The application of isothermal microcalorimetry for studying mixed probiotic cultures

MANSA FREDUA-AGYEMAN

Thesis submitted in accordance with the requirements of UCL

School of Pharmacy for the degree of Doctor of Philosophy

2014

UCL SCHOOL OF PHARMACY

29-39 Brunswick Square

London WC1N 1AX

1

Thesis declaration form

I confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signature:

Name: MANSA FREDUA-AGYEMAN

Date:

Dedication

This thesis is dedicated to my two beautiful daughters, Nana Serwaa Nhyira Fredua-Agyeman and Nana Konadu Aseda Fredua-Agyeman with love. You are now too young to read this thesis and may never read it even when you are old enough to read. If you are reading this thesis because you are considering a PhD, well done! (even if it is not your immediate step). Mind you, the road ahead is rough but you are more than capable. I love you and will support whatever you choose to be.

Your dearest mum,

M.D. Fredua-Agyeman

Acknowledgements

"And if by grace, then it cannot be based on works; if it were, grace would no longer be grace". Romans 11:6. Lord Jesus, thank you for your grace that has seen me through this PhD. Your grace was, is and will always be greater than my weaknesses.

I would like to express my deepest gratitude to my supervisors, Dr. Simon Gaisford and Prof. Abdul Basit for their support throughout this PhD. I am very grateful for their encouragement and the opportunities to present my work at different national and international conferences. I am also deeply grateful to Dr. Paul Stapleton and Prof. Anthony Beezer for their continuous support, useful advice and discussions that kept me going.

My heartfelt thanks to the Commonwealth Scholarship Commission for financial support and to the School of Pharmacy, University of Ghana, Legon for discharging me to pursue this PhD.

Special thanks goes to the following people: Isabel Gonçalves, Catherine Baumber, John Frost, Dr. David McCarthy, Kate Keen, Owen Shepherd, members of the registry of UCL School of Pharmacy, the library team and the school's technicians without whom, research and teaching in the school will not be possible.

I would also like to thank my fellow colleagues, particularly the members of Simon's and Abdul's research group: Luis, Jawal, Asma, Jip, Rin, Lily (adopted), Mustafa, Cornelius, Alex, Garima, Fozia, Jesse, Vivienne, Francisco, Hamid, Jie, Alvaro, Sarit and Vipul for their friendship, treats, and encouragement. Also, to Ramesh, Fouad, Naba, Zeneh, Mohammed, Magarida, Hanieh, Funmi, Mary, Athmar, Sahar, Sheliza, Laxmi, Samuel, Eranka, Tasnuva, Miao, Lin, Seen-Wai and all fellow PhD students kind enough to spare an instrument, ingredient or time.

I am appreciative of the brilliant MPharm and MSc students I was privileged to work with: Christine, Susan, Tahir, Shruti and Neha.

I would also like to acknowledge Symprove Ltd., in particular Barry Smith, Mike Butler and Clare Gurton whom we collaborated with during the course of this PhD and for sponsoring the ECCMID conference.

Abstract

The main aim of this research was to explore the potential of the isothermal microcalorimeter to detect bacteria in mixed cultures; applied to investigate the antagonistic effect of commercial probiotics against pathogens and each other; and also the prebiotic potential of a substrate. Gastric tolerance of commercial probiotic products was also investigated with an improvement on current methods.

An initial mixed culture study with *Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli* in the microcalorimeter showed that the microcalorimeter could detect their growth in mixed cultures; *S. aureus* was always outcompeted in growth. Antagonistic activity of probiotic strains, *Lactobacillus acidophilus*, *Bifidobacterium lactis*, *Bifidobacterium bifidum* or commercial probiotic products against *P. aeruginosa*, *E. coli*, *S. aureus* and the clinically important gut pathogen, *Clostridium difficile* was demonstrated in the microcalorimeter and was shown to be pH-dependent using neutralized and unmodified cell free culture supernatant (CFS) produced by the probiotic strains. But concentrated CFS of the probiotics also inhibited the pathogenic species in a non pH-dependent manner, likely due to specific antimicrobial substances or bacteriocins. The result also demonstrated that probiotic strains could compete with each other in growth when put together. The prebiotic potential of inulin was demonstrated with the microcalorimeter using faecal slurry and pure probiotic strains. Gastric tolerance assay of commercial probiotic products in porcine gastric fluid, SGF (acidified NaCl solution) and FaSSGF (acidified NaCl solution with biorelevant amounts of bile salt, pepsin and lecithin) mimicking the fed and fasted states showed significant differences between the products and fluids.

In conclusion, the research showed that the microcalorimeter is a useful *in vitro* tool for detecting bacterial growth in mixed cultures and studying functional characteristics of probiotics and prebiotics; overcoming some of the limitations of the conventional methods.

6

Contents

List of Figures

13

17

List of Tables

Abbreviations

ASA	Aminosalicylic acid
AUC	Area under the curve
BHI	Brain Heart Infusion
CD	Crohn's Disease
CDAD	*Clostridium difficile* Associated Disease
CFS	Cell Free Supernatant
CFU	Colony Forming Unit
CMM	Cooked Meat Medium
DMSO	Dimethyl sulfoxide
DSC	Differential Scanning Calorimetry
FAO	Food and Agricultural Organization of the United Nations
FaSSGF	Fasted State Simulated Gastric Fluid
FISH	Fluorescent In Situ Hybridization
FOS	Fructo-oligosaccharides
g	Acceleration due to gravity
GI	Gastrointestinal
h	Hour
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
IC	Isothermal Calorimetry
ISA	Iso-sensitest Agar
ISAPP	International Scientific Association for Probiotics and Prebiotics
J	Joules
kDa	Kilodalton
MCA	MacConkey Agar
min	Minute
MMC	Migrating Myoelectric Complex
MRS	deMan Rogosa Sharpe
MSA	Mannitol Salt Agar
NB	Nutrient Broth
nm	Nanometres
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PCR-DGGE	Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis

