ml Tween 80, 0.05 g Hemin (dissolved in a few drops of 1 M NaOH), 10 µl vitamin K, 0.5 g L-cysteine HCl, 0.5 g bile salts and 4ml of resazurin solution (0.02 %)). Before addition to the vessel, the medium was adjusted to pH 7.0 and autoclaved. The sterile medium was gassed overnight with O_2-free N_2 (15 ml / min) to establish anaerobic conditions. To mimic the distal region of the human large intestine, pH was held in the range of 6.7 - 6.9 by automatic addition of 0.5 M NaOH or 0.5 M HCl and controlled via pH meter controllers (Electrolab, UK) and the temperature was kept at 37 °C. Faecal samples were collected from three healthy female donors, who were between 25 and 40 years old, with no history of bowel disorders, who had not received antibiotic treatment for at least 6 months before the study and had not consumed pre- or probiotic supplements one month before the study.

All donors were informed of the study aims and procedures, and provided their verbal consent for stool samples to be used for the experiments in compliance with the ethics procedures required at the University of Reading. Samples were collected in anaerobic jars and used within one hour of collection. Samples were diluted 1:10 (w/v) with anaerobic phosphate buffer (0.1 M, pH 7.4) and homogenised in a stomacher for 2 mins; the batch culture systems were inoculated with 15 ml faecal slurry from an individual sample.

The vessels were treated with the following substrate: 1 % w/v, 1.5 g digested oat bran (OAT1.5); 3 % w/v, 4.5 g digested oat bran (digestibility of oats see McCane et al.[194]) (OAT4.5); 0.12 % w/v, 180 mg 94 % β-glucan extract (BG); 0.01 % w/v, 1.7 mg polyphenol mix (same profile as 4.5 g digested oats) (POLY); 1 % w/v, 1.5 g Synergy1 (positive control, PC); an additional vessel was prepared under the same conditions but without any substrate, negative control (NC) (Table 6). The Synergy 1 is an inulin enriched with oligofructose with proven prebiotic effects [195]. The 3 % w/v oat was chosen as the highest dose since it would be the concentration reached by an average person consuming 60 g oat bran (i. e. assuming 30 g oat bran reaches the colon and colonic total volume is 1L) [194]. Based on this calculation, β-glucan extract and polyphenol mix treatments were matched to the dose present in 3 % w/v oat bran. Treatment with OAT1.5 was added to the experiment to monitor the impact of the oat dose on the prebiotic effect. Five millilitre samples were collected at 0, 5, 10, 24 h, of which 1 ml aliquots were centrifuged at 13,000 *xg* for 10 min. Supernatants and pellets were stored separately at -20°C until analysis.

2.2.7. High throughput sequencing of the gut microbiota

The GM compositions and dynamics were determined using tag-encoded 16S rRNA gene MiSeq-based (Illumina, CA, USA) high throughput sequencing. DNA was extracted from 2 ml of faecal samples from fermentation vessel using Power Soil DNA isolation kit (Mo Bio). The bead-beating was performed in 3 cycles of 15 second each at 6.5 pulse / s.

(The FastPrep-24™ 5G Instrument, MP Biomedicals). DNA concentrations and purity were determined using Nanodrop 1000 (ThermoScientific, USA). The V3 region of the 16S rRNA gene was amplified using primers compatible with the Nextera Index Kit NXt_338_F: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACWCCTACGGGWGGCAGCAG -3' and NXt_518_R: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATTACCGCGGCTGCTGG -3'" [196]. PCR and library preparation were conducted as described in [197]. Briefly, PCR containing 12 µl AccuPrime SuperMix II, 0.5 µl of each primer (10 µM), 5 µl of genomic DNA (~10 ng / µl), and nuclease-free water to a total volume of 20 µl were run on a SureCycler 8800 (Agilent, CA, USA). Applied cycling conditions were: denaturation at 95 °C for 2 min; 35 cycles of 95 °C for 15 s, 55 °C for 15 s and 68 °C for 40 s; followed by final elongation at 68°C for 5 min. To incorporate primers with adapters and indices, PCR reactions contained 12 µl Phusion High-Fidelity PCR Master Mix, 2 µl P5 and P7 primer, 2 µl PCR product and nuclease-free water for a total volume of 25 µl.

Cycling conditions applied were: 98°C for 1 min; 12 cycles of 98°C for 10 s, 55°C for 20 s and 72°C for 20 s; elongation at 72°C for 5 min. The amplified fragments with adapters and tags were purified using AMPure XP beads. Prior to library pooling, clean constructs were quantified using a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA) and mixed in approximately equal concentrations to ensure even representation of reads per sample. 180 bp pair-ended MiSeq (Illumina) sequencing was then performed according to the instructions of the manufacturer. The raw dataset containing pair-ended reads with corresponding quality scores was trimmed using CLC Genomic Workbench (CLC bio, Arhus, Denmark). Trimming settings were set to a low-quality limit of 0.01, with no ambiguous nucleotides allowed, and trimming off the primer sequences.

Merging overlapped reads was performed using the "Merge overlapping pairs" tool using default settings. The Quantitative Insight Into Microbial Ecology (QIIME) tool (version. 1.8.0; Open source software) was used for further analysis [198].

Purging the dataset from chimeric reads was performed using USEARCH, while the usearch61 method was used for Operational Taxonomic Units (OTUs) selection [199]. The Greengenes (version 12.10) 16S rRNA gene database and EzTaxon were used as reference [200, 201]. High throughput sequencing yielded 12465219 sequences free from chimeric reads, providing an average ± SD of 178202 ± 80036 sequences per sample (Range 292-447040), five baseline samples and one 10h sample were excluded due to low reads (292-509).

2.2.8. Short-chain fatty acid analysis

The defrosted supernatant samples were filtered (0.22 µm, Millipore) and spiked with 50 µl internal standard (2-ethyl butyric acid, 100 mM). SCFAs were measured in an ion exclusion HPLC system (Agilent 1100 Series) equipped with DAD detector (G-1315B), autosampler (G1316A) and Aminex HPX-8711 column (300 x 7.8mm) heated to 84°C. Samples (20 µl) were injected in duplicate, and UV absorption was measured at 214 nm. The mobile phase was 2.5 mM aqueous sulphuric acid run at a flow rate of 0.6 ml/min. Peaks were integrated using Agilent ChemStation software (Agilent Technologies, Oxford, UK) and single point internal standard method. Peak identity and quantification were determined using a mixture of standards of acetic, butyric, iso-butyric, propionic, lactic, formic, valeric and isovaleric acids. Quantification was based on the 10-point calibration curve of analytical standards ($R^2 \geq 0.99$).

2.2.9. Statistics

For calculation of alpha and beta diversity measurement of the sequencing data, the d- and e-values were set to 9800 reads per sample (85 % of the sequence number of the most indigent sample). Alpha diversity measures expressed with an estimated total species, Chao1, the sequence similarity 97 % OTUs value were computed for rarefied OTU tables using the alpha rarefaction workflow. Differences in alpha diversity were determined using a t-test-based approach employing the non-parametric (Monte Carlo) method (999 permutations) implemented in the compare alpha diversity workflow with QIIME.The relative distribution of the genera registered was calculated and summarised at the genus level OTU tables, followed by Principal Coordinates Analysis (PCoA) plots generated with the Jackknifed beta diversity workflow based on 10 distance matrices calculated using 10 subsampled OTU tables with QIIME. The *p*-value and the conservative FDR-corrected *p*-value for multiple comparisons are reported. 3D plots were constructed from the three primary PCs from the PCoA of the MiSeq analysis to visualise group differences in the GM composition.

For the rest of the data analysis, GraphPad Prism statistics software package version 7 was used. One-way ANOVA was used to determine differences between fermentation treatments (OAT1.5, OAT4.5, POLY, BG) at the same time point (0, 5, 10 or 24 h), followed by the least significant difference (Bonferroni) post hoc test. A repeated measures ANOVA was used to explore the differences within the same treatment (OAT1.5, OAT4.5, POLY, BG) with all the time points (0, 5, 10 and 24 h) with Bonferroni as the post hoc test. In addition to these analyses, the *p* values were corrected using false discovery rate (FDR), $p \leq 0.05$ was considered statistically significant.

2.3. Results

2.3.1. The composition of undigested and digested oat bran

The macronutrient, fibre and phenolic content of undigested and digested oat bran are reported in Table 5. The *in vitro* oral, gastric and small intestine digestion reduced the carbohydrate content by 53 %, available carbohydrate by 62 %, while the dietary fibre and β-glucan content remained stable, losing only 2 % and 12 %, respectively. Free and conjugated, bound polyphenol compounds decreased by 48 % and 26 %, respectively.

Table 5. Macronutrient, fibre and phenolic content of oat bran before and after in vitro digestion and bioaccessibility (%) through digestion*

Components	**Before digestion**	**After digestion**	**Bioaccessibility %**
Amount (g)	60	40	33
Total Carbohydrate (g)	37.8	17.6	53
Available Carbohydrate (g)	32.6	12.5	62
Total dietary fibre(g)	5.2	5.1	2
β-glucan (g)	1.7	1.5	12
Total protein (g)	7	5	29
Fat (g)	3	1.1	63
Free+conjugated polyphenols	6.2	3.2	48
Hydroxybenzoic acids (mg)	1.2	1.1	8
Hydrocinnamic acids (mg)	4.6	2.1	54
Avenanthramides (mg)	0.4	0.1	75
Bound polyphenols	16.4	12.2	26
Hydroxybenzoic acids (mg)	1	0.4	60
Hydrocinnamic acids (mg)	15.4	11.8	23
Avenanthramides (mg)	ND	ND	-
Total polyphenols (mg)	22.6	15.4	32

ND, not detected

**Bioaccessibility - evaluated following in vitro upper gut digestion procedures, we have used the term bioavailability to indicate the percentage of compound remaining, thus available to the large gut microbiota. This term was first defined by Dall'Asta et al. in Nutrients 2016, 8(1), 42.*

2.3.2. Polyphenols and β-glucan content in oat bran and extract

The OAT4.5 contained 1.73 mg polyphenols (total amount of free + conjugated and bound), the POLY intervention were matched to OAT4.5 (Table 6). OAT1.5 and OAT4.5 delivered 56.67 mg and 170 mg β-glucan, respectively; the BG vessel to match OAT4.5 180 mg of 94% β-glucan extract was added (Table 6). The molecular weight of the β-glucan in the digested oat bran and the extract were similar (335.5 ($_{SD}$ 1.0) kDa and 387. ($_{SD}$ 1.0) kDa; $p > 0.05$).

***Table 6**. In vitro fermentation treatments*

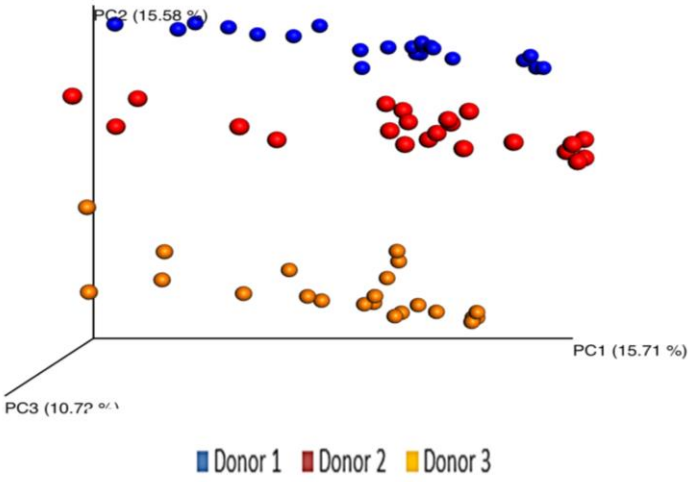
Treatment	β-glucan (mg)	Polyphenol (mg)
OAT1.5	56.67	0.58
OAT4.5	170	1.73
BG (94% of extract)	180	0.1
POLY	ND	1.73
PC	ND	ND
NC	ND	ND

Digested oat bran 1.5g (OAT1.5) and 4.5g (OAT4.5), β-glucan extract (BG), polyphenol mix (POLY), Synergy 1 positive control (PC) and negative control (NC), not detected (ND)

2.3.3. Changes in alpha and beta diversity

Independent of the donor, alpha diversity decreased with oats treatments over the 24h fermentation period (Chao1 2618 ($_{SD}$ 15) at 0h vs. 1577 ($_{SD}$ 22) at 24h for OAT1.5) but was constant over time in the negative control (Chao1 2630 ($_{SD}$ 87) at 0h vs. 2488 ($_{SD}$ 77) at 24h) and POLY (Chao1 2655 ($_{SD}$ 138) at 0h vs. 2241 ($_{SD}$ 320) at 24h). PCoA analyses based on unweighted UniFrac distance matrices of all samples showed a clear donor effect (Figure 5. A). However, the donor effect was no longer significant when using weighted UniFrac distance matrix analysis (ANOSIM test, Donor 1. vs. Donor 2 $p = 1$, Donor 1 vs Donor 3 $p = 1$, Donor 2. Vs. Donor 3 $p = 1$, Figure 5. B).

A



B

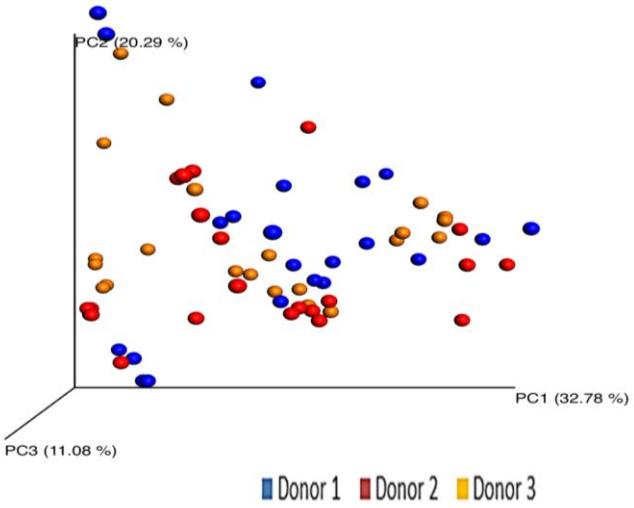


Figure 5. *Principal coordinates analysis (PCoA) plots of 16S rRNA gene profiles based on (A) unweighted and (B) weighted phylogenetic Unifrac distance matrices calculated from 10 rarefied OTU tables (9800 reads per samples) unweighted (A) showing clear clustering according to donors (ANOSIM test, p = 0.01). Weighted (B) quantitative information used to generate the bacterial relative abundance of donors showed no clear clustering (ANOSIM test, p >0.05) for a whole dataset (24h in vitro batch culture fermentation inoculated with 3 healthy donors faeces and administered with digested oat bran 1.5g (OAT1.5) and 4.5g (OAT4.5), β-glucan extract (BG), polyphenol mix (POLY), Synergy 1 (PC) and negative control (NC) as the substrates). Each colour represents a different donor*

Across all samples, seven bacterial phyla were classified and one phylum designated as unassigned. Phyla composition was similar for all donors ($p < 0.06$), although at baseline the third donor had lower Tenericutes ($p < 0.01$), while the second donor had higher Actinobacteria ($p < 0.05$). At baseline, the bacterial communities, were dominated by Firmicutes (57-67 %) and Bacteroidetes (32-41 %), while the remaining five phyla including Actinobacteria (0.8-1.4 %), Verrucomicrobia, Cyanobacteria, Tenericutes and Proteobacteria (0.4-0.9 %) constituted < 1.5 % of the community (Figure 6).

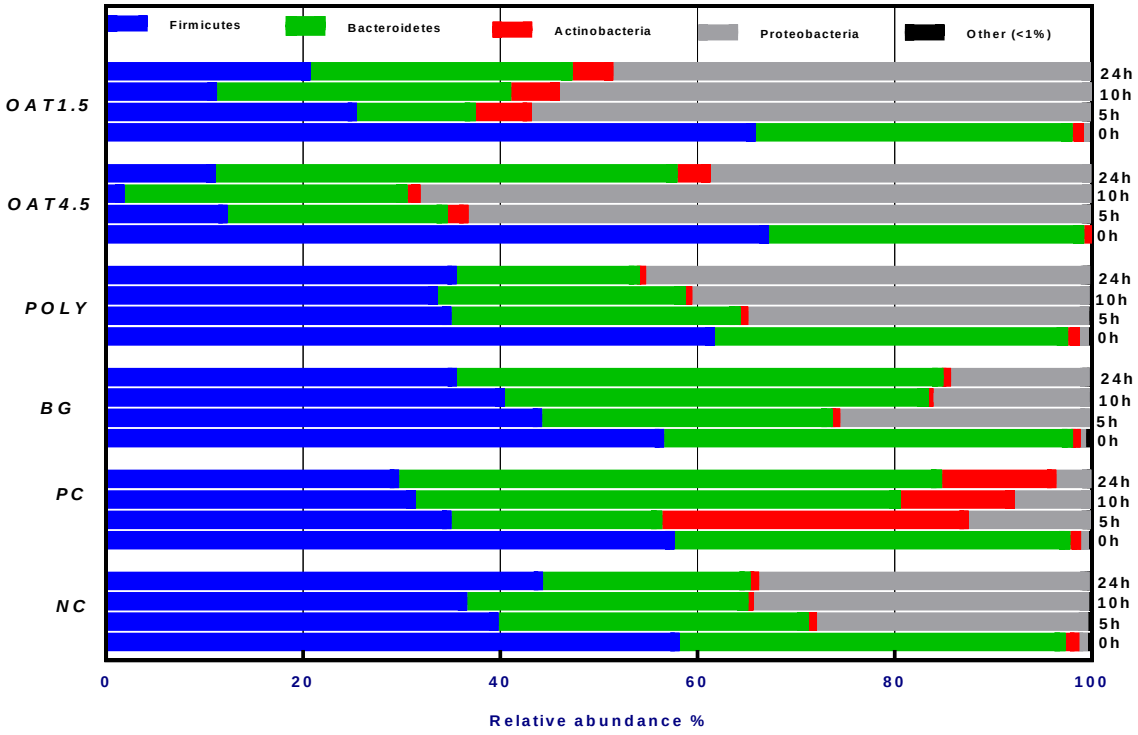


Figure 6. *Changes in bacterial phyla (relative abundances (%)) 0, 5, 10 and 24 h in vitro batch culture fermentation. This experiment was conducted three times, with a different faecal donor used for each run. The media was supplemented with digested oat bran 1.5g (OAT1.5) and 4.5g (OAT4.5), β-glucan extract (BG), polyphenol mix (POLY), Synergy 1 (PC) and negative control (NC). Samples were analysed at 0, 5, 10, 24 h. Values are mean (%).*

2.3.4. Compositional Shifts

Supplementation with OAT4.5 led to a lower proportional abundance of Firmicutes (p < 0.02) at 5 to 24 h and to an increased proportional abundance of Proteobacteria (p < 0.01) and Bacteroidetes at 24 h compared to NC. A similar trend was observed for OAT1.5 (p values) (Figure 6). The OAT1.5 treatment had a significant effect on the relative abundance of Actinobacteria at 10 h. The relative abundance of Actinobacteria differed between OAT1.5 and OAT4.5 at baseline (1.1 % and 0.8 %, respectively). Following 10 h fermentation, Actinobacteria were almost four times higher in OAT1.5 compared to OAT4.5 (4.9 % and 1.3 %, respectively), however, differences did not persist over 24 h (4.2 % vs. 3.4 %, respectively). The treatment of BG and POLY did not promote the growth of Actinobacteria, even though their dose was matched to the OAT4.5 treatment. The PC, Synergy1 resulted in the strongest bifidogenic effect and the relative abundance of Actinobacteria (baseline 1.1 % vs. 5 h at 31.1 %, 10 h at 11.5 %, p < 0.05) (Figure 6).