PFGE	Pulsed Field Gel Electrophoresis
PGF	Porcine Gastric Fluid
PPI	Proton Pump Inhibitor
PVP	Polyvinylpyrrolidone
qPCR	Quantitative Polymerase Chain Reaction
qT-RFLP	Quantitative Terminal Restriction Fragment Length Polymorphism
RAPD	Randomly Amplified Polymorphic DNA
RCM	Reinforced Clostridial Medium
RT	Room Temperature
s	Second
SCFA	Short Chain Fatty Acids
SGF	Simulated Gastric Fluid
SHIME	Simulator of the Human Intestinal Microbial Ecosystem
TAM	Thermal Activity Monitor
T_g	Glass Transition Temperature
TSB	Tryptone Soya Broth
UC	Ulcerative Colitis
WHO	World Health Organisation
µW	Micro-Watt

Chapter 1 Introduction

1.1 Overview

Probiotics are bacteria related to those found naturally in the human gut. It is claimed that ingesting probiotics could have beneficial effects in several biomedical conditions: diarrhoea, constipation, colitis, flatulence, gastroenteritis, gastric acidity, immunostimulation, hypercholesterolemia, hepatic encephalopathy and carcinogenesis (Gibson and Roberfroid, 1995). The belief that these positive effects of probiotics can be attained is based on the knowledge of beneficial effects of intestinal microbiota (Fuller, 1991). The mechanism by which their beneficial effects could be achieved has been proposed to include production of antimicrobial substances, competition for adhesion receptors, competition for nutrients and stimulation of immunity (Fuller, 1991, O'Toole and Cooney, 2008, Bermudez-Brito et al., 2012).

There is a large market for probiotics worldwide. In Western Europe alone, the consumer market for probiotic food is more than 1.4 billion euros (Saxelin, 2008). A global sale of probiotics amounted to $24.23 billion in 2011 and has been forecasted to rise steadily (Pedretti, 2013). The commercial growth of probiotics is largely attributed to their claimed health benefits.

To have a potential health benefit in a host, probiotics require certain functional characteristics apart from satisfying safety and technological characteristics (Sanders and Huis in't Veld, 1999, FAO/WHO 2001, Kailasapathy and Chin, 2000, Iannitti and Palmieri, 2010). The functional characteristics required include their adhesion to gut cells, ability to modulate immune responses and production of antimicrobial compounds (Sanders and Huis in't Veld, 1999, FAO/WHO 2001, Kailasapathy and Chin, 2000, Ouwehand et al., 2002). These functional characteristics could be absent in some strains, which satisfy safety characteristics and could also be compromised during industrial production (Gismondo et al., 1999, Annuk et al., 2003). Apart from these functional characteristics, it is important that probiotic products have sufficient numbers of viable cells and must be resistant to gastrointestinal barriers to bring about beneficial health effects (Sanders and Huis in't Veld, 1999, FAO/WHO 2001, Minelli and Benini, 2008, Champagne et al., 2011).

In vitro methods for characterizing most of these functional properties are conducted using conventional culture techniques (Charteris et al., 1998, Annuk et al., 2003, Mishra and Prasad, 2005, Masco et al., 2007, Kotsou et al., 2008, Bao et al., 2010, Tham et al., 2012). Methods based on conventional culture techniques are labour intensive and time consuming. For instance, for the determination of the antagonistic effect or antimicrobial production of potential probiotics, the probiotic species or their bioproducts are cultured together with pathogenic species, and changes in population determined by plating out (Annuk et al., 2003, Trejo et al., 2006, Schoster et al., 2013). When complexity of the gut is simulated through use of faecal components or multi probiotic species and pathogenic species are encountered, the workload increases due to the need for multiple sub-cultures to distinguish growth inhibition amongst the species (Tejero-Sarinena et al., 2013). This may require further biochemical assays for identification of species which could take several days or require the use of molecular techniques, in which identification is also a multi-step process requiring specialized reagents, equipments, trained personnel, and sometimes unable to distinguish viable and non-viable cells (Spiegelman et al., 2005, O'Mahony and Papkovsky, 2006, Nagarajan and Loh, 2014).

Thus, in the assessment of some functional characteristics, probiotics may be encountered as mixed cultures: with themselves or with pathogenic organisms. In microbiological assays where differential enumerations of multiple species are encountered, such assays could be tedious, requiring multiple subcultures and techniques (Spiegelman et al., 2005, Schmidt et al., 2007).

The work reported in this thesis explored the use of isothermal microcalorimetry to study some characteristics of probiotics, particularly the antagonistic effect of probiotics; where probiotics are encountered as mixed cultures and there was a need to differentially detect them in mixed cultures to determine their relationship with other species. Other functional aspects of probiotics were characterised using isothermal microcalorimetry.

Isothermal microcalorimetry is a technique based on the principle of measurement of heat and can be used for real-time monitoring of growth of bacteria. In an appropriate medium, bacteria utilize the energy sources provided for respiration and fermentation to form new cellular materials. These metabolic processes are heat evolving or consuming resulting in the exchange of heat energy to or from the environment and this can easily be detected by the microcalorimeter. It is a very simple, sensitive and fast technique, and

analyses heterogeneous systems non-destructively. Although it is not a very common technique among microbiologists, it is an established method that has been successfully applied in various disciplines for the detection and characterization of bacteria and other microorganisms mostly in pure culture (Zhao et al., 2000, O'Neill et al., 2003, Gaisford et al., 2009, Xing et al., 2011) and complex polymicrobial systems (Beaubien et al., 1987, Braissant et al., 2010b, Bravo et al., 2011). However its potential use for the study of differential growth of systems consisting of defined multiple species of bacteria has been just explored (Vazquez et al., 2014). Its use for characterizing potential health benefits of probiotics has also not been pursued. It must be mentioned that the capacity of isothermal microcalorimetry to discriminate between species of bacteria has been established (Newell, 1980). It was therefore hypothesized in this work that such discrimination could be used for the dectection of growth in mixed bacterial cultures for the study of some functional properties of probiotics.

This introductory chapter provides relevant background information to probiotics, the principle of isothermal microcalorimetry and its application to microbial systems. For the understanding of the scope of this research, which focuses on probiotics and their association to human health, some ecological considerations on the gut microbiota are also given.

1.2 Gut microbiota-the source of probiotics

1.2.1 Composition of the human gut microbiota

The human gastrointestinal (GI) tract (Figure 1.1), sub-divided into the mouth, oesophagus, stomach, small intestine and large intestine harbours a complex ecosystem consisting of trillions (approximately 10^{13} to 10^{14}) of microbes (Ley et al., 2006a) with approximately 500 to 1000 bacterial species (Xu and Gordon, 2003) and an estimated weight between 200 g and 1 kg (Gibson and Williams, 1999, Liddell, 2014). These microbes, together with those that inhabit the skin and mucosa of human outnumber the human cells by a factor of ten (Hooper and Gordon, 2001, Dethlefsen et al., 2006) and have a collective genome termed microbiome (Ley et al., 2006a, Ley et al., 2008). The human gut microbiota contains at least 100-fold more genes as the human genome and is often referred to as the hidden metabolic 'organ' of the body due to its enormous impact on ones wellbeing, including ones metabolism, physiology, nutrition and immune function (Ley et al., 2006a, Guinane and Cotter, 2013).
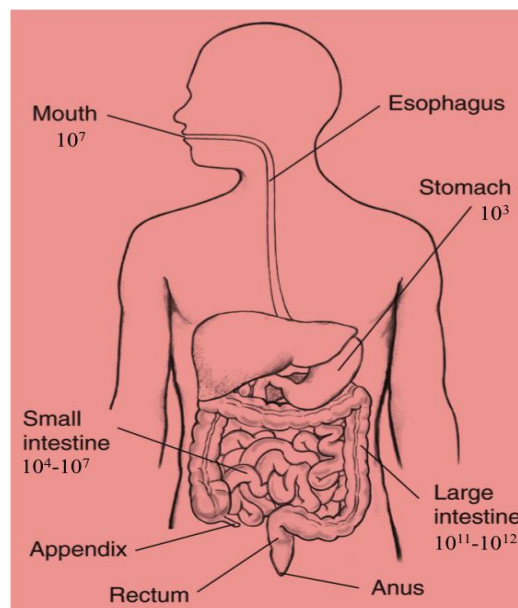
Figure 1.1. Anatomy of the human gastrointestinal tract showing the amount of bacteria in each region, figure adapted from (NIH. September 2013. Colonoscopy. [online]. Available from: http://catalog.niddk.nih.gov/ImageLibrary/detail.cfm?id=1450 [Accessed 7 May 2014])

The human gut microbiota contains three dominant bacterial phyla distributed throughout the gut: the Gram-positive Firmicutes, the Gram-negative Bacteroidetes, and

the Gram-positive Actinobacteria. The Firmicutes consist of over 200 genera including *Lactobacillus*, *Bacillus*, *Clostridium* and *Mycoplasma* (Vrieze et al., 2010). This phylum has been identified as the most prevalent in the human gut. The Bacteroidetes also consists of about 20 genera and include *Bacteroides*, *Prevotella* and *Porphyromonas* making up 9 to 42% of total bacteria in the gut (Dore and Corthier, 2010). The Actinobacteria, also a dominant phylum of the gut microbiota, consists of *Bifidobacterium* and *Colinsella-Atopobium* (Dore and Corthier, 2010, Vrieze et al., 2010).

Although these microbes are distributed throughout the gut, their relative numbers in different parts of the gut differ. This is because of the variations of the physiological condition along the GI tract. The variations are mainly pH, redox potential, transit time, presence of proteolytic enzymes and bile (Macfarlane and Macfarlane, 2004, McConnell et al., 2008b). The luminal pH influences bacterial concentration in each section of the gut. Generally, a low pH kills most bacteria whilst the redox potential is influenced by the bacterial concentration in each of the GI sections. High bacterial concentrations are responsible for lower redox potentials (Sousa et al., 2008). Transit time as earlier mentioned can influence bacterial activity. Movement of digestive material through the stomach and the small intestine is relatively quick, with mean transit time of approximately 4 to 6 h coupled with the presence of gastric acid, bile, pancreatic and intestinal secretions makes the establishment of microbes in this region of the gut very difficult (Gorbach et al., 1967, Savage, 1977) whereas the flow of digestive material in the colon is markedly slow (with mean transit time of approximately 60 to 70 h) facilitating the establishment of a complex microbial ecosystem (Macfarlane et al., 1997, Macfarlane and Macfarlane, 2004).

The mouth has around 10^7-10^9 bacteria per ml of saliva with approximately 10 times more anaerobic bacteria than aerobic (Ross, 1971, Evaldson et al., 1982). Aas et al., (2005) has estimated 700 different species of bacteria in the mouth with 9 bacterial phyla and 1 archaeal phylum. *Streptococcus* is the most dominant genus in the mouth (Drasar et al., 1969a, Aas et al., 2005). Pei et al., (2004) has also established that there are 95 species of bacteria from 6 phyla in the oesophageal mucosa.