Focusing on changes over 24 h, the abundance of Bacteroidetes increased with BG (49.3 % vs. NC for 21 %), whereas Proteobacteria significantly decreased in comparison to the NC (14 % vs. 33.5 %). POLY treatment, the proportional abundance of Proteobacteria increased over the course of fermentation (45 % vs. NC for 33.5 %) and decreased for Bacteroidetes (p< 0.04) (18.6 % vs. 46.8 % for OAT4.5). The oats and BG treatments promoted the growth of Bacteroidetes (Figure 6).

At the lower taxonomic level and across all samples, 59 genera and 69 species were identified when setting the cut-off at a relative abundance above 0.1 % of the community (Table 7). After 5 h, the OAT4.5 treatment led to a significantly lower proportional abundance of *Ruminococcus* (0.1 %), and *Coprococcus* (0.1 %) genus and, at the same time, increased the proportion of unassigned *Enterobacteriaceae* (61.2 %) compared to NC (25.8 %) (Table 7).

Most notably, OAT1.5 increased the abundance of *Bifidobacterium unassigned* (1.5 % vs. 0.2 for NC, $p < 0.05$) at 10 h and *Bifidobacterium adolescentis* at 10 h (1.9 % vs. 0.3 % for NC, $p < 0.04$) and at 24 h (2 % vs. 0.4 for NC, $p < 0.02$) (Table 7), whereas OAT4.5 did not significantly impact the relative abundance of these species due to the high standard deviation among donors. Using EzTaxon database, we were able to further identify the species designation of several OTUs at 24 h with BG and POLY treatments. At 24 h, the BG treatment had higher abundances in *Bacteroides ovatus* (13.5 %)*, B. uniformis* (12.9 %) and *B. xylanisolvens* (3.7 %) species, while POLY led to the most abundant levels of the *Enterobacteriaceae* species, *Hafnia alvei* (21.7 %). However, these changes mediated by BG and POLY treatments were not significantly different ($p > 0.05$) compared to NC.

Table 7. *Significant changes (FDR p<0.05) in relative abundance (%) of bacterial taxa at 5 h, 10 h and 24 h of in vitro batch culture fermentation inoculated with faeces and administered with digested oat bran 1.5g (OAT1.5) and 4.5g (OAT4.5), β -glucan extract (BG), polyphenol mix (POLY), Synergy 1 (positive control PC) and negative control (NC) as substrates. This experiment was conducted three times, with a different faecal donor used for each run. Mean values with their standard errors*

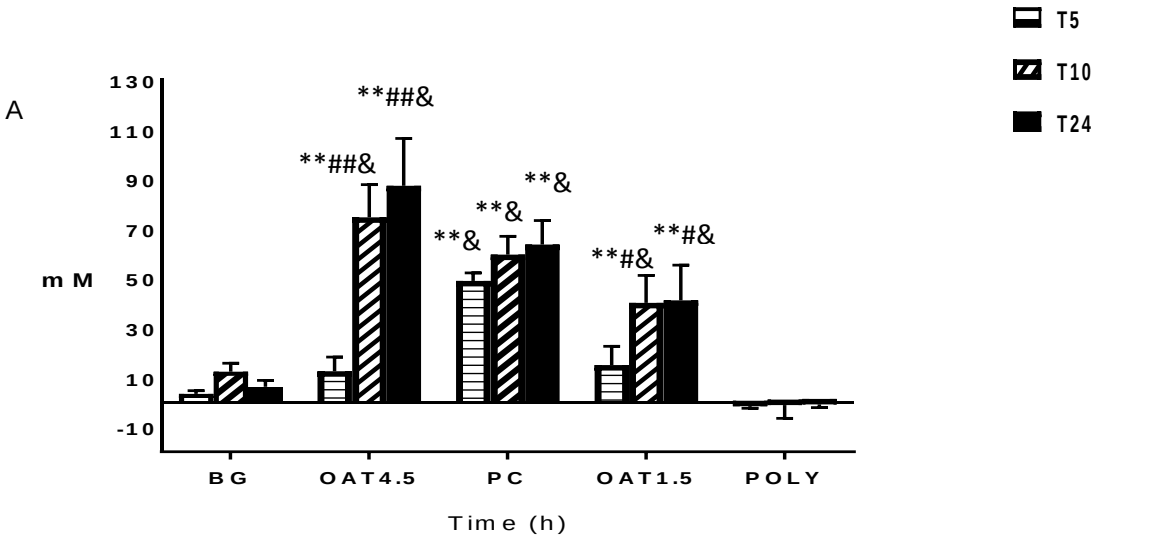
Phylum	Family	Genus	Species	Time	Treatment[1]						
					OAT 1.5	OAT 4.5	BG	POLY	PC	NC	*p value*
Actinobacteria	Bifidobacteriaceae	*Bifidobacterium*	*adolescentis*	5h	2.73±1.9	0.82±0.7	0.31±0.1	0.38±0.1	18.48±3.5*	0.39±0.2	0.001
Firmicutes	Ruminococcaceae	*Ruminococcus*	*Unassigned*	5h	0.2±0*	0.1±0.1	0.52±0.1	0.67±0.1	0.32±0.1	0.74±0.1	0.03
Firmicutes	Lachnospiraceae	*Coprococcus*	*Unassigned*	5h	0.2±0.1*	0.12±0*	0.48±0.2	0.44±0	0.76±0.3	0.54±0	0.02
Proteobacteria	Enterobacteriaceae	*unassigned*	*Unassigned*	5h	55.46±8.4*	61.2±9*	23±6	31.9±8.6	11.33*±3.7	25.9±11	0.05
Actinobacteria	Bifidobacteriaceae	*Bifidobacterium*	*Unassigned*	10h	1.17±10.1*	1.1±0.9	0.19±0.1	0.17±0.1	4.12±2.1*	0.27±0.1	0.03
Actinobacteria	Bifidobacteriaceae	*Bifidobacterium*	*adolescentis*	10h	1.93±0.5*	0.66±0.5	0.19±0.1	0.27±0.1	6.7±2.6*	0.26±0	0.02
Firmicutes	Ruminococcaceae	*unassigned*	*Unassigned*	10h	4.26±5	0.05±0*	8.96±2.8	8.68±1.7	9.76±10	8.85±0.8	0.03
Firmicutes	Lachnospiraceae	*unassigned*	*Unassigned*	10h	5.14±4	0.08±0*	10.1±2.4	8.14±5	5.11±1.2	10.1±0.2	0.03
Firmicutes	Lachnospiraceae	*Blautia*	*Unassigned*	10h	0.35±0.1*	0.02±0*	1.39±0.3	1.21±0.4	1.15±0.8	1.14±0.4	0.03
Proteobacteria	Enterobacteriaceae	*unassigned*	*Unassigned*	10h	50.2±12.2	67.2±14*	12.75±6*	36.1±9.2	6.47±3.7*	29.2±1.4	0.02
Actinobacteria	Bifidobacteriaceae	*Bifidobacterium*	*adolescentis*	24h	2.03±0.1*	1.91±1.8	0.31±0.1	0.31±0.2	6.87±3.7	0.41±0	0.05

[1] The difference in relative abundance of taxa between treatments within the same time points was assessed by ANOVA The p-value after correction for multiple tests (69taxa) with the FDR method

**Mean values were significant different (p<0.05) to NC at the same time point*

2.3.5. Production of SCFA

OAT4.5 led to a high production of total SCFA compared to NC at 10 h (98.2 ($_{SD}$ 22) mM vs 12.8 ($_{SD}$ 4) mM respectively) and 24 h (151.5 ($_{SD}$ 43) mM vs 28.1 ($_{SD}$ 5) mM). The concentration of butyric acid was significantly increased after 24 h fermentation of OAT1.5 ($p < 0.05$), and PC at 10 h ($p < 0.05$) and 24 h ($p < 0.01$) compared to NC (Figure 7). Acetic acid was the most abundant SCFA. Both oat treatments resulted in a significant increase of acetic acid at 10 h ($p < 0.01$), and 24 h ($p < 0.01$) compared with baseline and NC (Figure 7). At 24 h, OAT4.5, OAT1.5 and PC significantly increased the concentration of propionic acid compared to NC (48 ($_{SD}$ 24) mM, 16.7 ($_{SD}$ 3) mM, 21 ($_{SD}$ 9) mM and 4.5 ($_{SD}$ 0.3) mM, respectively $p < 0.01$, 0.05 and 0.05, respectively). Lactic and formic acids increased upon fermentation of OAT4.5 after 10 h compared to baseline, but did not reach significance due to the large variation among donors. BG led to a small production of acetic and butyric acids (not significant) whereas POLY did not induce SCFA production. Overall, there was a dose-response effect between the two oats doses in total SCFA at 10 h (98.2 ($_{SD}$ 22) mM vs 58 ($_{SD}$ 19) mM respectively, $p < 0.05$) and 24 h (151.5 ($_{SD}$ 43) mM vs 86.1 ($_{SD}$ 27) mM, $p < 0.05$).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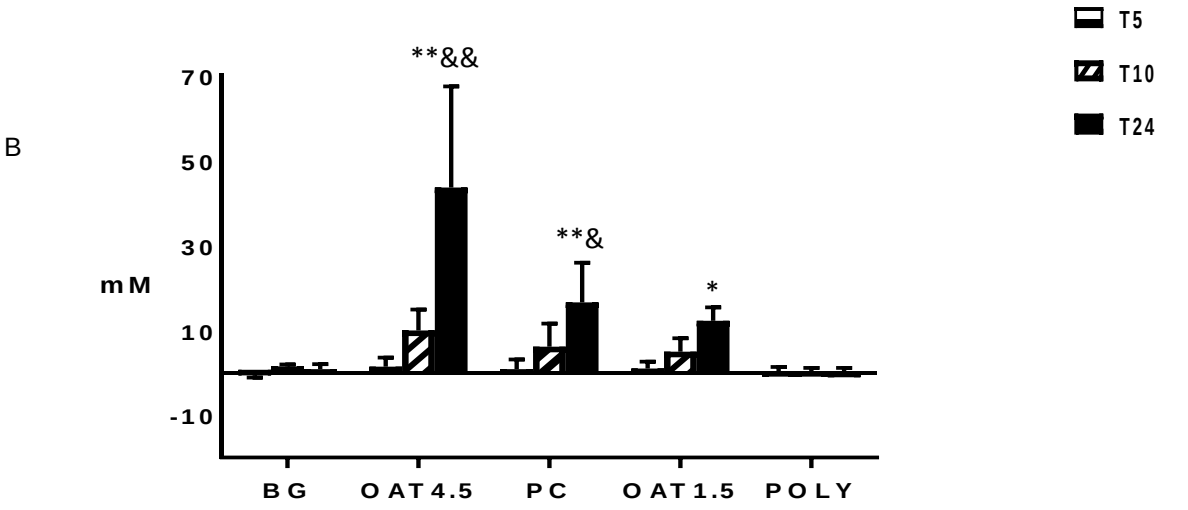
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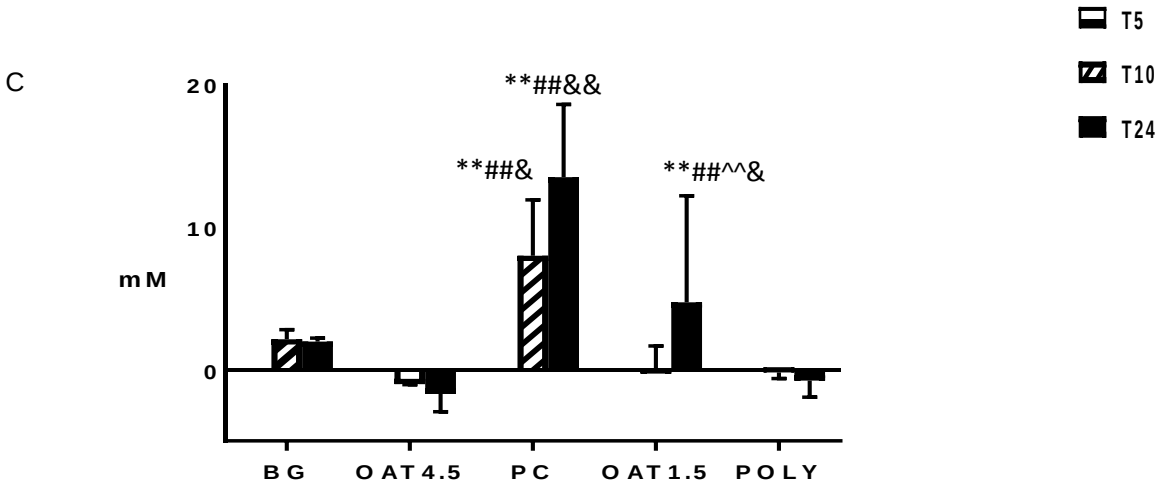
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POLY

*Values are reported after subtracted of negative control value. Significant from initial value *p < 0.05, **p< 0.01; Significant from 5h value #p < 0.05, ##p< 0.01, Significant from 10h value ^p < 0.05, ^^p< 0.01; Significant from negative control value at the same time point &p < 0.05, &&p< 0.01*

Figure 7. *Change in concentrations of acetic acid (A), propionic acid (B), butyric acid (C) from negative control (mM) throughout 24h in vitro batch culture fermentation. This experiment was conducted three times, with a different faecal donor used for each run. The media supplemented with digested oat bran 1.5g (OAT1.5) and 4.5g (OAT4.5), β-glucan extract (BG), polyphenol mix (POLY), Synergy 1 (positive control, PC) as the substrates. Samples were analysed at 0, 5, 10, 24 h*

2.4. Discussion

This study aimed to identify the impact of different doses of oats and its isolated bioactive compounds (i.e. β-glucan or polyphenols) on the faecal gut microbiota using in vitro systems. Digested oat bran decreased alpha diversity and had a bifidogenic effect but isolated β-glucan or polyphenol mix given at a matched dose did not induce a similar effect (Figure 6). This is likely to be because these compounds alone did not provide enough energy for bacterial growth. We have demonstrated that OAT1.5 significantly increased the proliferation of *Bifidobacterium adolescentis* (Table 7). An increase in *Bifidobacterium* (genus level) was reported by Connolly et al. [136] and Kedia et al. [132] after fermentation of 1 % and 5 % oat grains in similar *in vitro* studies. *B. adolescentis* has a number of benefits including prevention of the development of diabetes by stimulating insulin secretion [202]. Furthermore *B. adolescentis* has the ability to synthesise and secrete the neuroactive substance gamma-aminobutyric acid (GABA). GABA facilitates communication between bacteria and the human nervous system, enabling release of other neurotransmitters from specific epithelial intestinal cells [203, 204]. Our main findings suggest that oat bran could have beneficial effects on the host through increasing the relative abundance of *B. adolescentis.*

In accordance with the literature [205, 206], the compositional analysis of digested oat bran showed limited degradation of β-glucan in the stomach and the small intestine (Table 5). BG (180 mg – 387 kDa) did not introduce changes to Actinobacteria phyla, which is supported by previous studies on β-glucan [133, 134]. However, BG tended to promote the growth of beneficial *Bacteroides uniformis, Bacteroides ovatus* and *Bacteroides xylanisolvens* compared with NC (Table 7), which is in line with reports of Wang *et al*. [207]. Additionally, Hughes *et al.* [133] used a similar anaerobic batch culture system for fermentation of 0.5 g β-glucan and also showed an increase in a member of Bacteroidetes, specifically in the *Bacteroides-Prevotella* group following 24 h fermentation. However, the dose of β-glucan used by Hughes *et al*., was almost three times higher in concentration compared with what was found in 60 g oat bran (containing 0.17 g β-glucan), which would equate to consumption of 150 g oat bran.

While human digestive enzymes cannot degrade plant cell wall polysaccharides, gut xylanolytic bacteria (e.g. *B. xylanisolvens, B. uniformis* and *B. ovatus)* can, while producing SCFA with potential health-beneficial effects [208-210]. Certain strains of *B. uniformis* downregulate gene and protein expression of pro-inflammatory cytokines, notably iNOS and PPAR-γ, IFN-γ, resulting in reduced inflammatory status [211], suggesting that oat β-glucan could have beneficial effects on human health. More powered studies should be applied to confirm this effect.

In vitro upper gut digestion (mouth to small intestine) reduced polyphenols in the free - conjugated, bound fractions by 48 % and 26 %, respectively (Table 5). This is in agreement with findings by Dall`Asta *et* al. [212] on phenolic bioaccessibility in durum wheat aleurone fractions where caffeic and sinapic acids appeared as the most bioaccessible (83.3 % and 79.5 %) while total ferulic acid and *p*-coumaric acid were less bioaccessible (29.5 % and 40.7%) with *in vitro* digestion.

Moreover, several phenolic metabolites, including vanillic acid, 4 - and 3 - hydroxyhippuric acids, sulfate-conjugates of benzoic and ferulic acids are derived from the hepatic and microbial metabolism of oat brans [115]. Schar et al. [115] found relevant concentrations in urinary excretion of 30 different phenolics, and amounted to a total excretion of 33.7 ($_{SD}$ 7.3) μmol, suggesting that a high proportion of oat phenolics are bioavailable with absorption occurring both in the small intestine and then in the large intestine within 8 h of consumption.