The normal concentration of bacteria in the stomach is between 10^1-10^3 CFU/ mL (Finegold, 1969, O'Hara and Shanahan, 2006). This lesser population as mentioned

previously is due to the lower gastric pH of the stomach and the relatively faster movement of digestive material through the stomach making the establishment of microbes in this region of the gut very difficult (Savage, 1977). However when food is ingested, the gastric pH increases and bacterial population increases to 10^4–10^8 CFU/mL. The pH falls down again once food mixes with the gastric juice, and only the acid-resistant bacteria and those that are able to associate with the epithelial surfaces survive (Drasar et al., 1969a). In the stomach the rate of passage of microbes exceeds their rate of multiplication (Savage, 1977). A 16S rRNA survey by Bik et al., (2006) found 128 bacterial species from 8 phyla, which they suggested could be residents (*Helicobacter pylori* was the only definite resident) or ingested strains (Bik et al., 2006). Most of the bacteria in the stomach are predominately Gram-positive and aerobic. The most commonly isolated genera of bacteria in the stomach include *Streptococcus*, *Staphylococcus* and *Lactobacillus* (Finegold, 1969, Savage, 1977). Yeasts are also often isolated (Drasar et al., 1969a).

Bacterial population in the small intestine (from the end of the stomach to the beginning of the colon) is estimated to be approximately 10^4 to 10^7 CFU/mL with an increasing population of anaerobic bacteria (Hayashi et al., 2005). The microbiota of the duodenum is known to resemble that of the stomach whereas, that of the ileum resembles that of the colon. In the duodenum, the bacterial concentration is around 10^3–10^4 CFU/mL and the major species are aerobic and Gram-positive bacteria. The microbial population of the duodenum includes *Lactobacillus*, *Streptococcus*, *Staphylococcus*, and anaerobic *Veillonella*. Anaerobic bacteria can be isolated in the duodenum but in very low concentrations. Very few microorganisms are found in the jejunum and the upper ileum. Organisms present in this area of the small intestine include *Lactobacillus* and *Enterococcus*. In the distal ileum, bacterial concentrations are much higher and Gram-negative bacteria are known to outnumber the Gram-positive organisms. Anaerobic bacteria present in high concentrations include *Bacteroides, Bifidobacterium*, *Fusobacterium* and *Clostridium*. In addition, *Streptococcus* (*Streptococcus faecalis), Staphylococcus, Lactobacillus, Clostridium perfringes, Veillonella* and occasionally *Escherichia coli* can be isolated (Drasar et al., 1969a, Drasar and Shiner, 1969b, Gorbach et al., 1967, Gorbach, 1971, Thadepalli et al., 1979).

The colon is the most densely colonized part of the gut. Its bacterial population is around 10^{11} to 10^{12} CFU/mL containing approximately 800 species representing 9 bacterial phyla and 1 archaeal phylum (Ley et al., 2006a). The high bacterial population in this

region of the gut can be credited to the almost neutral pH as a result of the neutralisation of the contents of the bowel by the juice of the intestine as well as the decreased rate of movement of the contents in the large intestine (Savage, 1977, Macfarlane et al., 1997). This part of the GI tract primarily functions as a site for reabsorption of water from chyme, with less mixing and propulsive movements; functioning as a reservoir. The sluggish rate of movement of the luminal contents in the colon exceeds the doubling times of bacteria hence they proliferate (Savage, 1977). The main bacterial population present in this section of the GI tract is that of oxygen-intolerant anaerobic bacteria of various types (Gorbach, 1971) reflecting the fact that the oxygen concentration in the colon is very low (Roediger, 1980, Quigley, 2013). It is estabalished that the anaerobes outnumber the facultative anaerobes by a factor of 10^3 to 10^4 in the colon (Gorbach, 1971). The prevalent species isolated include *Bacteroides, Bifidobacterium* and *Eubacterium* (Gorbach et al., 1967, Hill and Drasar, 1975). Also anaerobic Gram-positive cocci*, Clostridium, Enterococcus* and other species of *Enterobacteriaceae, Diphtheroides,* coliforms*, Staphylococcus, Lactobacillus, Spirochetes,* yeasts, *Proteus, Pseudomonas, Bacillus subtilis, Actinomyces, Borrelia*, *Fusobaterium* and *Clostridium* species have also been found (Moore et al., 1969, Moore and Holdeman, 1974, Hill and Drasar, 1975, Moore et al., 1975, Savage, 1977).

Overall, *Bacteroides* species have been noted to account for nearly 32% of all organisms isolated from the GI microbiota. Some studies have pointed to the fact that the Gram-negative rods belonging to the *Bacteroides fragilis* group are numerically the predominant organisms and *account* for up to 30% of total anaerobes (Moore and Holdeman, 1974, Macy and Probst, 1979). Bifidobacteria have also been reported to account for up to 25% of the total cultivable gut flora (Mitsuoka, 1984). It has also been claimed that the bifidobacteria are the dominant species of the microbiota of healthy new born infants (Long and Swenson, 1977, Kawase et al., 1983, Heine, 1996) but then the bifidobacteria population decreases progressively as the infant ages (Thompson-Chagoyan et al., 2007).

It must be mentioned that much of the information regarding the composition of the gut microbiota has come from studies that used culture-based techniques (Guarner and Malagelada, 2003a). As a result, a thorough description of the human gut microbiota has not been established yet (Quigley, 2013) since several bacteria (more than 30%) that can be seen and counted by direct microscopic examination of faecal samples are not

recoverable by culture-based techniques (Holdeman et al., 1976, Guarner and Malagelada, 2003a). However the advent of non-culture based, high-throughput sophisticated methodologies is now facilitating the detailed description of the composition of the human gut microbiota in a way that has never been possible before (Quigley, 2013).

1.2.2 Significance

The gut microbiota are known to play vital roles in the human body. They are known to contribute towards human nutrition by synthesizing and secreting vitamins in excess of their own needs, which are absorbed as nutrients by humans (Hill, 1997). Evidence of this has been demonstrated with the requirement of supplementation with vitamins B and K for rodents reared in a sterile environment (germ free rodents) consuming standard chow compared with conventional rodents (Savage, 1986). They are also responsible for fermenting parts of the diet, which are not digested in the upper part of the digestive system. The products of carbohydrate fermentation of these bacteria are mainly short-chain fatty acids (SCFA), essentially acetate, propionate and butyrate and other metabolites such as lactate, pyruvate, ethanol and succinate (Cummings, 1981, Macfarlane and Macfarlane, 1997). The products of fermentation manipulate intestinal motility, aiding in digestion and peristalsis (Dass et al., 2007) as well as contribute to the energy requirement of the host (Saulnier et al., 2009). Acetate is a primary substrate for cholesterol synthesis (Ukai et al., 1976, Wong et al., 2006). It is metabolised in the kidney, heart, and muscles (Lindeneg et al., 1964). Butyrate is metabolised by the colonic epithelium where it serves as its major energy source (Cummings, 1981). It is known for its prevention of colon cancer by stimulating apoptosis and modulating the immune system (Roberfroid et al., 2010). Propionate, a gluceogenic substrate, is also important in glucose and lipid metabolism (Reshef et al., 1967). It inhibits cholesterol synthesis in hepatic tissue (Wong et al., 2006).

The gut microbiota also contribute towards metabolism of xenobiotic compounds including drugs (Ilett et al., 1990). While this has had toxicological implications of drugs, it has also been exploited pharmaceutically for applications such as site specific delivery and prodrug approach (McConnell et al., 2008a, Sousa et al., 2008).

The gut microbiota also functions to protect the gut against colonization by pathogens (Fuller, 1991). This is achieved by competitive exclusion such as occupation of attachment sites, consumption of nutrient sources and production of antimicrobial compounds (Sekirov et al., 2010). This protective function has been demonstrated by the fact that germ free animals are more susceptible to infections than their conventional counterparts. This difference was shown by infections caused by *Salmonella enteritidis* (Collins and Carter, 1978) and *Clostridium botulinum* (Moberg and Sugiyama, 1979). In humans, this has been evidenced from infections with *Clostridium difficile* characterised by pseudomembranous colitis after antibiotic treatment which is reversed by administration of faecal enemas from healthy adults (Schwan et al., 1984, Fuller, 1991).

The gut microbiota also play important role in the development of tissues like the caecum and development of both the intestinal mucosal and systemic immune systems (Macpherson and Harris, 2004, Sekirov et al., 2010). This again has been demonstrated in studies in which germ free animals showed deficits in local and systemic lymphoid structures; poorly formed spleens and lymph nodes and smaller Peyer's patches (Macpherson and Harris, 2004).

The gut microbiota has been known to be important in diseases such as inflammatory bowel disease, allergies, obesity, diabetes, non-alcoholic fatty liver diseases, cancers and other metabolic and degenerative disorders (Savage, 1977, Ley et al., 2005, Dethlefsen et al., 2006, Dore and Corthier, 2010).

1.2.3 Acquisition

Shortly after birth, microorganisms colonize the human gut, which is initially known to be sterile. These microorganisms are derived from the mother's faecal and vaginal microbiota, maternal milk (West et al., 1979, Perez et al., 2007) as well as the environment. It is thought that colonization starts during the process of childbirth. Some researchers have suggested that colonization is complete after 1 week, reaching levels of 10^{11} bacteria per gram of stool (Hooper and Gordon, 2001, Bjorksten, 2006, Dore and Corthier, 2010). Vrieze et al., have also mentioned that the microbiota changes after the initial colonization as a result of changes in diet and that an individual's microbiota is fully matured at 4 years (Vrieze et al., 2010).

It has been proposed that the type of delivery: vaginal verses caesarean, hygiene (Adlerberth et al., 2006), geographical location (Fallani et al., 2010); all play significant roles in the dynamics of colonization. For example, it is known that births by caesarean section are associated with high colonization by *Klebsiella, Enterobacter* and clostridia (Conroy et al., 2009) which are organisms usually found in the hospital surroundings and are acquired from the air, equipment, nursing staff, the mother and other infants (Tennyson and Friedman, 2008). On the other hand, vaginal birth is often associated with a higher colonization by bifidobacteria (Chen et al., 2007a) which is acquired mainly from maternal flora. Tennyson and Freidman (2008), have suggested that the longer the birth process, the greater the exposure of the maternal bacteria to the infant. The type of feeding of infants also affects the colonization pattern. Formula fed infants have shown an increased amount of clostridia and *Bacteroides* compared to breastfed infants (Conroy et al., 2009) who have high amounts of bifidobacteria (Tennyson and Friedman, 2008). Tennyson and Friedman have also established that after the introduction of solid food, the intestinal microbiota of both breastfed and formula fed infants becomes similar.

The gut microbiota of humans evolves during different stages of life, from infancy through adulthood to old age. The transformation from infant to adult-type microbiota has been proposed to be triggered by numerous host and external factors; stabilizing thereafter until the 7th decade albeit there will be inconsequential fluctuation of the established colonisers of the individual (Brown et al., 2007, Ley et al., 2006b). There are also significant fluctuations in disease states and during treatments with drugs. For example, short term treatment with a single dose of an oral antibiotic has led to changes of the composition of the gut microbiota for as long as 4 weeks before returning to the original composition (Dethlefsen et al., 2008b).

The ultimate composition of the gut microbiota of an individual is known to be determined by: his or her physiology, colonisation history, environment and genetic makeup (Gordon et al., 2009). Some of these factors are more important than others. For example, the genetic makeup of an individual is more important in determining the composition of his or her microbiota than the environment. Zoetendal et al., (2001), were able to establish that the microbiota of identical twins living separately was similar than the microbiota of unrelated individuals. In contrast, a couple living in the same

environment with similar dietary habits did not show similarity in their microbiota making environment a less important factor in gut colonisation.