In the current study, POLY did not change the abundance of the Actinobacteria phyla, which is similar to the research conducted by Gwiazdowska *et al.* [213], which showed that 20 μg/ml polyphenols had an effect on *Bifidobacterium* after 1 h incubation, but no effect at 24 h. The effects of polyphenols on microbial composition, may also be related to the fact that there is no carbohydrate energy available for this fermentation. Therefore, at least part of the change may also be due to utilisation of protein as an energy source. However, in the current study a POLY- induced a proportional increase in Enterobacteriaceae family and *Hafnia alvei* species was detected [214]. Several studies connected this group with polyphenol-degrading metabolism [215, 216]. While Wen Gu et al. [217] and Hunter et al. [218] observed that Enterobacter strains could transform ferulic acid to vanillin via the non-oxidative decarboxylation, Kuntz et al. [219] believed that the *H. alvei* significantly attenuated the expression of adhesion molecules and cytokine secretion (IL-8 and IL-6), resulting in reduced inflammation [219]. The higher relative abundance of Enterobacteriaceae family and *H. alvei* species in POLY and digested oat bran treatments might be associated with the metabolic activity of these microbial groups towards plant-derived polyphenols and saccharides [220].

Previous oat fermentation studies have not shown significant increases in the growth of Enterobacteriaceae family and *H. alvei* species [132], most likely because the fluorescence *in situ* hybridisation (FISH) method used did not target Enterobacteriaceae or allow analysis at the species level.

We have detected an increased presence of Proteobacteria phyla (Figure 6) in all 3 vessels including NC, which might be due to the artificial conditions of batch culture systems [221, 222]. Facultative anaerobic microbes are indeed less abundant in the human colon [223] but this batch culture model may not be able to achieve a strict anaerobic environment, causing an increase in Proteobacteria phyla [222].

Despite *in vitro* enzymatic digestion (mimicking the digestion in the upper intestine prior to colon fermentation), the available carbohydrate content of the oat bran remained high (62 %) (Table 5). Wholegrain oats are a source of starch (60 % of the total dry matter of the oat grain), consisting of 7 % rapidly digestible starch, 22 % slowly digestible and 25 % resistant starch [85] all of which contribute to the available carbohydrates. The findings of Englyst & Cummings [224] suggest that human digestive enzymes do not break down dietary resistant starch. Strikingly, 30 % resistant starch type 4 also led to species level alterations in a colon and increases in *B. adolescentis* [225]. A previous study [190] reported that 1 % (w/v) arabinoxylan, a dietary fibre found in wholegrain including oats, has a bifidogenic effect using a similar 24 h batch culture fermentation in vitro model, yet oats generally have about 3.8 – 13.2 % of arabinoxylan [95]. This, in combination with our findings, suggests that interactions among a wide range of dietary polysaccharides may explain the bifidogenic effect of oats [226].

The current results indicate that digested oat bran treatments increase SCFA production dose-dependently, with a higher dose inducing higher acetate and propionate production but lower butyrate. Lactic acid disappeared at 24 h, possibly because it can be further metabolised by bacteria to acetic and propionic acids [73]. Acetate and lactate are widely produced by different bacteria; however, bifidobacteria have a distinct pathway, transforming glucose to acetate and lactate [227].

SCFA can create acidic conditions in the human colon inhibiting the growth of pathogens and reducing harmful enzyme activities in the human intestine, while they also act as an energy source for gut epithelial cells [75, 228]. Furthermore, propionate derived from carbohydrate fermentation is a substrate for gluconeogenesis in humans, and also inhibits the utilisation of acetate for cholesterol synthesis in the colon and liver [229, 230]. Therefore oat bran may have a beneficial impact on human health through SCFA production.

In previous studies [154, 231] differences were observed between the activity of pure and of food matrix derived β-glucan on small intestine digestion and lipolysis. Purified β-glucan was observed to have less gel forming capacity and a lesser effect on lipolysis and cholesterol metabolism, than the matrix derived β-glucan. In addition, dietary fibre induced SCFA production has been shown to interact with phenolics [174], where butyric acid increased the ferulic acid absorption in the colon, potentially translating to higher concentrations of ferulic acid in systemic circulation *in vivo*. These findings indicate positive interactions of fibres and polyphenols within the food matrix on cholesterol metabolism and the bioavailability of phenolics, and also highlights the importance of considering the structure and physicochemical properties of foods, and not just the nutrient content.

In conclusion, our study has shown that oat bran as a complex food matrix beneficially increases the proportion of *B. adolescentis* and the amount of SCFA production. In contrast, a matched dose of the isolated bioactive compounds, β-glucan and other polyphenols did not show any effect on the proportional abundance of Actinobacteria. However, by regulating the Bacteroides genus and Enterobacteriaceae family, they may also contribute to further health benefits.

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The authors declare no conflict of interest

CHAPTER 3

Bioconversion of oat phenolic acids and avenanthramides following i*n vitro* digestion: are fibre bound phenolic acids bioavailable?

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Abstract

Scope Oat bran may represent a valuable dietary source of phenolic acids, such as ferulic acid and avananthramides. However, their bioaccessibility, and thus potential to influence human physiology, may be limited by their binding to fibre in the grain. It has been suggested that the microbiota is capable of releasing such compounds from this fibre-bound state, and the aim of this study was to provide further evidence of their release and their subsequent fate.

Methods A simulated *in vitro* upper gut digestion was utilized to pre-digest oat bran and HPLC was used to assess resultant phenolic acids and avenanthramides. Upper gut digested oat bran (DOB) was added to an *in vitro* colonic batch culture model of the human large intestine (3 % w/v, 4.5 g; 1.7 mg total phenolics). 1.7 mg of pure oat phenolic acids was run to provide a parallel digestion of oat-bound phenolics over the 24 h. The release of bound phenolic acids during bacterial fermentation and their subsequent metabolites was assessed using UPLC-MS/MS and targeted metabolomics.

Results Following upper GI tract digestion, the DOB contained 0.3 mg of free and conjugated phenolic acids (21%) and 1.4 mg of bound phenolics (79%). Levels of free and conjugated phenolics, including avananthramides and *p*-coumaric acid, in DOB were reduced in concentration by 88 % and by 69%, respectively, whilst the bound phenolic fraction persisted (pre-digestion 16.4 mg vs. post-digestion 12.1 mg). In the bacterial fermentation the bound phenolics remained intact up to 4h, which was followed by a significant increase of free phenolic metabolites only at 24 h (4.9 ($_{SD}$ 0.1) μM at 0.5 h to 39.8 ($_{SD}$ 9.7) μM at 24 h, post-hoc P-value < 0.01). In contrast, exposure to pure phenolic acids mixture in fermentation model resulted in high levels of microbial metabolites at 0.5 h (51 ($_{SD}$ 0.7) μM) and a lower level at 24 h (35.7 ($_{SD}$ 15) μM). The major phenolic metabolites present at 24 h were similar for both treatments, including 4-hydroxyphenylacetic dihydoferulic acid 4-hydroxybenzoic acid and protocatcheuic acid

Conclusion: The data presented here suggest that bound phenolic acids act as a reservoir of phenolic acid, which are released over a 24 h period. Overall, oat bran may represent a sustainable and relatively inexpensive route to provide nutritionally valuable phenolic components to consumers.

3.1. Introduction

Epidemiological studies indicate that diets rich in whole grain products contribute to a reduced risk of developing chronic diseases, including cardiovascular disease, cancer and diabetes [2, 10, 232, 233]. Whole grain oats (*Avena Sativa*) are considered to be a relatively high source of proteins, minerals, vitamins, soluble β-glucan fiber and phenolic compounds [85]. Oat phytochemicals comprise a diverse group of phenolic acids such as hydroxycinnamic acids, benzoic acid derivatives and avenanthramides [95, 234]. They are found in three different forms within the oat food matrix: as soluble free acids; as soluble conjugates; and as insoluble bound acids esterified to high molecular weight components including cell wall polysaccharides, arabinoxylan [96]. The main hydroxycinnamic acids presents in oats include, ferulic acid, caffeic, *p*-coumaric, *o*-coumaric and sinapic acids. Hydroxybenzoic acid derivatives are protocatechuic, syringic, vanillic, *p*-hydroxybenzoic and gallic acids [98, 100]. Avenanthramides total contents range from 42-91 μg/g [95, 192]. Analytical data indicate that, on average, 19% of these phenolic acids (range: 50-110 μg/g) are present in the soluble state, 34% as conjugates (range: 111-314 μg/g) and 47 % bound (range: 131- 640 μg/g) [95].

The benefits traditionally attributed to the intake of oats have been suggested to reside mainly in their β-glucan content. Nonetheless, these effects may also partially be mediated by (poly)phenols [235], which benefits are likely to be dependent on their bioavailability, including their liberation from the food matrix [184]. To assess the release of bioactive components from the food matrix, including from the fibre-bound state, *in vitro* digestion procedures, generally simulating gastric, small and large intestinal digestion, are used [236]. Phenolic compounds can be delivered from the food matrix in the gastrointestinal tract by enzymes and pH conditions. The released free phenolics are absorbed in the small intestine, followed by conjugation with other compounds, leading to their introduction in the blood circulation system [237].

However, the fibre-phenolic compounds are not bioaccessible, and pass undissolved and unaltered through the upper intestine [128, 238]. Nonetheless, the bound-phenolic compounds, including ferulic acid and *p* – coumaric acid, may be substrates for bacterial polysaccharide hydrolyses and esterase activity, resulting in the slow and continuous release of phenolics in the colon [21].

Though many insights have been gained regarding the metabolism of (poly)phenols [239-243], the fate of fibre-bound oat phenolic acids in the lower gut remains unclear. The primary aim of this *in vitro* study was to determine the bioaccessibility of phenolic acids from whole grain oats and their degradation by the gut microbiota.

3.2. Materials and Method

Contributions

The authors' contributions were as follows: AK, MYS designed the study; GS analysed the HPLC results, AK conducted the *in vitro* fermentation experiments; GC run the samples on UPLC-MS/MS, AK conducted the statistical analyses. AK, MYS, GW, GK, GS and JS interpreted the data and AK, GC, GW and JS drafted the manuscript.

3.2.1. Chemicals and reagents

The oat macronutrient composition was measured by Campden BRI laboratories, UK, whereas the detailed phenolic acids content was analysed in our laboratory in the University of Reading based on Schar *et al.* [115]. All chemicals and reagents were purchased from Sigma-Aldrich Chemical Co Ltd. (Pool, Dorset, UK) or Fisher (Loughborough, Leics, UK) unless stated otherwise. The anaerobic jar (AnaerojarTM 2,5L) and gas-generating kit (AnaeroGen TM) were purchased from Oxoid Ltd (UK), the dialysis tube from Spectrum (VWR International). Media and instruments were autoclaved at 121°C for 15 min. The oat bran was obtained from White's (UK).

3.2.2. In vitro upper gut digestion of oat bran (mouth to small intestine)

The method employed was adapted from Mills *et al.* [191] and describe in previous publication [244]. In brief: oat bran was digested *in vitro* in three phases: the oral phase, the gastric phase and the small intestinal phase. 60 g of oat bran was mixed with 150 ml of sterile and distilled water and homogenized. In the oral phase, 20 mg of α-amylase was dissolved in 6.25 ml $CaCl_2$ (1 mM, pH 7.0) and added to the solution, then incubated at 37°C for 30 minutes on a shaker set at 120 *x g*. After incubation, the pH was adjusted to 2.0 with 6 M HCl and the gastric phase introduced by adding 2.7 g pepsin in 25 ml HCl (0.1 M) and further incubated for 2 hours under the same conditions. In the small intestinal phase, 560 mg pancreatin and 3.5 g bile were mixed with 125 ml $NaHCO_3$ (0.5 M) and dispensed into the mix. The pH was adjusted to 7.0 then incubated for 3 hours at 37 °C with shaking. The sample solution was transferred to a seamless semi-permeable molecular weight cut-off 100-500 Dalton regenerated cellulose dialysis tubing and dialysed against NaCl (0.01 M at 5°C). After 15 hours, the dialysis fluid was changed and the process continued for an additional 2 hours. The digested oat bran mix was collected and transferred into several 250 ml clear plastic containers, frozen to -80 °C and freeze-dried for 5 days to remove all fluid content.

Following freeze drying the 60 g average serving portion of oat bran was reduced to 40 g, a 20 % loss through digestion; as such we take account in the following calculation of bioaccessibility:

$$\frac{\text{Phenolic amount before digestion (60 g)} - \text{Amount of residue after digestion 40 g}}{\text{Amount of phenolic before digestion (60g).}} \times 100$$

3.2.3. Extraction and analyses of phenolic acids resulting from the small intestinal model

Soluble and bound phenolic fractions were extracted from oat bran using the method of Schar *et al.* [115] and describe in previous publication [244]. In brief: after hexane defatting, were extracted with acidified ethanol and subsequently the supernatant 4 h (free and conjugates fraction) and residue 17 h (bound) an alkali based hydrolysis. The phenolic compounds were identified and quantified in oat extracts using a high-performance liquid chromatograph (HPLC) Agilent 1100 series (Agilent Technologies Ltd) equipped with a quaternary pump, autosampler, column thermostat, sample thermostat and photo diode array detector. Compound separation was achieved by a Kinetex biphenyl column (100 A 250x4.6 mm length, 5 uM particle size; Phenomenex) and using a gradient elution. Mobile phase A consisted of 0.1 % (v:v) formic acid in HPLC water (A), while mobile phase B was 0.1 %(v:v) formic acid in methanol. The flow rate of the mobile phase was 1.0 ml/min and the sample injection volume was 20 µl [192]. The absorbance was recorded at 254, 280 and 320 nm and quantification was based on 12 point linear calibration curves (mean R^2 > 0.99) and as a ratio to the internal standard (i.e. 3,5-dichloro-4-hydroxybenzoic acid) to account for losses during extraction ($R^2 \geq 0.99$). The total concentration was given in µg per gram oat bran (µg/g).

3.2.4. pH controlled faecal batch culture fermentation

The method were described in previous publication [244]. In brief: The anaerobic, stirred, pH and temperature controlled faecal batch culture fermentation vessels (300 ml volume: one vessel per treatment) were sterilised and filled with 135 ml of sterile basal medium (g/l: 2 g peptone, 2 g yeast extract, 0.1 g NaCl, 0.04 g K_2HPO_4, 0.04 g KH_2PO_4, 0.01 g MgSO4·7H_2O, 0.01 g CaCl2·6H_2O, 2 g $NaHCO_3$, 2 ml Tween 80, 0.05 g Hemin (dissolved in a few drops of 1 M NaOH), 10 µl vitamin K, 0.5 g L-cysteine HCl, 0.5 g bile salts and 4ml of resazurin solution (0.02 %). Before addition to the vessel the medium was adjusted to pH 7.0 and autoclaved. The sterile medium was gassed overnight with O_2-free N_2 (15 ml/min). The pH was held in the range of 6.7 - 6.9 to mimic the distal region of the human large intestine, and controlled by addition of 0.5 M NaOH or 0.5 M HCl, the temperature was kept at 37 °C. Faecal samples were collected from three healthy female donors, BMI between 19 and 23 Kg/m^2, 25 to 40 years of age. Donors had no history of bowel disorders and had not received antibiotic treatment for at least 6 months before the study or pre- or probiotic supplements one month before the study. The compliance with the ethics procedures required at University of Reading, all donors were informed of the study aims and procedures and provided their verbal consent for stool samples to be used for the experiments. Samples were collected in anaerobic jars and used within one hour of collection, diluted 1:10 (w/v) with anaerobic phosphate buffer (0.1 M, pH 7.4) and homogenised in a 158 stomacher (Serward, Worthing, UK) for 2 min at 460 paddle-beats per min. The batch culture systems were inoculated with 15 ml faecal slurry from an individual sample. The vessels were treated with the following substrate: 3 % w/v, 4.5 g digested oat (DOB); 0.01 % w/v, 1.7 mg phenolic acids mix (pure phenolic acids), same profile as 4.5 g digested oats including ferulic acid , 4-hydroxybenzoic acid, vanillic acid, 4-hydroxy benzaldehyde, syringic acid, p coumaric acid, sinapic acid, avenanthramides A and B (Table 8).

The 3% w/v oat was chosen because it relates to the concentration reached by an average person consuming 60 g oat bran (i.e. assuming 30 g oat bran reaches the colon and colonic total volume is 1 L) [194]. Based on this calculation the pure phenolic acid mix were matched to the dose present in 3 % w/v oat bran. Samples were collected at 0.5, 4, 6, 8, 10, 24 h, 1 ml aliquots were centrifuged at 13,000 ×*g* for 10 min, and the supernatants were stored separately at -20°C until analysis.

Table 8*. In vitro* fermentation treatments

	DOB	**Pure phenolic acids mix**
	µg/4.5g	
Free+Conjugated		
4-hydroxybenzoic acid	**64.4**	**76.1**
vanillic acid	**27.1**	**53.7**
4-hydroxy benzaldehyde	**9.4**	**17.1**
syringic acid	**23.4**	**23.8**
p coumaric acid	**64**	**294.4**
ferulic acid	**99**	**1136.4**
sinapic acid	**68.9**	**113.9**
avenanthramides A	**1.3**	**1.3**
avenanthramides B	**4.5**	**4.5**
Total Free+Conjugated	**362**	**1732.4**
Bound		
4-hydroxybenzoic acid	**11.7**	**-**
vanillic acid	**26.6**	**-**
4-hydroxy benzaldehyde	**7.7**	**-**
syringic acid	**0.4**	**-**
p coumaric acid	**230.4**	**-**
Vanillin	**11.3**	**-**
ferulic acid	**1037.3**	**-**
sinapic acid	**45**	**-**
Total Bound	**1370.4**	**-**
Total Phenolic acids	**1732.4**	**1732.4**

Digested oat bran (DOB)

3.2.5. Solid phase extraction

Phenolic acids were extracted from batch culture supernatant using a validated method [245] with minor modifications. Briefly, 1 ml of supernatant was spiked with an internal standard (i.e. 3,5-dichloro-4-hydroxybenzoic acid) and subsequently extracted using solid phase extraction cartridges (Strata-X columns 500 mg / 6 ml; Phenomenex). These were washed with 12 ml of 0.1/99.9 v/v hydrochloric acid/water, dried for 30 min under vacuum, soaked in 0.1/99.9 v/v hydrochloric acid/methanol for 10 min and eluted into glass vials with 7 ml 0.1/99.9 v/v hydrochloric acid/methanol. Samples were evaporated to complete dryness under speedvac at room temperature. The dried samples were resuspended in 250 µl of mobile phase (0.1/5/94.9, v/v/v, formic acid/methanol/water) by 30 s vortexing, 15 min ultrasound sonicating and 1 h shaking. Samples were stored at - 80 °C until analysis. For phenolic acids the method has a mean and SD extraction efficiency of 88.3 ($_{SD}$ 17.8) %, the extractions were performed in triplicate for each oat sample.