The microbiota diversity and distribution has been related with certain conditions and diseases. However, there is generally a huge variability of microbiota between individuals (Ley et al., 2006b, Zoetendal et al., 2006) and this variability sometimes complicates studies attempting to establish the relationships between disease or host health and specific gut microbial population.

1.2.4 Homeostasis and Dybiosis

Even though the microbiota of the gut may vary considerably from one individual to another, the dynamics and homeostasis of the gut microbiota may be considered specific for a given individual. Thus, the composition of the microbiota can be said to be consistent in an individual over time. Under normal physiological conditions, the dominant phyla are stable throughout an individual's life. Even, dominant species, specific to an individual will remain dominant throughout life (Zoetendal et al., 1998, Vanhoutte et al., 2004). However, the same cannot be said for individual strains, as not much research has been done in this area. Although, the environment, presence of exogenous substrates and diet may cause changes in the microbiota of an individual as mentioned earlier, these changes are sometimes not remarkable and even if they tend to be, they only occur over a short period and sometimes has no impact on the individual. For example, a modified microbiota caused by the use of antibiotics can see a return to the normal dominant profile within a month without causing a pathological state (Dethlefsen et al., 2008a, De La Cochetiere et al., 2005). Thus, the microbiota of each individual has got a resilience factor that allows it resist changes induced by stress (De La Cochetiere et al., 2005, Dore and Corthier, 2010). It has been implied that genotype could play a role in resilience of the microbiota and also in the development and structuring of the microbiota and this has been linked to the fact that the faecal microbiota profile of monozygote twins are similar than the microbiota of an unrelated individual (Zoetendal et al., 2001, Dore and Corthier, 2010). However, excessive stress could disturb the equilibrium of an individual's microbiota, resulting in an irreversible perturbation (Dore and Corthier, 2010). The threshold above which one's microbiota

loses its ability to return to its original balance has however not been clearly defined (Dore and Corthier, 2010).

Dysbiosis is the term used when the composition of the microbiota of the gut is thrown out of a state of equilibrium (due to excessive stress) leading to a state of imbalance, which could consequently affect a host metabolically or immunologically (Round and Mazmanian, 2009). The microbiota has been described as a balanced composition of different classes of bacteria consisting of symbionts, commensals and pathobionts (Round and Mazmanian, 2009). Symbionts are those bacteria of the microbiota, which have health promoting effect. Commensals are those that have neither health promoting nor depreciating effect and pathobionts are those bacteria of the gut, which have the potential to cause diseases (Round and Mazmanian, 2009). A balanced composition of these different classes of bacteria in the GI is responsible for the known health effects of the microbiota (Figure 1.2; Roberfroid et al., 2010). In dysbiosis, it has been proposed that there is either a reduction in the number of symbionts or an increase in the number of pathobionts (Round and Mazmanian, 2009).
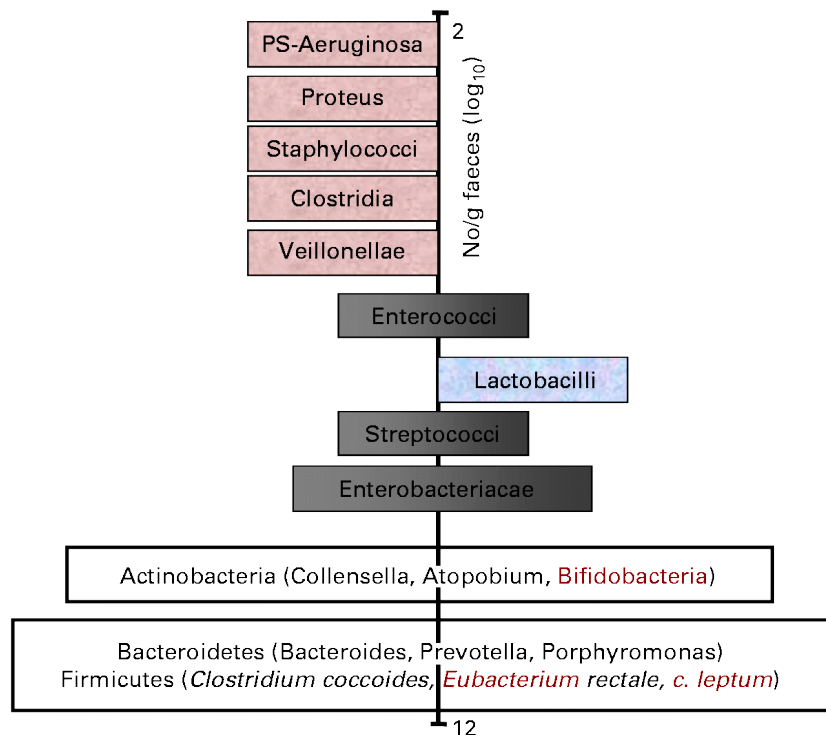
Figure 1.2. A scheme describing the hypothesis of a balanced microbiota of an adult proposed by Gibson and Roberfroid, (1995) and revised by Roberfroid et al., (2010). The figure shows the number of CFU/g of faeces of major phyla/genera. The bacteria are divided into three groups: those groups that have harmful or pathogenic influences on human health (located on the left), those that have beneficial effects (located on the right), and those that may have both (located on both sides, also contain genera/species yet to be classified). Obtained from Roberfroid et al., (2010).

Dysbiosis could be caused by, for example, excessive lifestyle of host, host genetics, medical practices: overuse of antibiotics and vaccination (Round and Mazmanian, 2009). It is believed that certain diseases are the result of dysbiosis. Diseases that have been linked to dysbiosis include celiac disease (De Palma et al., 2010), type 2 diabetes (Larsen et al., 2010), obesity (Ley et al., 2005, Ley et al., 2006b, Turnbaugh et al., 2009, Zhang et al., 2009), IBD (Tannock, 2008, Seksik, 2010) and chronic diarrhoea (Swidsinski et al., 2008). Table 1.1 lists some members of the microbiota that are known to increase or reduce in diseases associated with dysbiosis.

Table 1.1. Diseases associated with dysbiosis and members of the microbiota implicated

Human disease	Abundance	Reduction
Celiac disease	Gram-negatives, *Bacteroides-Prevotella*, *Escherichia coli*	Gram-positives, *Bifidobacterium*, *Clostridium histolyticum* and *C. liteseburense*, *Faecalibacterium prausnitzii*
Inflammatory bowel diseases	*Enterobacteriaceae*, *Bacteroidetes*, *Enterococcus*, *Clostridium difficile*, *Escherichia coli*, *Shigella flexneri*, *Mycobacterium paratuberculosis*, *Listeria monocytogenes* and paramyxovirus	*Firmicutes*, *Eubacterium rectale*, *Bacteroides fragilis*, *B. vulgatus*, *Ruminococcus albus*, *R. callidus*, *R. bromii*, and *Fecalibacterium prausnitzi*
Obesity	*Firmicutes*	*Bacteroidetes*, *Bifidobacterium*, *Staphylococcus aureus*
Type 2 diabetes	*ß-Proteobacteria*, *Bacteroides*, *Parabacteroides*	*Firmicutes*, *Clostridium*, *Bifidobacterium*, *Bacteroides vulgatus*
Chronic diarrhoea	*Enterobacteriaceae*, *Eubacterium cylindroides*, *Clostridium histolyticum*, and *Clostridium lituseburense*.	*Eubacterium rectale*, *Bacteroides* and *Faecalibacterium prausnitzii*

Despite the inter-individual variation of the microbiota, a number of recurring features have also been mentioned to generally characterise dysbiosis (Walker and Lawley, 2013). For example, the overall diversity of the microbiota is known to reduce. Likewise, irrespective of the disease state, there are also reductions in the population of obligate anaerobic members of the Firmicutes, and a corresponding increased population of facultative anaerobes, especially members of the Enterobacteriaceae family of the Proteobacteria phylum which consists of a number of strict and opportunistic pathogens such as *Salmonella* spp., *Shigella* spp., *Klebsiella* spp., *Proteus* spp. and *E. coli* (Walker and Lawley, 2013).

1.3 Probiotics

Since it is a balanced composition of one's microbiota that is responsible for the known health effects of the microbiota (Round and Mazmanian, 2009), there has been much interest in deliberately manipulating the normal microbiota to accrue health benefits through using "probiotics". Probiotics are bacteria related to those found naturally in the human gut, notably those supposedly found in the gut of breastfed infants because they are known to be significant in contributing towards the health effects of the microbiota (Iannitti and Palmieri, 2010). When ingested, they are claimed to prevent or treat dysbiosis by changing the ecology of the gut microbiota towards a healthier composition. Its concept dates back to over 100 years when the Russian Nobel laureate, Elie Metchnikoff linked the longevity of Bulgarian peasants to their consumption of a fermented milk product that contained lactic acid bacteria. He therefore suggested that ingesting these "useful microbes" could replace harmful microbes and consequently have beneficial effects for man (Metchnikoff, 1908). During the time of Metchnikoff's discovery, a French paediatrician, Henry Tissier also noticed that children with diarrhoea had lower number of peculiarly Y-shaped bacteria "bifid bacteria" in their stools compared with healthy children who had an abundant amount of those bacteria (Tissier, 1906). Tissier therefore suggested that these bacteria could be given to patients with diarrhoea to treat them. Even though these two scientists introduced the concept of probiotic, the term was only coined in 1965 by Lilly and Stillwell (Lilly and Stillwell, 1965) to describe substances secreted by one organism, which stimulated the growth of another. This description has however been rephrased/ redefined a number of times (Fuller, 1989, Fuller, 1991, Havenaar and Huis In't Veld, 1992, Guarner and Schaafsma, 1998) but the World Health Organisation (WHO) and Food and Agricultural

Organization of the United Nations (FAO) have jointly adopted the definition of probiotics as "live microorganisms, which, when administered in adequate amounts, confer a health effect on the host." Recently, the International Scientific Association for Probiotics and Prebiotics (ISAPP), have made minor grammatical correction to the definition as "live microorganisms that, when administered in adequate amounts, confer a health effect on the host" (Hill et al. 2014). Microorganisms used as probiotics are usually species of lactic acid bacteria and bifidobacteria. They generally include members of the genera *Lactobacillus* and *Bifidobacterium*. Also, members of the genera *Streptococcus* and *Enterococcus and Lactococcus* spp*., Bacillus* spp. and some yeast, for example *Saccharomyces boulardii*, have been identified as probiotics (Salminen et al., 1998, FAO/WHO 2001, Iannitti and Palmieri, 2010). These organisms are generally applied in food (yogurt, cheese, fermented milk products) or used as dietary supplements (for example, capsules, tablets, powders and solutions).

1.3.1 Properties of probiotics

For an organism to be considered a probiotic, and used in food, the WHO/FAO guideline stipulates that it should be safe and non-pathogenic. It must survive passage through the digestive tract and be capable of proliferation in the gut. Which according to the guideline, means they must be "resistant to gastric juices and be able to grow in the presence of bile under conditions in the intestines, or be consumed in a food vehicle that allows it to survive passage through the stomach and exposure to bile." Apart from these properties, it is also proposed that all probiotics either included in food or used as supplement must also reduce pathogenic adherence and be able to adhere to cells, persist, multiply and produce acids, peroxides and bacteriocins that are antagonistic to pathogenic adherence and should co-aggregate to form a normal balanced flora and demonstrate health benefits. They must also be amenable to industrial processes and remain viable in a product throughout its shelf life (Collins and Gibson, 1999, Gismondo et al., 1999, Kailasapathy and Chin, 2000, Iannitti and Palmieri, 2010).