3.2.6. UPLC-MS/MS analysis

The UPLC-electrospray ionisation-MS/MS system consisted of an Aquity UPLC H - class (Waters) coupled to a Xevo TQ-S micro electrospray ionisation mass spectrometer (Waters) operated using MassLynx software (V4.1, Waters Inc, USA). Compound separation was achieved using an Aquity UPLC HSS T3 1.8 µm column (2.1 x 100 mm) attached to a Van guard pre-column of the same material and pore size, maintained at 45°C with a flow of 0.65 ml/min and a sample injection volume of 2 µl. The mobile phase consisted of 0.1/99.9 v/v formic acid/water (A) and 0.1/99.9 v/v formic acid/acetonitrile (B); and a mobile phase gradient consisting of: 1 % B at 0 min, 1% B at 1 min, 30 % B at 10 min, 95 % B at 12 min, 95 % B at 13 min, 99 % B at 13.10 min, 99 % B at 16 min. Quantification was established using the most intense sMRM transition and 11-14 point calibration curves of analytical standards.

The limit of detection was established for each compound as the concentration of a peak with a signal to noise ratio of 3. Blank and quality control samples were run every 10 injections and the quality control indicated a between-run coefficient of variation of less than 10 %. Sample acidification using 5 % formic acid did not significantly affect phenolic compound peak areas (established in n = 3 samples; data not shown) and therefore non-acidified supernatant was used for the complete analysis. Results were expressed as milligrams per 1 gram of sample on a dry weight basis (mg / g DW).

3.2.7. Statistical Analysis

Data are presented as means and SEMs. Normality test and two – way analyses of variance (ANOVA): post hoc multiple comparisons was used to identify differences between the treatment, p-values of < 0.05 were considered statistically significant. Statistical analysis was performed by using Graphpad Prism statistics software package version 7.

3.3. Results

3.3.1. Small intestinal digestion

Simulated *in vitro* upper gut digestion resulted in an overall reduction in both oat bran mass and free phenolics. The initial 60 g of oat bran (OB), containing 16.4 mg bound and 6.2 mg free and conjugated phenolics was reduced to 39.5 g in digested oat bran (DOB), containing 12.2 mg of bound (74% of original) and 3.2 mg free and conjugated phenolics (52% of original material) (Table 9). Regarding free phenolics, the highest losses were avananthramides (88 % reduction, 114.6 µg to 13.6 µg) and *p*-coumaric acid (69 %, 1877.4 µg to 569.2 µg). In contrast, the bound phenolic acids persisted in the upper gut model, with the exception of syrengic acid (98 %, 161.8 to 2.8 µg) and synapic acid (70 %, initial 1357.2 µg to 401.2 µg) levels (Table 9).

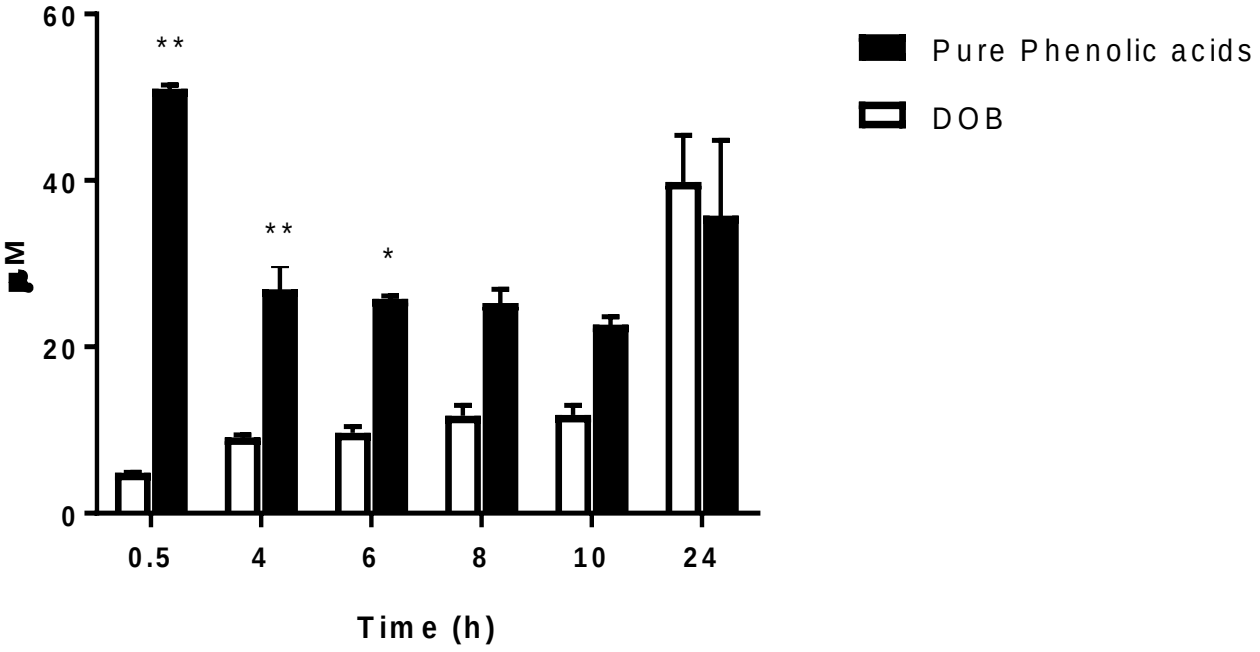
Table 9. *Phenolic composition of oat bran and digested oat bran and their bioaccessibility. Data are expressed both as content of oat bran (OB) and digested oat bran (DOB) of µg/g mean and SEM of 3 replicate and percentage contribution of individual phenolic acids to total content, and content of µg/60 OB and µg/40g DOB, and as percentage of their bioaccessibility*

	OB			DOB			OB	DOB	Bio accessibility
	µg/g	SEM	%	µg/g	SEM	%	µg/60g	µg/40g	%
Free+Conjugated									
4-hydroxybenzoic acid	6.3	0.2	6	14.3	0.1	18	377.4	571.2	- 51
vanillic acid	7.0	0.7	7	6.0	0.5	7	420.0	240.0	43
4-hydroxy benzaldehyde	1.9	0.1	2	2.1	0.1	3	115.2	82.0	29
syringic acid	4.4	0.6	4	5.2	0.2	6	266.4	206.8	22
p coumaric acid	31.3	2.9	30	14.2	2.3	18	1877.4	569.2	70
Vanillin	ND	-	-	ND	-	-	ND	ND	-
ferulic acid	32.9	3.8	32	22.0	3.4	27.9	1973.4	881.6	55
sinapic acid	12.7	0.1	12	15.3	1.0	19	761.4	612.8	19
avenanthramides A	1.9	1.7	2	0.3	0.1	0.1	114.6	13.6	88
avenanthramides B	5.5	2.4	5	1.0	0.1	1	332.4	41.6	87
TIP* Free+Conjugated	104	12		80.5	7.7		6238.2	3218.8	48
Bound									
4-hydroxybenzoic acid	4.4	0.7	2	2.6	0.1	1	266.4	105.2	60
vanillic acid	7.8	0.9	3	5.9	0.5	2	469.2	236.8	49
4-hydroxy benzaldehyde	1.8	0.2	1	1.7	0.1	1	107.4	66.8	38
syringic acid	2.7	1.5	1	0.1	0.6	0.1	161.4	2.8	98
p coumaric acid	31.6	4.6	12	51.2	3.8	17	1898.4	2050.0	- 8
Vanillin	2.2	0.3	1	2.5	0.2	1	129.6	101.2	22
ferulic acid	200.3	12.7	72	230.5	12.7	74.9	12016.8	9221.6	23
sinapic acid	22.6	2.2	8	10.0	0.5	3	1357.2	401.2	70
avenanthramides A	ND	-	-	-	-	-	ND	ND	-
avenanthramides B	ND	-	-	-	-	-	ND	ND	-
TIP* Bound	273.4	23.1		304.6	18.6		16406.4	12185.6	26
Total Free+Conjugated+Bound									
4-hydroxybenzoic acid	10.7	0.7	3	16.9	0.1	4	643.8	676.4	- 5
vanillic acid	14.8	1.5	4	11.9	0.2	3	889.2	476.8	46
4-hydroxy benzaldehyde	3.7	0.2	1	3.7	0.1	1	222.6	148.8	33
syringic acid	7.1	1.8	2	5.2	0.3	1	427.8	209.6	51
p coumaric acid	62.9	2.4	17	65.5	1.7	17	3775.8	2619.2	31
Vanillin	2.2	0.3	1	2.5	0.2	1	129.6	101.2	22
ferulic acid	233.2	15.3	62	252.6	21.0	66	13990.2	10103.2	28
sinapic acid	35.3	2.3	9	25.3	1.5	7	2118.6	1014.0	52
avenanthramides A	1.9	1.7	1	0.3	0.1	0.1	114.6	13.6	88
avenanthramides B	5.5	2.4	1	1.0	0.1	0.1	332.4	41.6	87
TIP*	377.4			385.1			22644.6	15314.0	32

**TIP -total individual phenolic acids, sum of the individual phenolic acids*

3.3.2. Human gut microbiome digestion

A total of 22 phenolic acids were quantified in DOB Table 10. Microbial fermentation of DOB indicted that bound phenolics remain in that state for up to 4 h, after which a significant increase in total free phenolic metabolites were detected (4.9 ($_{SD}$ 0.1) μM, 0.5 h; 9.13 ($_{SD}$ 0.5) μM, 4 h; 11.4 ($_{SD}$ 2) μM, 10 h; 39.8 ($_{SD}$ 9.7) μM, 24 h; post-hoc p-value < 0.01) (Figure 8). In contrast, when pure free phenolic acids were added to the model, the amounts of phenolic metabolites more rapidly (51 ($_{SD}$ 1) μM, 0.5 h; 26.9 ($_{SD}$ 4.9) μM, 4 h; 35.7 ($_{SD}$ 15.6) μM, 24h (Figure 8).



*Significant from DOB value *p < 0.05, **p < 0.01 between the two intervention*

Figure 8. *Changes in level of total phenolic acids (μM) throughout 24 h in vitro batch culture fermentation inoculated with 3 healthy donors faeces in media supplemented with either digested oat bran (DOB) or pure phenolic acids. Samples were analysed at 0.5, 4, 6, 8, 10, 24 h*

When pure free phenolic acid was added to the model, notably ferulic acid was almost completely metabolised (97%) by 4 h (post-hoc P-value < 0.001) (Table 10), with dihydoferulic acid the predominant phenolic detected from 4 to 24 h (Figure 9B).

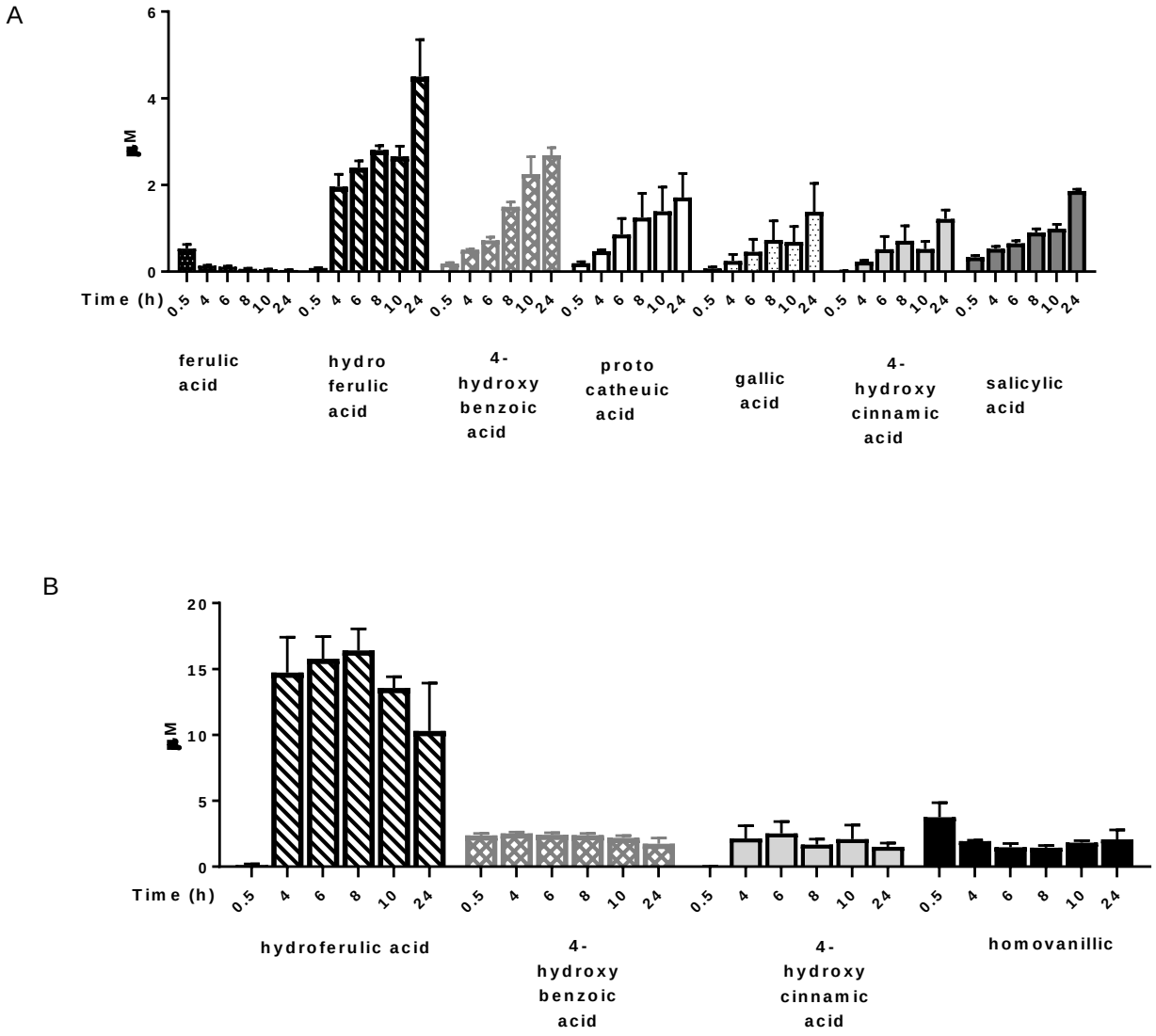


Figure 9. *Change in levels (mean and SEM) of main phenolic acids metabolites throughout 24 h in vitro batch culture fermentation inoculated with 3 healthy donors faeces in media supplemented with either digested oat bran - DOB (A) or pure phenolic acids mix (B). Samples were analysed at 0.5, 4, 6, 8, 10, 24 h*

When individual phenolic metabolites were monitored there was no 4-phenylacetic acid detected until after 10 h fermentation, then at 24 hours there was 22.7 μM within the DOB vessel, and 17.6 μM within pure phenolic acid vessel (Figure 10).

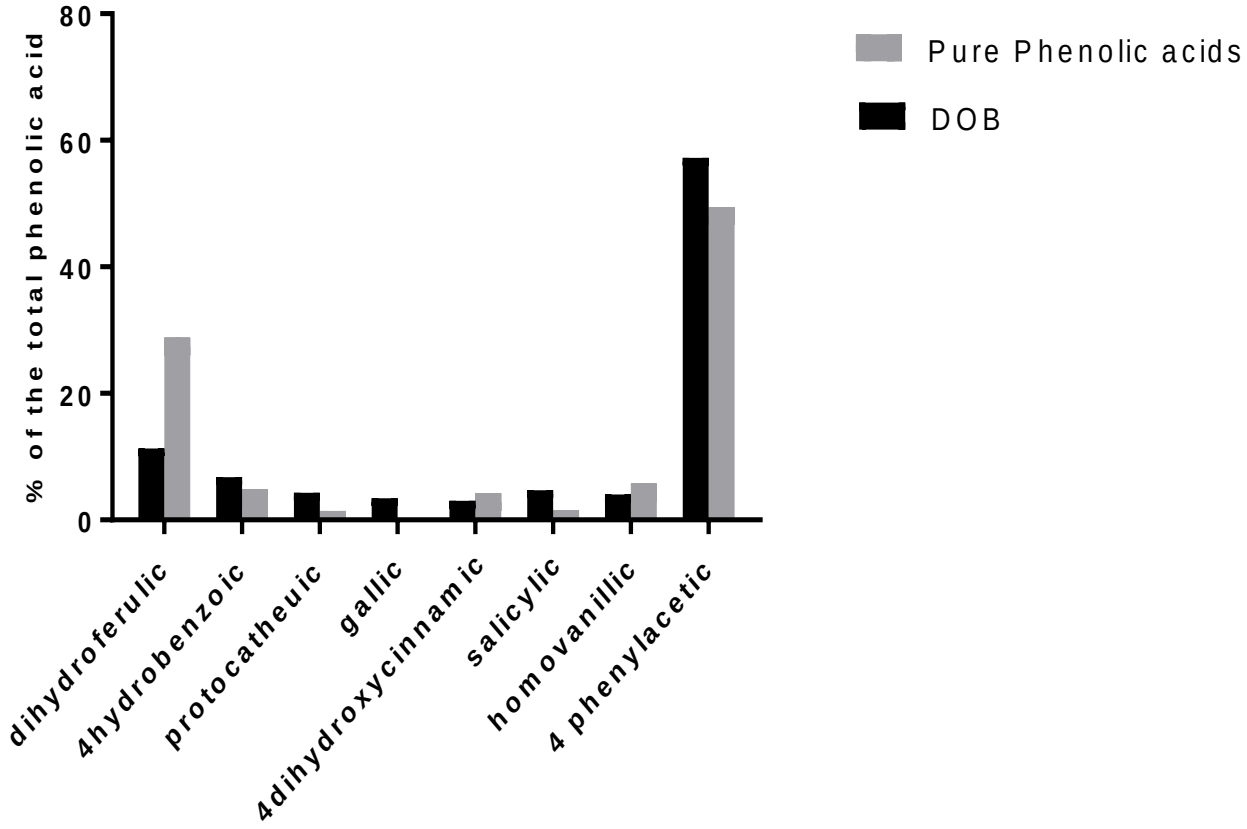


Figure 10. The main (mean) phenolic acids metabolites percentage of the total phenolic acids at 24 h in vitro batch culture fermentation supplemented with either digested oat bran (DOB) or pure phenolic acids

Table 10. *Phenolic acids composition of digested oat bran (DOB) and pure phenolic acids. Data are expressed in µM mean and SEM of 3 replicates at 0.5, 4, 6, 8, 10 and 24 h*

		0.5 h		4 h		6 h		8 h		10 h		24 h	
Compound	**Treatment**	**Mean**	**SEM ±**	**Mean**	**SEM ±**	**Mean**	**SEM ±**	**Mean**	**SEM ±**	**Mean**	**SEM ±**	**Mean**	**SEM ±**
ferulic acid	DOB	0.53	0.22	0.14	0.05	0.11	0.10	0.06	0.12	0.05	0.02	0.04	0.07
	Pure phenolic acids	28.68	0.23	0.72	0.76	0.08	0.12	0.11	0.19	0.12	0.10	0.08	0.06
isoferulic acid	DOB	0.08	0.08	0.03	0.07	0.05	0.13	0.04	0.47	0.05	0.18	0.05	0.58
	Pure phenolic acids	1.91	0.53	0.07	0.19	0.01	0.03	0.01	0.04	0.01	0.00	0.02	0.01
dihydroferulic acid	DOB	0.08	0.06	1.96	0.35	2.40	0.17	2.80	0.11	2.66	0.27	4.50	0.88
	Pure phenolic acids	0.13	0.94	14.73	1.49	15.77	0.67	16.41	0.70	13.56	0.43	10.31	1.72
p-coumaric acid	DOB	0.08	0.03	0.12	0.06	0.01	0.01	0.00	0.04	0.00	0.00	0.01	0.03
	Pure phenolic acids	7.21	0.22	0.21	0.45	0.02	0.14	0.01	0.01	0.02	0.07	0.01	0.09
4-hydroxybenzoic acid	DOB	0.19	0.08	0.50	0.04	0.72	0.14	1.50	0.15	2.25	0.49	2.68	0.18
	Pure phenolic acids	2.37	0.17	2.53	0.10	2.42	0.18	2.40	0.14	2.19	0.19	1.73	0.49
protocatechuic acid	DOB	0.19	0.11	0.47	0.08	0.86	0.70	1.25	0.83	1.39	0.73	1.71	0.61
	Pure phenolic acids	0.18	0.18	0.24	0.11	0.25	0.07	0.28	0.09	0.33	0.10	0.51	0.73
gallic acid	DOB	0.07	0.16	0.25	0.65	0.46	0.88	0.73	0.99	0.69	0.70	1.38	0.82
	Pure phenolic acids	0.07	0.19	0.18	0.31	0.16	0.31	0.19	0.40	0.09	0.17	0.06	0.17
2.5 dihydroxybenzoic acid	DOB	0.03	0.31	0.06	0.24	0.10	0.18	0.15	0.14	0.15	0.13	0.28	0.09
	Pure phenolic acids	0.03	0.11	0.03	0.04	0.03	0.07	0.04	0.04	0.05	0.06	0.07	0.13
4 dihydroxybenzoic acid	DOB	0.11	0.08	0.21	0.35	0.23	0.34	0.27	0.13	0.35	0.27	0.45	0.29
	Pure phenolic acids	0.05	0.04	0.07	0.10	0.06	0.06	0.08	0.11	0.10	0.10	0.11	0.12

caffeic acid	DOB	0.21	0.13	0.45	0.87	0.09	0.37	0.06	0.13	0.06	0.13	0.04	0.02
	Pure phenolic acids	1.92	1.86	0.41	0.50	0.04	0.09	0.04	0.07	0.04	0.07	0.04	0.07
sinapic acid	DOB	0.12	0.04	0.43	0.62	0.46	1.08	0.43	1.59	0.26	0.88	0.32	2.75
	Pure phenolic acids	1.02	0.41	0.64	0.55	0.33	0.40	0.28	0.35	0.17	0.33	0.01	0.11
4-hydroxyaldehyde	DOB	0.43	0.22	0.55	0.01	0.32	1.02	0.12	0.14	0.09	0.19	0.05	0.05
	Pure phenolic acids	1.09	0.16	0.61	0.12	0.32	0.06	0.20	0.05	0.09	0.08	0.04	0.06
4-dihydroxycinnamic acid	DOB	0.01	0.06	0.23	0.10	0.52	1.50	0.71	1.60	0.52	0.74	1.22	0.40
	Pure phenolic acids	0.02	0.40	2.14	3.22	2.52	1.72	1.67	0.80	2.08	1.97	1.51	0.38
vanillic acid	DOB	0.10	0.08	0.31	0.13	0.22	0.19	0.24	0.61	0.19	0.39	0.22	1.47
	Pure phenolic acids	0.78	0.15	0.84	0.13	0.91	0.31	0.94	0.32	0.80	0.15	0.68	0.50
hippuric acid	DOB	0.37	0.02	0.32	0.07	0.30	0.09	0.25	0.06	0.23	0.03	0.12	0.24
	Pure phenolic acids	0.22	0.12	0.11	0.15	0.07	0.17	0.05	0.16	0.04	0.12	0.08	0.78
AVA A	DOB	0.02	0.07	0.03	0.01	0.02	0.06	0.02	0.05	0.01	0.06	0.00	0.04
	Pure phenolic acids	0.01	0.06	0.01	0.02	0.00	0.03	0.01	0.04	0.00	0.04	0.00	0.03
salicylic acid	DOB	0.33	0.13	0.53	0.12	0.65	0.15	0.90	0.16	0.99	0.18	1.86	0.05
	Pure phenolic acids	0.51	0.29	0.50	0.14	0.50	0.20	0.50	0.22	0.64	0.28	0.55	0.21
syrengic acid	DOB	0.20	0.05	0.66	0.07	0.54	0.47	0.45	1.12	0.46	1.30	0.51	5.03
	Pure phenolic acids	1.02	0.10	0.88	0.20	0.76	0.28	0.62	0.19	0.51	0.22	0.19	0.41
homovanillic acid	DOB	1.63	0.13	1.80	0.40	1.58	0.65	1.68	0.99	1.42	0.99	1.60	1.99
	Pure phenolic acids	3.76	1.12	1.94	0.08	1.48	0.49	1.44	0.28	1.82	0.21	2.07	1.03
4-phenylacetic acid	DOB	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	22.77	1.61
	Pure phenolic acids	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	17.69	1.69

3.4. Discussion

The present study has examined the bioaccessibility of oat bran phenolics during *in vitro* small intestinal digestion and large intestinal fermentation. The study observed that upper gut digestion results in the reduction of 48 % and 26 % of free and bound phenolic acids, respectively (Table 9). Furthermore, the microbial fermentation in the large intestine model showed significant increase in total free phenolic metabolite formation in oat bran (Figure 8). The 4-phenylacetic acid, hydroferulic acid, 4-hydroxybenzoic acid, protocatcheuic acid, gallic acid, 4-hydroxycinnamic acid and salycilic acid were the predominant metabolites, accounting for 95% of total phenolics (Table 10).

There is some evidence to suggest that nutritionally-significant amounts of phenolic acids are bound to cell walls in plants potentially restricting bioavailability in the small intestine and, as a consequence, delivering phenolics to the large intestine for fermentation and metabolism by gut bacteria [21, 246]. The combined action of carbohydrate-degrading enzymes of gut microbiota such as β-glucosidase, β-glucuronidase and esterases in the colon is able to release the bound phenolic acids from oats [21, 246, 247]. A previous research study confirmed that the catabolism of the free phenolic compounds is rapid, finalised over 6 h [248]. In contrast, the metabolism of the bound phenolics in wheat bran and grape seeds was completed over a longer period, 10‑24 h of fermentation [241, 249]. In the present study, the metabolism of the oat bound ferulic acid to dihydroferulic acid started as early as 4 h of fermentation and was completed throughout the 24 h, a significantly longer period than the metabolism of the pure phenolic mix (6 h). The fermentation studies with varied microbiome confirmed the metabolisation of ferulic acid to protocatechuic acid via vanillin, *p*-coumaric acid via p-hydroxybenzaldehyde, p-hydroxybenzoic acid, and protocatechuic acid [250-252].

In our group, we recently observed increased bioavailability of 33 phenolic metabolites within 0-2 and 4-8 h, following 60 g oat bran intake in healthy men [115]. The European Prospective Investigation into Cancer and Nutrition cross-sectional study measured urinary excretion of 34 dietary polyphenols and the highest median levels were observed for phenolic acids such as 4-hydroxyphenylacetic acid (157 μmol / 24 h) [253]. In the current study, at 24 h the main metabolite from the DOB was 4-dihydroxyphenylacetic acid (22 μM / 24 h), 57 % of the total phenolics.

Evidence from multiple studies indicates that conjugation of phenolic acids greatly strengthens their biological activities due to their longer bioavailability in plasma [254]. An animal study with a single dose of wheat bran or an equivalent amount of 4.04 mg / kg body weight of free ferulic acid administration proved that wheat bran ingestion through release of bound phenolic acids resulted a constant concentration in plasma and more effective antioxidant activity of the ferulic acid. In contrast, free ferulic acid was rapidly detectible in plasma at 1 h and completely disappeared at 4 h [113]. In addition, the single administration of 250 mg of oat bran, containing 40 μmol total phenolics compounds in hamsters showed a high relative bioavailability of *p*-coumaric, sinapic and syrengic acids at 40 min after the intake, due to its free phenolic contents. However these phenolics did not change plasma antioxidant capacity or the LDL oxidation resistance *ex vivo* [117].

Studies have indicated that the appearance of oat-derived phenolic acids in the circulation following high oat phenolic intake can have health benefits [103, 151, 255, 256]. Ingestion of avenanthramides from oats, is associated with the production of dihydrocinnamic acids metabolites, which exert antioxidant and antigenotoxic activities in the *in vitro* / animal models [255, 257].

The hydroxylated phenolics from ferulic and *p* - coumaric acids have been shown to reduce the inflammatory response of human peripheral blood mononuclear cells through reduced secretion of IL-6, IL-1, and TNF-α [258, 259]. Larrosa *et al.* [260] found that dihydroxyphenylacetic and hydroferulic acid reduced prostaglandin E production by at least 50% in colon fibroblast cells stimulated with IL-1β; the same compounds were also shown to decreased oxidative DNA damage [261]. Moreover, protocatechuic acid inhibits *in vitro* carcinogenesis and exerts antiproliferative effects in different tissues [262], inhibits monocyte adhesion to tumor necrosis factor-alpha-activated mouse aortic endothelium and decreases cholesterol levels [263], and as low as 1 μM was able to reduce VCAM-1 protein secretion [264].

In conclusion, our data suggest that oat bran phenolics are more accessible than previously detected [97, 112] and characterised by high bioaccessibility of avanenthramides (88 %) for the small intestine and prolonged release of bound phenolic acids by the gut microbiome in the large intestine. Future investigations must address to establish the detailed pharmacokinetics of circulating concentrations of oat bran-derived phenolic compounds, and to define their biological activities and contributions to the human health.

CHAPTER 4

Effect of phenolic acids and avenanthramides rich-wholegrain oats on gut health and inflammation: a human intervention study

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This trial is registered at clinicaltrials.gov as 211656

Abstract

Background: High dietary intake of wholegrain cereals has been found to lower the risk of chronic diseases, such as cardiovascular disease, as well as play an important role in gut heath and inflammation management. Whole grain oats are distinguished for their unique combination of high level of soluble fibre (β-glucan) and potential bioactive compounds, such as phenolic acids and avenanthramides. Whilst fibre is known to impact on the gut microbiome, further randomized control trials are necessary to confirm the role of oat polyphenols in the management of gut heath and inflammation.

Objective: To investigate whether the intake of high or moderate avenanthramide and phenolic acid wholegrain oats alters the gut microbiome and associated inflammatory biomarkers.

Design: 28 adults with prehypertension (i.e. average systolic 130 mmHg and diastolic 80 mmHg) were subjected to a randomised, cross-over, intervention trial with three 4-week dietary intervention periods, comprising of a high (H - 68.1 mg of phenolic acid, 4.8 g of β - glucan), moderate (M - 38.9 mg of phenolic acid, 4.8 g of β - glucan), or a fibre matched control diet, separated by a washout period of ≥ 4 weeks. The response of the gut microbiota composition and inflammatory markers to the interventions was assessed with rRNA gene sequencing (v4 region) using Illumina MiSeq and Flow cytometry, respectively.

Results: The high avenanthramide/phenolic acid rich-wholegrain oat diet significantly increased the relative abundance of the Proteobacteria phylum ($p = 0.04$) and *Sutterella* genus ($p = 0.02$), relative to moderate phenolic and control interventions. Investigation of microbial modulation over time within each treatment indicated that there were significant increases in the relative abundance of the Actinobacteria phylum ($p = 0.03$) and *Bifidobacterium adolescentis* ($p = 0.04$) after M intake, but not after H. In addition, in treatment H, only volunteers who had *Prevotella copri* at baseline showed statistical significant enrichment in *Prevotella copri* ($p = 0.04$) after treatment. No alteration in the serum inflammatory markers was detected compared with the fibre matched control.

Conclusion: Compared with fibre matched diet, high avenanthramide/phenolic acid rich-wholegrain oat diet increased the relative abundance of the Proteobacteria phylum, specially the *Sutterella* genus, without changing the inflammatory markers.

4.1. Introduction

High dietary intake of wholegrain cereals has been found to lower the risk of chronic diseases such as cardiovascular disease [2, 9, 233, 265] and diabetes [16], as well as play an important role in the management of gut heath [1, 266]. The health benefits associated with wholegrains might be due to improved glucose metabolism [6, 10, 267] and lipid absorption [5, 268], and lower inflammatory protein concentration [232, 269]. Alterations of gut microbiota by whole grain oats have been observed in a number of *in vitro* [24, 130-132, 136], animal [137-140] and human studies [23, 148].

Current thinking prescribes these effects to fermentable dietary fibre to induce the growth of health promoting bacterial genera within the colon, such as *Bifidobacterium* and *Lactobacillus* [92-94, 181], and additionally lead to higher production of beneficial short chain fatty acids. For example, a daily intake of 45 g wholegrain oats for 6-week led to the increase of faecal bifidobacteria and lactobacilli ($p = 0.001$) and confirmed the cholesterol lowering effect of oats for total and LDL-cholesterol ($p = 0.02$) compared to non-whole grain treatment [23]. The presence of soluble β-glucan in wholegrain oat has been found to contribute to its cholesterol-lowering benefits through the reduced reabsorption of bile acids, by either entrapping the bile acids in the viscous chyme matrix of the upper gut [107] and/or by direct binding of cholesterol or bile acids [14-17]. A 5-week, 3-3.5 g/day of native β-glucan human intervention study induced reduction in total cholesterol by 6% ($p = 0.022$) and increase in faecal bifidobacteria ($p = 0.012$) [148]. Soluble dietary fibre can also increase gastrointestinal transit time and might increase the availability, fermentation and uptake of polyphenols in the small intestine [270]. However, wholegrain oats are also distinguished for their unique combination of high amounts of soluble fibre (β-glucan) and polyphenols, such as phenolic acids and avenanthramides [271, 272], and these have also been previously shown to impact specific gut microbial populations such as Bifidobacterium and Bacteroidetes.

The aim of the current study was to assess this contribution to better understand the overall impact of wholegrain oats on the human microbiota.

Dietary polyphenols belong to the large family of phytochemicals, natural compounds, occurring in plants, including foods such as fruits, vegetables, and cereals [261]. Epidemiological studies and meta-analyses have proved that a diet rich in fruits and vegetables can reduce the incidence of several chronic diseases, including cardiovascular diseases [270]. There is still a knowledge gap on the mechanisms underpinning the observed health effects of a diet rich in fruits and vegetables with the consumption of dietary polyphenols [273]. The bioavailability of polyphenols depend on a variety of factors related to diet and food matrix [270]. In oats, for example, the entire food matrix plays an important role in the release and absorption of phenolic compounds [274]. Oat phytochemicals comprise a diverse group of phenolic acids, such as avenanthramides, hydroxycinnamic acids (e.g. ferulic acid) and benzoic acid derivatives [95, 234]. A large proportion of oat phenolics are covalently linked *via* ester bond to lignin and cell wall polysaccharides, a matrix that reduces phenolic acid absorption in the small intestine and increase their bioavailability for bacterial catabolism in colon [246, 249]. As there are no esterases in the human genome capable of cleaving these ester links, and the main site for metabolism is in the colon, where cinnamoyl esterase producing bifidobacteria have been identified [186, 187]. While the effect of wholegrain on microbiome composition is well known, some studies suggest that phenolic acids also may contribute towards a *Bifidobacterium* population increase [110].

Furthermore, dietary polyphenols have been proposed to play a role in the improvement of host immunological response, due to polyphenol-induced modulation of the gut microbiome [18, 35, 275].

For example, rats fed with 1 mg of resveratrol/kg/day for 25 days showed increased lactobacilli and bifidobacteria levels, as well as protection of the colonic mucosa architecture, due to the reduction of systemic inflammation markers, colonic mucosa prostaglandin E2, cycloxygenase-2, prostaglandin E synthase and nitric oxide levels [276]. In addition, consumption of cocoa-derived polyphenols (494 mg cocoa flavanols / d) for 4-weeks has been associated with significant increases in bifidobacteria and lactobacilli populations and reductions in C-reactive protein concentrations [50]. In the metabolic syndrome patients, red wine polyphenols (381.9 mg / d) significantly increased the number of faecal bifidobacteria, lactobacilli and butyrate-producing bacteria, *Faecalibacterium prausnitzii* and *Roseburia* [277].

Overall, these recent findings suggest that the microbiota modulating effects of wholegrains may be mediated by the combination and interaction of both fibre and polyphenols. Nevertheless, to date and to our knowledge, no human trials have investigated the direct effects of oat phenolic acids and avenanthramides on the growth of the microbiota and inflammation. The current chronic human trial was conducted to assess the effect of either high or moderate avenanthramides and phenolic acids rich-wholegrain oats diet on gut health and inflammatory markers.

4.2. Subjects and Methods

Contributions

The authors' contributions were as follows: AK, MYS, GS and SA designed the study; AK and MYS, IH, GS and SA conducted the human trial and collected the samples. Phenolic acid, avenanthramide, β-glucan contents of treatments were measured by AK, GS, MYS. The monosaccharides profiles and beta-glucan`s molecular weight were analysed at Rothamsted Research Institute by dr Alison Lovegrove and dr Ondrej Kosik respectively.

Treatments macronutrients, total fibre and sodium, energy contents quantified by Campden BRI group, UK. AK conducted the DNA extraction of faecal samples, Centre for Genomics Research in Liverpool University performed further sample preparation, the next generation sequencing and data preparation, AK and GM conducted the data analyses and statistical analyses. AK, GW, GK and JS interpreted the data and AK, GM, GW, GK and JS drafted the manuscript. AK had primary responsibility for the final content. All authors agreed on the final version of the manuscript. None of the authors had a conflict of interest.

4.2.1. Study subjects

This study was conducted according to the guidelines laid down in the Declaration of Helsinki following Good Clinical Practice. The study was approved by the University of Reading Research Ethics Committee as NCT02847312 and registered at www.clinicaltrials.gov (ID 211656 and REC reference 16/LO/1542). Volunteers were recruited from the University of Reading and surrounding area using the Hugh Sinclair Unit of Human Nutrition database, local media and local GP practices. Eligible volunteers were required to be in general good health, but have prehypertension (i.e. average systolic 130 mmHg and diastolic 80 mmHg). The inclusion criteria were non-smoking women taking the contraceptive pill or on hormone replacement therapy and aged 27 - 75 y. The exclusion criteria were: abnormal biochemical/haematological results assessed at the health screening, hypertension (i.e. SBP/DBP ≥ 160/100 mm Hg), BMI >35 kg/m^2, current smoker or ex-smoker ceasing <3 months ago, past or existing medical history of vascular disease, diabetes, hepatic, renal, haematological, neurological, thyroidal disease or cancer, prescribed or taking lipid lowering, anti-hypertensive, vasoactive, anti-inflammatory, antibiotic or antidepressant medication, allergies to whole grains, dairy and/or lactose intolerance, parallel participation in another research project, had the flu vaccination or antibiotics treatment within 3 months of the trial start, chronic constipation, diarrhoea or

other chronic gastrointestinal complaint (e.g. irritable bowel syndrome), on a weight reduction regime or taking food, probiotic or prebiotic supplements or laxatives within 3 months of the trial start, performing high levels of physical activity (i.e. ≥ 150min aerobic exercise/week), consumption of alcohol ≥ 21 units/week for men and ≥ 15 units/week for women, females who were breast-feeding, or who may be pregnant or, if of reproductive age, were not using a reliable form of contraception. Of the 84 volunteers screened at the Hugh Sinclair Unit of Human Nutrition at the University of Reading, 34 were recruited and randomized into the study, with 28 participants completing the study, 13 women and 15 men. The reasons given by the 6 participants who dropped out were: medical issues (n = 3) or could not commit the time for the visits (n = 3).

4.2.2. Study design and treatments

All volunteers signed an informed consent form before commencing the study. The study was a three-arm, double-blinded, placebo-controlled randomised crossover intervention, entirely carried out in the University of Reading. During a 2 week run-in period and the total study duration, volunteers were asked to completely refrain from eating oats (apart from the study interventions), taking dietary, probiotic or prebiotic supplements, drink no more than 400ml/d of tea and only polyphenol-low coffee (i.e. a highly roasted polyphenol-degraded commercial instant coffee provided by the researchers at the Hugh Sinclair Unit, University of Reading), maintain their habitual dietary and activity patterns and keep their body weight within 1 kg of their starting weight. The study lasted 20 weeks and consisted of 3 periods: subjects consumed any of the 3 randomised assigned treatment for 4 weeks, followed by 4-week washout period (without consuming any treatment products) and then switched to the next randomised assigned treatment (Figure 11). The study included six visits: baseline for each treatment (visit 1, 3 and 5) and at the end of the 4-week treatment (visits 2, 4 and 6). All visits were standardised: participants attended the Hugh Sinclair Unit of Human Nutrition at the University of Reading at 8 am after fasting overnight for 12 h (not eating or drinking anything but water). They were instructed to avoid flavonoid containing foods for 24 hours prior to every study visit and consume a standard low fat flavonoid-free meal (<15 g fat) the evening before the visit. Compliance to a 24-hour flavonoid free diet and 12-hour fasting was monitored by a 24-hour dietary recall taken on the morning of each study visit.

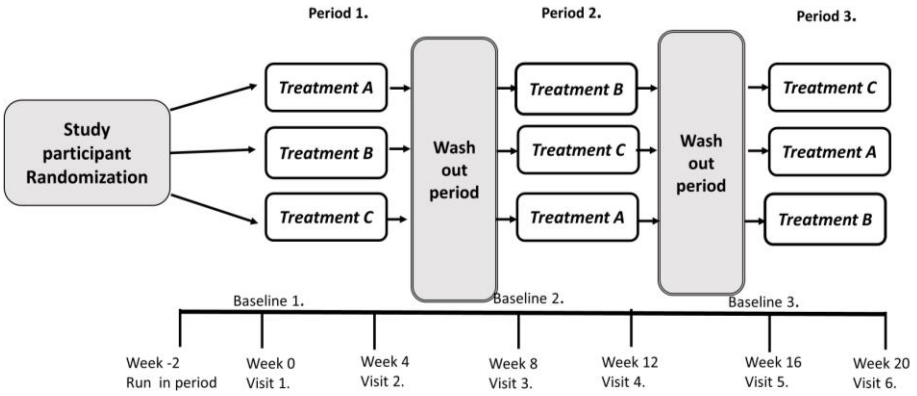
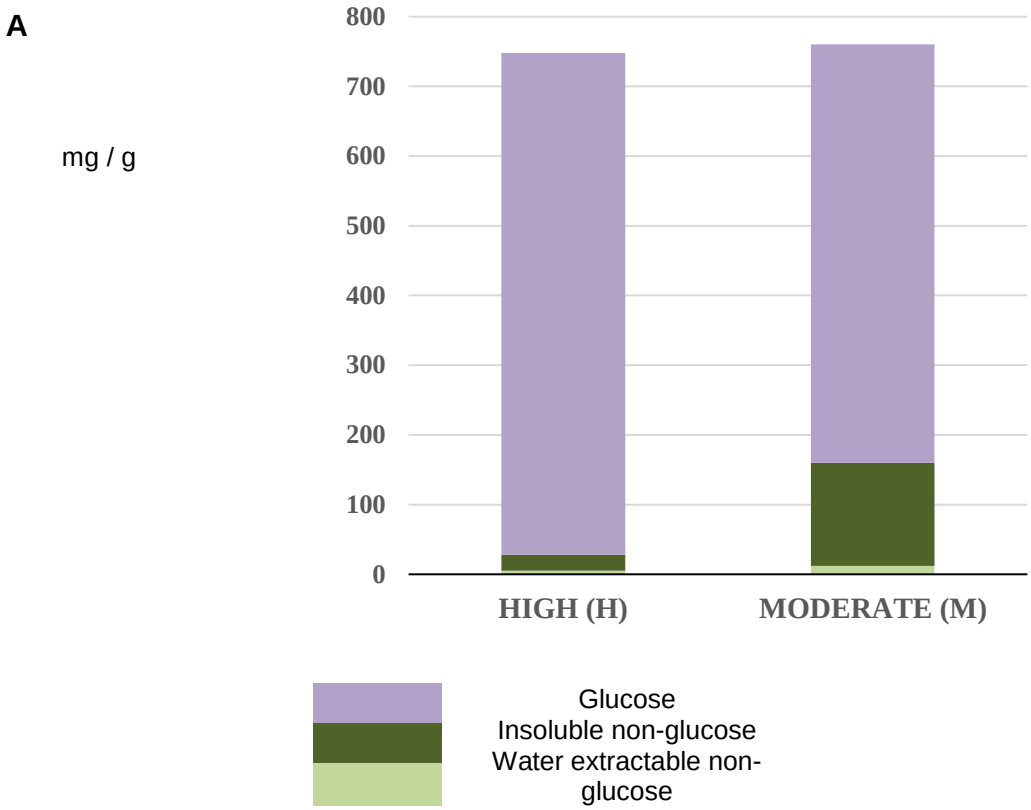
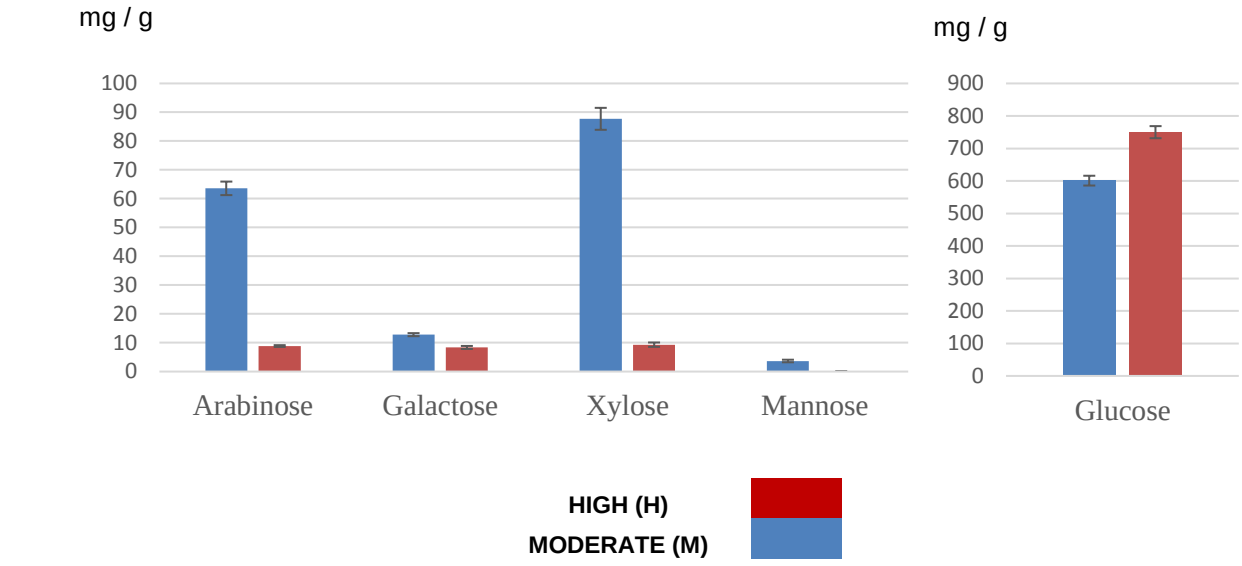


Figure 11. *Study design*

Time line of the randomised crossover trial; three 4-week dietary treatments were assessed in succession. The treatments were interspaced by 4-week washout period.

The subjects were randomized to one of the following 3 diets (Table 11): high dose of avenanthramides and phenolic acids-containing oat diet (H - 66.8 g of oatmeal and 60 g of oatcake / day), moderate dose of avenanthramides and phenolic acids-containing oat diet (M - 17 g of oatwell blend with 63.3 g cream of rice and 60g cream crackers) or control diet (C - 68.8 g cream of rice mixed with 8.1g of cellulose and 4.8 g of pectin, 60 g cream crackers). Participants were blinded to the diet allocation, dietary intakes were determined from 3-d diet diaries completed during the intervention, which were analyzed by using Dietplan 6.6 (Foresfield). Previous work (unpublished data) showed no significant differences in polyphenols levels of commercially available oat varieties in UK. To achieve the β-glucan matched moderate avenanthramides and phenolic acids oat treatment we used Oatwell, an oat bran concentrate product, containing 28% β-glucan [278]. Analysis of the intervention products, H and M showed a similar content of β – glucan (H - 3685 ($_{SD}$ 2) %, M - 3991 ($_{SD}$ 1) % kDa). The total monosaccharide levels in the H and M was also similar (H – 748 mg / g, M – 760 mg / g), but it differed in composition (Figure 12), with M having more insoluble non-glucose sugars (arabinose, galactose, xylose, mannose) than H.





Data from Dr Alison, Rothamsted Research

Figure 12. *Monosaccharides profile in high (H) and moderate (M) intervention: A, Insoluble and water extractable non - glucose and glucose ratios in H and M mg / g, B, Profile of total glucose and non – glucose monosaccharides in H and M intervention mg / g*

***Table 11**. Nutritional composition of diet interventions*

Quantities of the intervention materials, phenolic acid, avenanthramide and β-glucan, and their nutritional content are shown in grams (g), unless specified as mg, kcal or kDa, for the control, moderate and high phenolic-content oats interventions.

Macronutrients, total fibre and sodium contents quantified by Campden BRI group, UK. Phenolic acid, avenanthramide, β-glucan and energy contents were measured at the University of Reading.

Treatment	High avenanthramide and phenolic acid (H)	Moderate avenanthramide and phenolic acid (M)	Control (C)
Breakfast meal	Oatmeal – 66.8g	Oatwell–17g and Cream of Rice – 63.3g	Cream of rice – 69.8g Cellulose - 8.1g; Pectin - 4.8g
Afternoon snack	Oat cake – 60g	Cream Crackers – 60g	Cream Crackers – 60g
Energy	**500.1 kcal**	**500.2kcal**	**500.2kcal**
Carbohydrate	**68.7**	**97.9**	**101.0**
Fibre	**15.5**	**11.4**	**15.5**
Soluble fibre	**4.8**	**4.8**	**4.8**
Insoluble fibre	**10.7**	**6.6**	**10.7**
β-glucan **molecular weight (Mean and SD) &**	4.8 3685.1 (SD 2) % kDa	4.8 3991.6 (SD 1) % kDa	0
Protein	**16.9**	**14.7**	**11.2**
Fat	**14.1**	**2.3**	**1.6**
Monounsaturated fatty acids	**8.0**	**0.6**	**0.2**
Polyunsaturated fatty acids	**3.7**	**0.6**	**0.3**
Phenolic acids	48.9 mg	38.4mg	13.8mg
Avenanthramide	19.2 mg	0.5mg	0mg
Total Phenol	68.1 mg	38.9 mg	13.8 mg

&- Data from Dr. Kosik, Rothamsted Research

4.2.3. Sample collection

Participants were provided with anaerobic specimen containers, instructions for stool sample collection, and were advised to deliver the sample to the Hugh Sinclair Unit of Human Nutrition within 2 h from collection. From each stool sample, 3 aliquots of 2g were obtained and immediately stored at - 80 °C.

The blood samples were collected in EDTA-coated tubes (Greiner Bio-One, UK) after 12-h fasting, and processed within 2 h from collection for whole blood cytokine analysis.

4.2.4. Whole blood culture for cytokine analysis

The whole blood was diluted 6:10 with RPMI 1640 medium (Sigma-Aldrich, UK) supplemented with L-glutamine-Penicillin-Steptomycin solution (Sigma-Aldrich, UK) and MEM non-essential amino acids (Sigma-Aldrich, UK). The diluted blood (1 mL / well) was placed into 24-well tissue culture plates and 0.5 µg / mL lipopolysaccharides from *E. coli* (LPS) (Sigma-Aldrich, UK) was added to stimulate cytokine production. Cultures were incubated at 37 °C in a 5% CO_2 atmosphere for 24 h. At the end of the culture period, plates were centrifuged at 1000 rpm for 5 min. Culture supernatants were collected and stored in aliquots at −20 °C until analysis.

4.2.5. Measurement of cytokine concentrations

In the culture supernatants, concentrations of tumor necrosis factor alpha (TNF-α), Interferon γ (IFN-γ), Interleukins IL-2, IL-4, IL-6, IL-10, IL-17A, and intercellular-CAM1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) were measured using BD CBA Human TH1/TH2/TH17 Kit, sCD54 (ICAM-1) and sCD106 (VCAM-1) Flex Set Kit from BD Biosciences (Oxford, UK) according to the manufacturers' instructions.

The intensity of the fluorescence signal was acquired on a BD Accuri C6 Flow Cytometer (BD Biosciences, Oxford, UK) and data analysed using the BD FCAP Array v3 software (BD Biosciences, Oxford, UK). Excluding volunteers with missing visits and medication (5 volunteers).

4.2.6. High throughput sequencing of the gut microbiota

The faecal microbiota compositions and dynamics were determined using tag-encoded 16S rRNA gene HiSeq 2500 (Illumina, CA, USA) high throughput sequencing. DNA was extracted from 200 mg of faecal samples using QIAamp PowerFecal DNA Kit (Qiagen, Sweden). DNA concentrations and quality were determined using Nanodrop 1000 (ThermoScientific, USA) and agarose gel electrophoresis (Fisher Scientific, UK). Extracted DNA was then used to create an Illumina DNA library and sequenced using a HiSeq using the V4 chemistry (2 × 300 bp) at the Centre for Genomic Research, Liverpool University, UK. Briefly, the V4 region of the 16S rRNA gene was amplified using first round PCR with conditions 98 °C for 2 mins, 20 sec at 95 °C, 15 sec at 65 °C, 30 sec at 70 °C for 10 cycles then a 5 min extension at 72 °C.

F: 5'ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNGTGCCAGCMGCCGCGGTAA3'

R: 5'GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT3'

The Primer design incorporates a recognition sequence to allow a secondary nested PCR process. Samples were first purified with AMPure SPRI Beads before entering the second PCR to incorporate Illumina sequencing adapter sequences containing indexes (i5 and i7) for sample identification. PCR was performed using the same conditions as above for a total of 25 cycles in all. Samples were purified using AMPure SPRI Beads before being quantified using Qubit and assessed using the Fragment Analyzer. The amplicon libraries were sequenced on an Illumina® HiSeq 2500 platform with version 2 chemistry using sequencing by synthesis (SBS) technology to generate 2 x 300 bp paired-end reads.

4.2.7. 16S rRNA gene data bioinformatics analysis

Bioinformatics analysis was conducted at the Centre for Genomic Research, Liverpool University. Briefly, base-calling and de-multiplexing of indexed reads was performed by CASAVA version 1.8.2 (Illumina) to produce 168 samples data files, in FASTQ format. The raw FASTQ files were trimmed to remove Illumina adapter sequences using Cutadapt version 1.2.1 and Sickle version 1.200 with a minimum window quality score of 20. After trimming, reads shorter than 20 bp were removed. To improve base quality in both read pairs, sequencing errors were corrected in both forward and reverse reads using the error-correct module within SPAdes assembler, version 3.1.0 [279], then read pairs were aligned using PEAR (version 0.9.10; [280]). Fragmented PhiX phage genome was added to the sequence library to increase the sequence complexity. Any sequences passing the filters for each sample were merged into a single file. This final sequence file, plus its own metadata file describing each sample, was used for the analysis by using a custom pipeline based on Quantitative Insight Into Microbial Ecology (QIIME 1.9.1) tool [198] and GreenGenes database (version 13.8). To identify the sequence variability in each sample, the obtained amplicon sequences were sorted and assigned to groups (clusters) according to their sequence similarity using SWARM (version 2.2.1, [281]). Purging the dataset from chimeric reads was performed using VSEARCH2.6.2. The taxonomic assignment of each cluster (now referred to as operational taxonomic unit, OTU) was carried out using the QIIME script and the RDP classifier [282]. High throughput sequencing providing an average and SD of 573159 ($_{SD}$ 117090) sequences per sample, the identified clustered an average and SD of 2601 ($_{SD}$ 415). Excluding volunteers with missing visits (2 volunteers).

4.2.8. Sample-size calculation and random assignment

The power calculation was conducted for the primary clinical outcome of the study (flow-mediated dilation) to determine the minimum number of participants required for the study. Consequently, with a standard deviation of gut microbiota diversity within participants of 2.4 % (based on previous studies performed in our group), a significance level of $p \leq 0.05$ and a power of 80 %, 27 subjects were needed to determine a significant within-subject difference between interventions of at least 1.3 % of FMD. However, to provide power to use the secondary measures and achieve significant within-subject differences, we aimed for 30 participants to complete the trial, nevertheless with the drops out we reached 28 volunteers. Participants were randomly assigned to the treatment O, OW or C.

4.2.9. Statistics

Data were analysed using QIIME 1.9.1, Graphpad version 7 and SAS version 9.4 (SAS Institute). Data normality was tested with the Shapiro-Wilk test and log transformations were used as needed.

Metagenomics analyses (QIIME 1.9.1) of alpha diversity measures expressed with an observed species with Chao1, the sequence similarity 97 % OTUs value were computed for rarefied OTU tables using the alpha rarefaction workflow. Differences in alpha diversity were determined using a t-test-based approach employing the non-parametric (Monte Carlo) method (999 permutations) and corrected for multiple comparisons using Bonferroni FDR method. Statistical analyses of beta-diversity was performed using the Nonmetric Multidimensional Scaling (NMDS) analysis conducted with Bray-Curtis dissimilarity matrices then using adosin and ANOSIM test.

To sub analyse the microbial data (Graphpad) we used Wilcoxon test or paired t-test to identify the differences between the pre – treatment to post –treatment period. Effects were only considered when significant level was at a probability of (p value) ≤ 0.05.

Changes in inflammatory markers and microbial abundances measures at the end of each dietary treatment period were modelled using PROC MIXED in SAS 9.4 to fit a linear mixed model with fixed effects for a 3 period crossover design and participant as a random effect. Normality of residuals was assessed visually and logarithmic transformations were used as needed. A significance level of 5% was adopted.

Data presented in the text, tables, and figures represent the arithmetic means and SDs.

4.3. Results

4.3.1. Phenotype and microbiome characteristics of the study population at baseline

Twenty-eight volunteers, 13 females and 15 males, participated in the nutritional trial, and subject's baseline parameters are presented in Table 12.

Table 12. *Baseline characteristics of the 28 subjects*

Characteristic	Mean ($_{SD}$)	Range
Age (y)	**49.6 ($_{SD}$ 2.3)**	**26 – 68**
Body Mass Index - BMI (kg/m^2)	**26.7 ($_{SD}$ 0.7)**	**20.9 - 34.9**
Systolic blood pressure (mm Hg)	**129.7 ($_{SD}$ 1.9)**	**112-153**
Diastolic blood pressure (mm Hg)	**80.1 ($_{SD}$ 1.2)**	**65 – 96**
Microbial phyla	**Relative Abundance in % Mean ($_{SD}$)**	**Range**
Actinobacteria	**2.5 ($_{SD}$ 2.8)**	**0.2 – 12.8**
Bacteroidetes	**42.9 ($_{SD}$ 13.0)**	**12.5 – 64.6**
Firmicutes	**50.4 ($_{SD}$ 12.9)**	**26.1 - 78.3**
Proteobacteria	**2.3 ($_{SD}$ 1.8)**	**0.1 – 6.8**
Other	**1.6 ($_{SD}$ 1.2)**	**0.1 – 4.1**

The identified reads belonging to 12 bacterial phyla, 53 family and 97 genera were identified. The pre-treatment faecal microbiota was dominated by the phyla Firmicutes and Bacteroidetes, with lower proportion of Actinobacteria and Proteobacteria (Table 12).

The three most abundant phyla varied in relative abundance between volunteers: Actinobacteria (0.2 – 12.8 %), Bacteroidetes (12.5 – 64.6%) and Firmicutes (26.1 – 78.3%). The beta-diversity from four volunteers, suggest that differences between the volunteers gut microbiota were high (Figure 13).

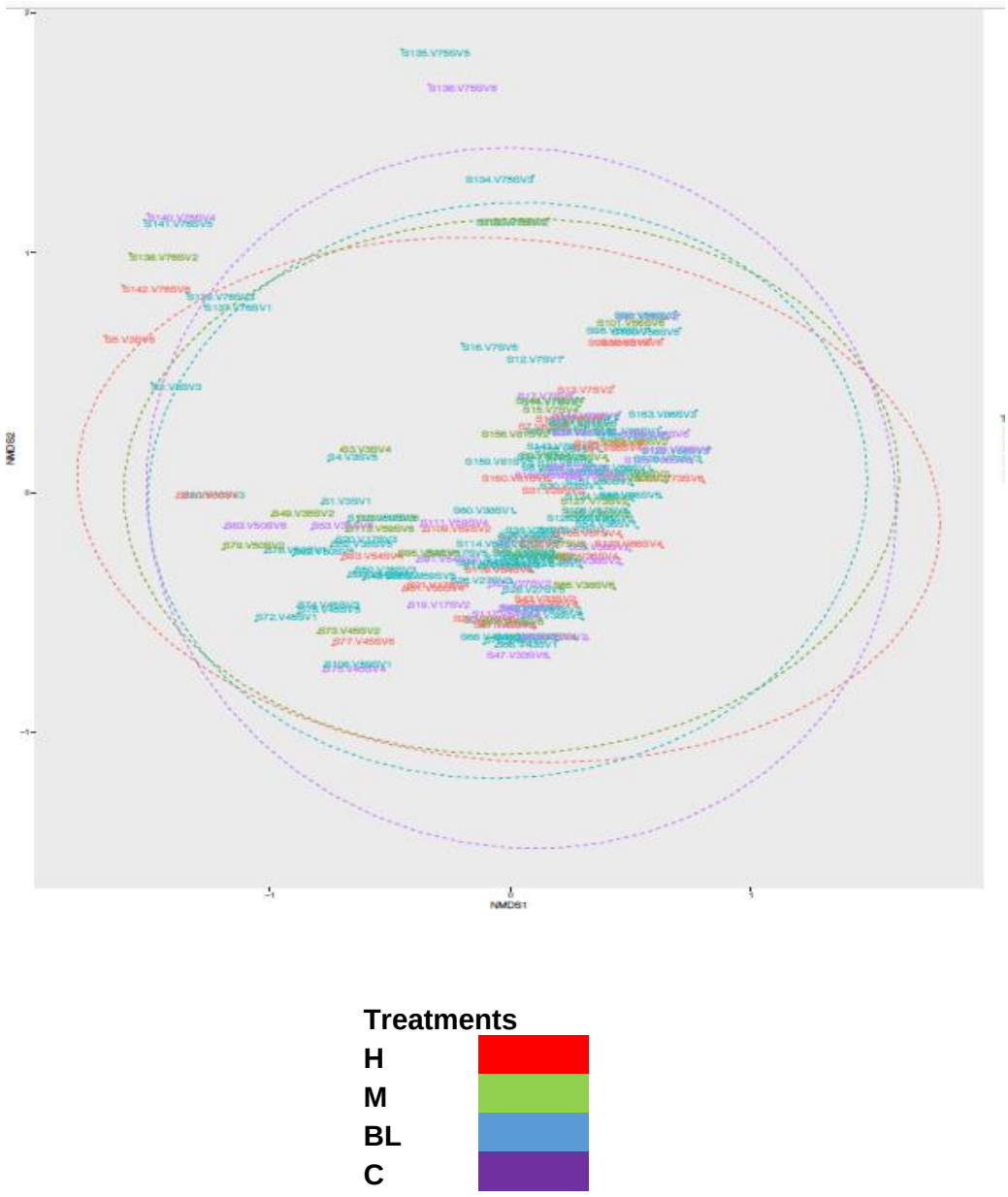


***Figure 13**. NMDS on the Bray-Curtis matrix analysis result: first and second components*

Each colour represents different treatments and each volunteers

4.3.2. Inflammatory markers in whole blood culture

There were no significant differences in the concentration of circulating TNF-α, IFN- γ , IL-2, IL-4, IL-6, IL-10, IL-17A and ICAM-1, VCAM-1 compared to baseline and intervention groups (Table 13).

Table 13*. Variation of plasma concentration of inflammatory markers over the study period (Excluding volunteers with missing visits and medication)*

	∆H	∆M	∆C	n	p
IL17 - A	13.2 ±54	9.9 ± 45	4.6 ± 60	23	0.50
INF - У	241.8 ± 935	241.1 ± 1348	-296.5 ± 716	23	0.30
TNF-α	1618 ± 2354	709 ± 3692	-162 ± 2078	23	0.20
IL - 10	81.6 ± 262	-59.8 ±150	26.4 ± 166	23	0.13
IL - 6	47.9 ± 112	35.4 ± 75	13.7 ± 86	23	0.15
IL - 4	21.6 ± 41	1.8 ± 42	-10.41 ± 51	23	0.62
IL - 2	22.4 ± 40	5.2 ± 42	-12.3 ± 53	23	0.49
ICAM	14.1 ± 77	-1.8 ± 54	-8.2 ± 32	23	0.54
VCAM	70 ± 123	22.4 ± 83	-8.6 ± 79	23	0.13

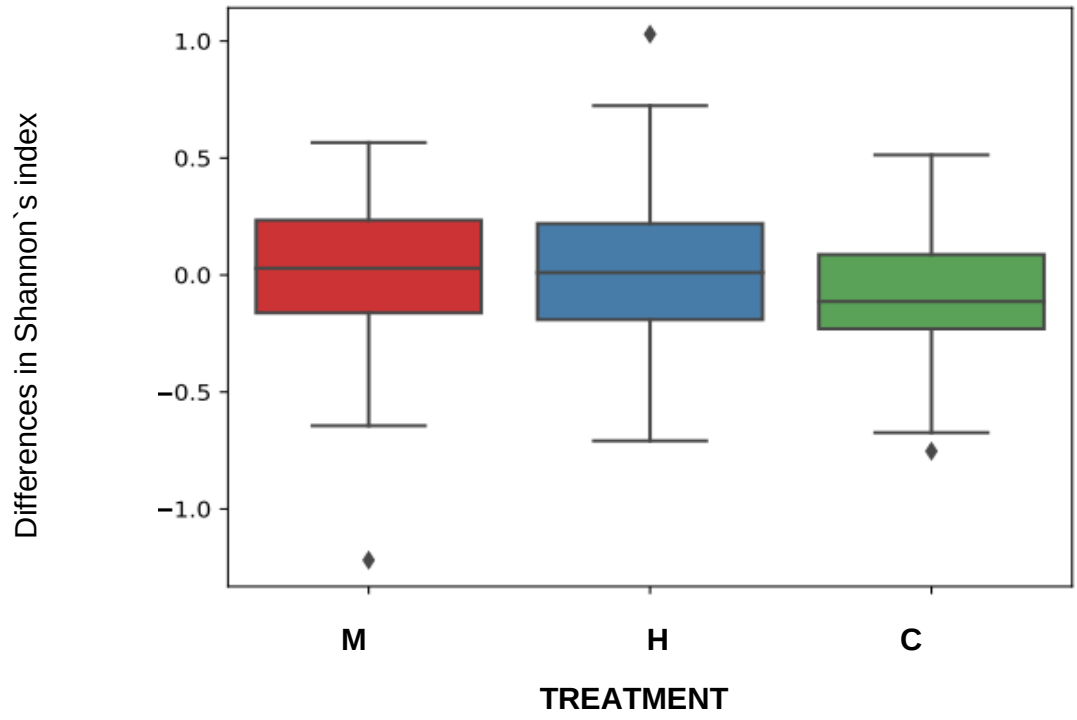
∆ - Treatment concentration extracted from Baseline concentration; values are in means and SDs; n= number of volunteers; IL ∆ - Treatment concentration extracted from Baseline concentration; values are in means ± SDs; n= number of volunteers; IL 17 - A, interleukin 17 –A; INF – У, interferon – У; TNF-α, tumor necrosis factor; IL-10, interleukin – 10; IL-6, interleukin – 6; IL-4, interleukin –4; IL-2, interleukin –2; ICAM, intercellular-CAM1; VCAM, vascular cell adhesion molecule-1

Values of IL17A, INF-У, TNF-α, Il-10, IL-4, IL-2 are in ng/1000 monocytes; values of IL-6, ICAM, VCAM are in pg/1000 monocytes

4 3.3. Effect of phenolic acids on faecal microbial communities

Sequence data were used to establish whether either a high dose (H) or a moderate dose (M) of oat avenanthramides and phenolic acids leads to dose-dependent chronic improvements of the gut microbiota composition compared to fibre matched control (C). None of the three treatments changed significantly the alpha richness measured by Chao 1 index (Group mean and SD – H 2633.9 ($_{SD}$ 348); M: 2636.6 ($_{SD}$ 345); C: 2633.2 ($_{SD}$ 462), p = 1) (Figure 14).

A



B

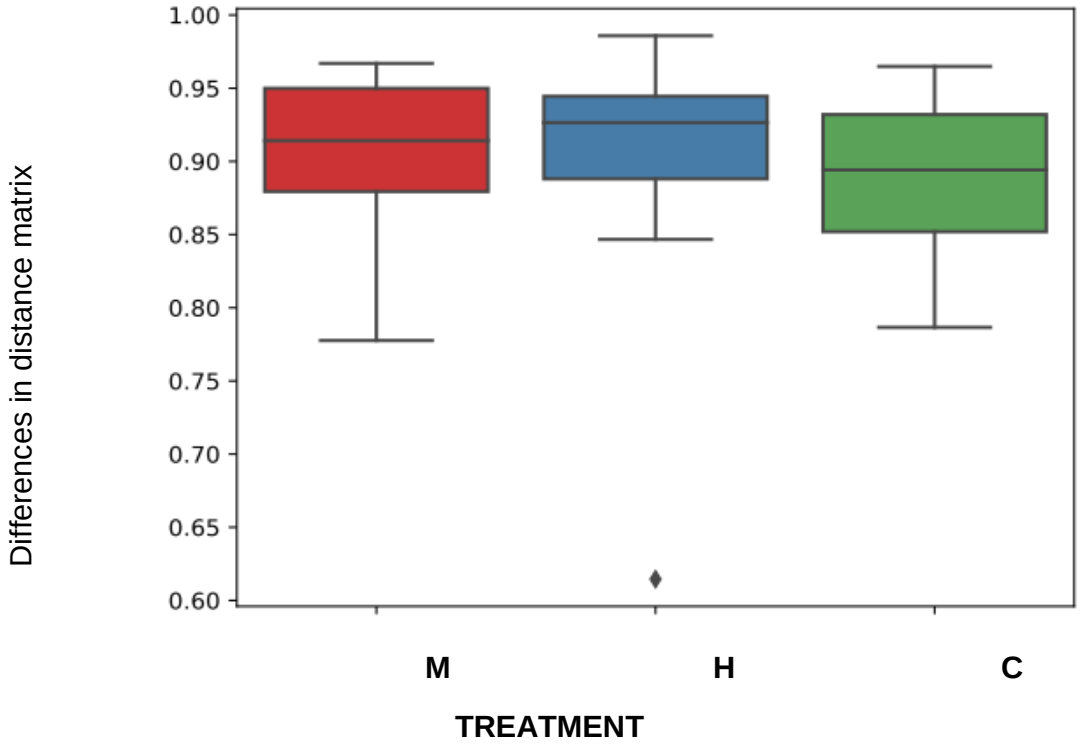


Figure 14. *A) Longitudinal analyses for the Shannon alpha – diversity index (H – high phenolic acids diet, M – moderate phenolic acids diet, C – control diet), B) Longitudinal analyses for the Bray-Curtis beta – diversity index (H – high phenolic acids diet, M – moderate phenolic acids diet, C – control diet)*

Analyses of beta diversity among the sample groups was based on the build pairwise sample distance matrices, using the Bray-Curtis and Weighted and Unweighted UniFrac dissimilarity measures. The test of the distance matrix compared to each group did not indicate any significant differences with Adonis (p = 1) and ANOSIM test (p =0.65). To understand the changes compared to baseline we performed longitudinal analysis, samples were reprocessed using QIIME2, after the identification and quantification the amplicon sequence variants, performed the taxonomical assignment the alpha and beta-diversity were assessed, rarefying the dataset at 50,000 sequences. The longitudinal pairwise distances [283] and longitudinal pairwise-differences (Bray – Curtis) were used to assess the differences in alpha and beta- diversity between each treatment and its baseline, for each volunteer. None of the pairwise comparisons showed a statistically significant difference for these analyses (Kruskal Wallis test p = 0.3 and p = 0.1, Figure 14).

The pre–treatment subtracted genus relative abundances are presented in Table 14. We observed (p = 0.04) statistically significant differences in the relative abundance of *Sutterella* genus in high phenolic compounds intervention compared to control, with SAS model adjusted for BMI, sex and age, Cohen's d is 0.505, which is considered to be a medium effect size. The rest of genus were no significant differences between treatments.

Table 14. *Variation of relative abundance (%) of the genus over study period (Excluding volunteers with missing visits), p values calculated by SAS*

Phylum	Family	Genus	∆M	∆H	∆C	p
Actinobacteria	Bifidobacteriaceae	**Bifidobacterium**	0.50 ±1.2	-0.43 ±1.6	0.57 ±3.3	> 0.05
Actinobacteria	Coriobacteriaceae	**Collinsella**	0.1 ±0.5	-0.09 ±0.6	-0.02 ±0.5	> 0.05
Bacteroidetes	Prevotellaceae	**Prevotella**	-2.92 ±8.0	5.43 ±10.5	2.71 ±9.7	> 0.05
Bacteroidetes	Bacteroidaceae	**Bacteroides**	0.42 ±15.3	-1.57 ±10.6	1.64 ±13.0	> 0.05
Bacteroidetes	Porphyromonadaceae	**Parabacteroides**	-0.41 2.1	-0.17 ±1.8	0.60 ±1.5	> 0.05
Firmicutes	Lachnospiraceae	**Roseburia**	0.56 ±1.6	-0.25 ±2.3	-0.84 ±2.2	> 0.05
Firmicutes	Clostridiaceae	**Clostridium**	0.05 ±0.3	-0.07 ±0.2	-0.06 ±0.3	> 0.05
Firmicutes	Lachnospiraceae	**Blautia**	0.11 ±2.2	0.30 ±1.6	-0.26 ±1.7	> 0.05
Firmicutes	Lachnospiraceae	**Coprococcus**	-0.38 ±2.0	0.14 ±1.3	-0.33 ±1.4	> 0.05
Firmicutes	Lachnospiraceae	**Dorea**	-0.11 ±0.6	-0.02 ±0.3	-0.10 ±0.3	> 0.05
Firmicutes	Lachnospiraceae	**Lachnospira**	-0.09 ±1.0	-0.26 ±1.1	-0.14 ±2.0	> 0.05
Firmicutes	Ruminococcaceae	**Faecalibacterium**	-0.82 ±2.4	-0.58 ±2.4	-0.38 ±2.2	> 0.05
Firmicutes	Ruminococcaceae	**Oscillospira**	0.09 ±3.9	0.53 ±2.9	0.59 ±4.3	> 0.05
Firmicutes	Ruminococcaceae	**Ruminococcus**	-0.07 ±4.2	-1.17 ±3.4	-1.31 ±2.0	> 0.05
Firmicutes	Veillonellaceae	**Phascolarctobactrium**	0.83 ±3.6	-0.88 ±3.4	-0.30 ±4.8	> 0.05
Firmicutes	Veillonellaceae	**Dialister**	0.38 ±2.1	0.94 ±4.0	-0.50 ±1.9	> 0.05
Proteobacteria	Alcaligenaceae	**Sutterella**	0.13 ±2.0	0.68 ±1.3	0.09 ±1.0	**0.04***
Proteobacteria	Desulfovibrionaceae	**Bilophila**	0.02 ±0.2	0.03 ±0.2	-0.05 ±0.2	> 0.05
Verrucomicrobia	Verrucomicrobiaceae	**Akkermansia**	0.05 ±0.7	-0.08 ±1.6	-0.17 ±1.2	> 0.05

∆ *- Treatment relative abundance extracted from Baseline relative abundance; values are in mean and SD, *- significant differences between treatment H and C*

Nevertheless, focusing on changes over time for each treatment separately, there were significant changes in relative abundance of *Bifidobacterium adolescentis* (p = 0.04) after M intake, but not after H, with respect to their pre – treatment level, Cohen's d is 0.64, medium effect size. Within, the Bacteroidetes phylum, in the H intake we found an increase in the relative abundance of *Prevotella copri* (p = 0.04) compared to pre – treatment (Table 15), the changes in responders Cohen's d is 0.862, which is considered to be a relatively large effect size.

Table 15. *Relative abundance (%) of Prevotella copri species with high phenolic (H) intervention, pre and post-treatment*

Volunteer ID	**Pre-treatment**	**Post -treatment**	Δ
NON – RESPONDER			
45	**16.34**	**6.80**	**- 9.54**
RESPONDERS			
17	**25.88**	**38.65**	**12.77**
35	**6.95**	**34.56**	**27.61**
50	**26.37**	**38.43**	**12.06**
54	**1.65**	**10.70**	**9.05**
59	**3.66**	**10.20**	**6.54**
76	**29.98**	**55.59**	**25.61**

4.4. Discussion

In order to explore the impact of either high or moderate avenanthramides and phenolic acids rich-wholegrain oats diet on gut health and inflammatory markers a double-blinded, placebo-controlled randomised crossover trial was conducted in healthy adults. A 4-week oat interventions involved daily consumption of 66.8 g of oatmeal and 60 g of oatcake (providing 68.1 mg of phenolic acids, 4.8 g of β - glucan and 10.7 g of insoluble fibre). Twenty eight volunteers completed this study.

Daily high avenanthramides and phenolic acids intervention led to increased relative abundance of *Sutterella* genus ($p = 0.04$, with medium (0.5) effect size). Similarly Proteobacteria was reported to increase following fermentation in our previous *in vitro* study (Kristek et al., 2018, chapter 2). In contrast to our results, a 6 - week human intervention trial with 45 g of whole grain oat - based granola breakfast, in volunteers at risk of developing cardio-metabolic disease showed an increase in proportion of *Bifidobacterium* [23]. The cardio-metabolic disease study used non – whole grain as a control, failing therefore to match the fibre intake. Furthermore, due to the cardio-metabolic disease the volunteers may have already had an altered gut microbiome composition. Finally, the fluorescence *in situ* hybridisation technique used to measure targeted microbial composition [23] did not targeted Proteobacteria, therefore this phyla would not have been assessed [284]. There is some evidence to suggest that polyphenols may be involved in the proportional increase of Proteobacteria. In rats, the polyphenol proanthocyanidin extract (500 mg per kg body weight) from grape seed has been shown to increase the abundance of this phylum and *Sutterella* genus [285]. In humans, after 20 days of red wine (369.5mg / d) intake the bacterial concentration of Proteobacteria, Fusobacteria, Firmicutes and Bacteroidetes phylum and *Prevotella* genus significantly increased compared with the washout period [51]. In another human study with polyphenols, 1000 mg of pomegranate extract with ellagic acid daily for a 4-week intervention [286] has increased the genera of *Butyrivibrio, Enterobacter, Escherichia, Lactobacillus*, and *Prevotella. Sutterella* species have been frequently associated with human diseases, such as autism, and inflammatory bowel disease, but the impact of these bacteria on health still remains unclear [287, 288]. Recent *in vitro* study proved that this bacteria group do not contribute significantly to the microbiota dysbiosis in the intestinal track [287].

Differences in the way β-glucans impact on the microbiota cannot be easily compared as the measurement of bound-polyphenols is usually ignored in the extracts and the purity of the glucan is not stated. Mice studies which focused on the impact of oat - derived β - glucan [289] or whole grain oat flour [139] on the gut microbiota reported a greater increase in relative abundance of Proteobacteria - *Sutterella* and *Prevotella* genus, respectively, compared to the control. Instead, an *in vitro* fermentation of β-glucan did not show changes in Proteobacteria phylum [290]. Nevertheless, the source of the oat derived β-glucan is not specified and polyphenol content of the extract was not measured in these studies. A study feeding 3 g high molecular weight barley β - glucan to humans for 5 weeks detected increased *Prevotella,* which was correlated ($P < 0.05$) with a reductive shift of CVD risk factors, including blood pressure and triglyceride levels [207]. During the period of the increased oat intake, within Bacteroides, we found an increase in the relative abundance of the *Prevotella copri* species ($p = 0.04$) with large effect size compared to pre-treatment levels, such an effect has been observed previously *in vitro* [291, 292] and animal studies [139]. Previous research reported [293] the patients with rheumatoid arthritis showed higher prevalence of *Prevotella copri* than healthy controls. In addition, individuals have responded to treatment with barley fibre, which has similar structure to oat fibre [294], with increased abundance of *Prevotella copri*, showed improvement in their glucose metabolism [295]. As such the increase in *Prevotella copri* potentially effects to the host require further research.

To date, no human intervention study has explored the impact of oatwell on gut microbiota and only an *in vitro* study showed no effect on gut microbiota composition, although samples from only three donors were used [157]. In our study, when we focused on changes over time for each treatment, after oatwell intake there were significant changes in relative abundance of Actinobacteria phylum ($p = 0.03$) and *Bifidobacterium adolescentis* ($p = 0.04$), but not with oat, with respect to their pre – treatment level.

The higher prevalence of more insoluble non – glucose (Figure 12) sugar such as arabinose, galactose and xylose in oatwell might have been responsible such increase, as shown in previous studies [296, 297]. Similar effect was observed in a previous *in vitro* study (Chapter 2) *B. adolescentis* has a number of health benefits including prevention of the development of diabetes by stimulating insulin secretion, synthesis and secretion of the neuroactive substance gamma-aminobutyric acid which facilitates the communication between the bacteria and the human nervous system. Future studies should examine further differences between oat and oatwell.

Consumption of oat was not associated with significant changes in plasma levels of inflammatory markers, although our study population was healthy adults without elevated inflammatory status. To date, only a few intervention studies in healthy humans have explored the impact of whole grain oats on inflammation [298, 299], the whole grain intake was associated with reduction of plasma C-reactive protein in study populations at risk of cardiovascular diseases [298]. In a larger scale parallel intervention study (n = 233), the daily intake of 1 serving of whole wheat foods and 2 servings of oats (30–40 g whole-meal bread + 70–80 g whole-grain cereals) for 16 – weeks found no effect on high-sensitivity C-reactive protein and IL-6 [300]. In contrast, a 24-h incubation *in vitro* study with oat avenanthramides mixture (20 and 40 μg / ml) significantly suppressed the expressions in human aortic endothelial cell monolayers of the intercellular-CAM1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), secretion of proinflammatory cytokines IL-6 and chemokines IL-8 [102, 301]. In an animal study, 10% oat bran, within a 28 day pig feeding trial showed decreased mRNA expression of IL-8 and TNF [302].

In conclusion, the current study clearly demonstrated that the high avenanthramides and phenolic acids rich-wholegrain oats diet significantly increased the relative abundance of the *Sutterella* genus compared to β-glucan matched control diet, without alteration in the serum inflammatory markers. The human gut is home to a multitude of bacterial strains, which co-exist in a dynamic eco-system, and are host-specific [303]. High interpersonal variability in gut microbiome across individuals is challenging to overcome [304, 305]. The wide inter-individual diversity in gut microbiome compositions at pre – treatment (Table 12), and the PCA plot (Figure 13) of beta-diversity from our volunteers, suggest that differences between the volunteers gut microbiota were bigger than the differences induced by the dietary treatments. Therefore, the diversity in the individual baseline microbiome composition might lead to a wide range of responses, possibly shading the effectiveness of any treatment.

The sample size in this study was calculated based on previous work conducted with the fluorescence *in situ* hybridisation technique, which does not detect the wide individual variation of gut microbiome as depth as non – targeted high throughput sequencing technique. Further metagenomic, metabolomic and proteomic integrated studies are required to obtain a more holistic understanding of oat derived polyphenol induced *Sutterella* and *Prevotella* genus microbial metabolic activities and their interaction with the host.

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CHAPTER 5

5.1. General Discussion

The main scope of this thesis was to investigate whether the avenanthramides and phenolic acids are the active moieties in whole grain oats if they exert their activities via the gut microbiota.

We have shown that *in vitro* gut model supplementation with 3% w/v oat bran increased the abundance of Proteobacteria and Bacteroidetes, with 1 % w/v having a bifidogenic effect, increasing the relative abundance of *Bifidobacterium adolescentis* and concentrations of acetic and propionic acid. In addition, we reported high bioaccessibility of avanenthramides and extended bioconversation of bound phenolics by gut microbiome to free phenolic metabolites, hydroferulic acid, 4-hydroxybenzoic acid, protocatcheuic acid and 4-phenylacetic acid via *in vitro* model. A human intervention study linked a high avenanthramides and phenolic acid wholegrain oat diet to an increase in the relative abundance of the *Sutterella* genus compared to a β-glucan matched control diet (medium effect size); and *Prevotella copri* species compared to pre-treatment level (large effect size), these effects were seen without alteration in the serum inflammatory markers.

The gut microbiome contains millions of different genes that encode various metabolic activities that influence host metabolism and impact on risk factors associated with chronic diseases [306]. Dietary changes are thought to be responsible for around 20% of the variation in the gut microbiota [40, 41]. Existing scientific data suggests that a high intake of wholegrain foods contributes to a reduced risk of cardiovascular disease [2] possibly due to an increase in beneficial gut microbiota and their metabolites in the colon [23], influencing lipid metabolism and plasma cholesterol levels [14, 126, 152]. Whole grain oats are one of the most popular breakfast cereals and are an excellent source of dietary fibre, in particular, soluble oat β – glucans, phenolic acids and avenanthramides [85].

There are largely inconsistent findings from *in vitro*, animal and human studies regarding the influence of oats on the gut microbiota and cardiovascular risk factors. Such discrepancies are possibly due to differences in experimental techniques, the use of the fluorescence *in situ* hybridisation technique to measure targeted microbial composition limits the range of detectable bacteria if probes for key microbial groups are not used. Furthermore, compounds in oats, other than β -glucans, have not been considered, as the measurement of bond-polyphenols is usually ignored in the extracts and the purity of the glucan is not stated. Little was known concerning the bioavailability of oat polyphenols and their effect on gut microbiota composition. To address this evidence gap *in vitro* batch culture experiments (**Chapter 2 and 3**) and a chronic human crossover randomised trial (**Chapter 4**) were performed to explore the effect of oat phenolic acids and avenanthramides on gut microbiota composition and metabolic activity.

To assess if the gut microbiota changes can be linked to one of the pure compounds (β -glucan extract or polyphenols) *in vitro* anaerobic batch - culture experiments (**Chapter 2**) were performed. This first study mimicked upper gut digestion then analysed the polyphenol profiles and β – glucan content and molecular weight of the digested oat bran. The lower dose (1 % w/v) of digested oat bran increased the relative abundance of *Bifidobacterium adolescentis* ($p < 0.05$) but isolated β – glucan or polyphenol mix given at a matched dose did not induce a similar effect. This is likely to be because these compounds alone did not provide enough energy for bacterial growth. Supplementation with 3 % w/v oats increased the abundance of Proteobacteria ($p < 0.01$), Bacteroidetes ($p < 0.05$) and concentrations of SCFA. One of the limitations of *in vitro* study was a high undigested protein in the oat bran treatments. The high protein content resulted increase in Enterobacteriaceae family due to this family has obligate protein fermenters which are the major producers of ammonia from protein fermentation [307]. Might it is the reason the in vitro experiment did not replicate well in the human study.

There is some evidence to suggest that nutritionally-significant amounts of phenolic acids bind to cell walls in plants potentially restricting bioavailability in the small intestine and, as a consequence, delivering phenolics to the large intestine for fermentation and metabolism by gut bacteria [21, 246]. To gain insight into the role of bound phenolics on the gut microbiota, simulated *in vitro* upper gut digestion and fermentation was used (**Chapter 3**). This is the first study that used the *in vitro* digestion to access the bioaccessibility of the oat polyphenols through the whole digestion. Such experiments showed that following upper GI tract digestion, levels of free, conjugated phenolics were reduced, whilst the bound phenolic fraction persisted. During microbial fermentation, the oat bran phenolic fraction largely remained intact up to 4h, then a significant increase in total free phenolic metabolites was observed at 24 h. In contrast, the levels of the matched pure free phenolic acid mixture resulted in high levels of microbial metabolites as fast as 0.5 h in the fermentation and their level lowered at 24 h. The major phenolic metabolites present at 24 h were similar for both digested oat bran and pure phenolic acid treatments, these were 4-hydroxyphenylacetic acid, dihydoferulic acid, 4-hydroxybenzoic acid, and protocatcheuic acid. Evidence from multiple studies indicates that conjugation of phenolic acids greatly strengthens biological activities by longer bioavailability in plasma [254]. Studies have indicated that the appearance of oat-derived phenolics in the circulation following high oat phenolic intake is linked to health benefits [103, 151, 255, 256]. The hydroxylated phenolics have been shown to reduce the inflammatory response of human peripheral blood mononuclear cells through reduced secretion of IL-6, IL-1, and TNF-α [258, 259]. These findings suggest that the circulating concentrations of oat bran-derived phenolic compounds are biologically active and could contribute to the anti-inflammatory effect of the oats.

A chronic crossover human trial (**Chapter 4**.) was conducted to assess the effect of either a high or moderate avenanthramides and phenolic acid rich-wholegrain oat diet on gut health and to assess their anti - inflammatory effects. A previous study [23] had focused on oat effects on the gut microbiota however without considering phenolics and fibre content within and using a non – whole grain control, therefore not matching fibre intake.

Consumption of oat was not associated with significant changes in the plasma level of inflammatory markers. In contrast, in our previous *in vitro* study (**Chapter 3**), we detected several hydroxylated phenolics which have been shown to reduce the inflammatory response through reduced secretion of IL-6, IL-1, and TNF-α [258, 259]. To date, only a few intervention studies in healthy humans have explored the impact of whole grain oats on inflammation [298, 299], mainly in study populations with a high risk of cardiovascular diseases. As such, it is likely that to see such changes, populations with inflammatory status would need to be studied.

In the current study daily consumption of 66.8 g of oatmeal and 60 g of oatcake (providing 68.1 mg of phenolic acids, 4.8 g of β - glucan and 10.7 g of insoluble fibre) was linked to increases in the relative abundance of the *Sutterella* genus ($p = 0.02$) with medium effect size (0.5). Some volunteer responded with a huge increase in *Prevotella copri* to a high polyphenol treatment, however, it was not significant compared to other treatments due to big variation between volunteers. The high interpersonal variability in gut microbiome across individuals is challenging to overcome in my research [304, 305]. The PCA plot of beta-diversity from our volunteers suggests that differences induced by the dietary treatments were smaller than the differences between the volunteers gut microbiota. Therefore, the diversity in the individual baseline microbiome composition might lead to a wide range of responses.

The research presented in this thesis has led to the assessment of active moieties in whole grain oats and exerted their activities via the gut microbiota and highlighted the importance of food matrix against pure extracts. Overall, further human randomised controlled trials are warranted to fully establish the health implications of oat bio actives with respect to CVD and gut health.

5.2. Future perspectives

In the thesis, we addressed a number of research questions, while highlighting some opportunities for further research. The uptake and utilisation of microbial phenolics metabolites by the host results in a highly dynamic system of metabolic fluxes and makes the determination of changes in concentrations of metabolites with time very difficult using current single snapshot analyses in faecal samples. The properly designed metabolic tracer experiments with the application of stable isotope probes in combination with high resolution mass spectrometry and mathematical modeling may prove valuable in this area.

The human intervention study clearly demonstrated that the high avenanthramides and phenolic acids rich-wholegrain oats diet significantly increased the relative abundance of the *Sutterella* genus compared to a β - glucan matched control diet without alteration in the serum inflammatory markers. However further metagenomics, metabolomics, and proteomics integrated studies are required to obtain a holistic understanding of oat matrix effect on the gut microbiome. Metagenomics study can be used to identify the intestinal microbiome diversity [308], metabolomics is the construction of a metabolic signaling network [309] and complete with proteomics, the characterization of proteins and the functional activities of human gut microbiota [310].

Further research required to examine the oat and oatwell, oat-derived polyphenol induced *Sutterella* and *Prevotella* genus microbial metabolic activities and their interaction with the host, as well as of polyphenols' health benefits.

Furthermore, future randomised controlled trials with oat or β – glucan as the intervention are needed to determine the detailed characteristics of β – glucan and phenolic components to further investigate whether there is a synergetic interaction. Moreover, we have shown a large degree of inter-individual variability in gut microbiome compositions in volunteers, more volunteers should be used in future interventions to able to identify responders and non-responders. It has been suggested the response of microbiota to the intervention was dependent on the initial microbiota state. These findings perhaps introduce the way to focus on a specific target group to identify the nutrition intervention beneficial effects. Personalised medicine aims to develop tailor-made nutrition prevention approaches using the individual characteristics of patients. The combination and integrative omics analysis drive the personalised medicine forward to use artificial intelligence to analyse and find health trends in sets of big data.

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