1.3.2 Health benefits of probiotics

Given the significant effect of the gastrointestinal microbiota on human health and disease, probiotic consumption has been linked to a number of health benefits. These

health benefits can be categorized into nutritional and therapeutic as likened to the benefits of the normal microbiota. Nutritionally, they are believed to improve digestibility by breaking down complex carbohydrates, (which are otherwise non-digestible) producing absorbable substrates and energy, also secreting β-D galactosidase, which metabolize lactose into glucose and galactose (Shah, 2007). They are also known to synthesize and excrete vitamins, which are used by man (Rossi et al., 2011). Therapeutically, they are known to prevent and alleviate complains of the GI tract and other ailments. For instance, ingestion of probiotics has been shown to prevent the occurrence of diarrhoea (Saavedra, 2000). The mechanism by which this is achieved has been suggested to be by competitive exclusion (Steer et al., 2000); they are thought to inhibit the adhesion of pathogens to intestinal mucosa and produce SCFA, organic acids, peroxides, and other antimicrobials, which kill off pathogens. In constipated patients, these, SCFA can stimulate intestinal peristalsis which help in normal bowel movement (Arunachalam, 1999). Probiotics are claimed to reduce cancer risk by reducing the concentration of cancer-promoting enzymes and/or putrefactive (bacterial) metabolites in the gut (Goldin and Gorbach, 1984, El-Nezami et al., 2000, Oatley et al., 2000). They are also believed to prevent or alleviate allergies and atopic diseases in infants (Majamaa and Isolauri, 1997, Kalliomaki et al., 2001, Isolauri et al., 2000, Rosenfeldt et al., 2003), prevent respiratory tract infections (common cold, influenza) and other infectious diseases as well as help in the treatment of urogenital infections (Rio et al., 2002, Hatakka et al., 2001, Reid, 2001b, Marelli et al., 2004). They are also known to improve autoimmune diseases like arthritis (Malin et al., 1997). Certain probiotics are believed to stimulate the immune system; they increase mucosal antibody production, boost pro-inflammatory cytokine expression, and enhance host defensin production (Sokolnicka, 2000). They are also considered to have a hypocholesterolemic effect and reduce the risk of ischemic heart diseases (Naruszewicz et al., 2002, Agerholm-Larsen et al., 2000). A good account of health benefits of probiotics are reviewed in these articles: Goldin, 1998, Saarela et al., 2000, Kailasapathy and Chin, 2000, Ouwehand et al., 2002, Salminen et al., 2005, Parvez et al., 2006, de Vrese and Schrezenmeir, 2008, Saulnier et al., 2009, Masood et al., 2011.

Although the claimed effects of probiotics are endless due to their relation with the gut microbiota, there are however differing degrees of evidence supporting their purported benefits. Nonetheless, there is also some clinical evidence to suggest that specific probiotics can be effective in the treatment of some dysbiosis-related diseases. Table 1.2

summarizes some studies on diseases which probiotics have been shown to evidentially alleviate.

Table 1.2. Proven health effects of probiotics

Disease	Probiotic	Study	Outcome	Reference
Diverticular disease	VSL#3 (Probiotic of different strains of *Bifidobacterium* and *Lactobacillus*)	To determine whether probiotic could prevent reccurence of disease and alter the colonic bacteria to metabolize balsalazide into 5-ASA and 4-aminobenzoil-β-alanine	Probiotic in combination with anti-inflammatory drug was found better in treatment and preventing relapse of Diverticular disease than single use.	(Tursi et al., 2007)
Irritable bowel syndrome (IBS)	*L. plantarum* *L. reuteri* *S. boulardiii*	To determine whether probiotic could improve symptoms of IBS	Flatulence, abdominal pain, pain frequency and overall symptoms of IBS improved for most of the probiotics studied.	(Guslandi, 2007)
Inflammatory bowel disease (IBD)	*B. longum*	Synbiotic of *B. longum* and prebiotic (inulin-oligofructose) given to patients with Ulcerative colitis	Improved inflammation in Ulcerative colitis patients.	(Furrie et al., 2005)
	S. boulardii	Probiotic added to baseline therapy in Crohn's disease	Improved intestinal permeability but not complete normalization of the disease.	(Vilela et al., 2008)
Colon cancer	*L. rhamnosus LC705 (LC705)* *Propionibacterium freudenreichii* ssp *shermanii JS (PJS)*	To examine whether the administration of *LC705* and *PJS* could decrease the activity of faecal β-glucosidase, β-glucuronidase, and urease	*LC705* and *PJS* were associated with a decrease in β-glucosidase activity.	(Hatakka et al., 2008)

Table 1.2. Proven health effects of probiotics (continued)

Disease	Probiotic	Study	Outcome	Reference
Diarrhoea	*Lactobacillus GG* *B. bifidum* and *S.thermophilus* VSL#3 Bifilac (synbiotic)	Different studies involving the use of these probiotics or synbiotic to evaluate their efficacy in reducing the frequency and duration of diarrhoea induced by rotaviral infection	All studied probiotic or synbiotic reduced the rate of rotavirus-associated diarrhoea by reducing either the volume or frequency of diarrhoea.	(Saavedra, 2000) (Dubey et al., 2008) (Narayanappa, 2008)
H. pylori eradication	*L. acidophilus HY2177, L. casei HY2743, B. longum HY8001* and *Streptococcus thermophilus B-1*	These probiotics (delivered in yoghurt) was added to a Proton Pump Inhibitor (PPI)-based triple therapy to determine whether the probiotic could increase success in eradication of *H. pylori*	Probiotic was shown to increase success of *H. pylori* eradication	(Kim et al., 2008)
Bacterial vaginosis	*L. rhamnosus GR-1* and *L. reuteri RC-14*	To find out if the probiotics could help in curing urogenital infection when taken concomitantly with single dose tinidazole	There was an improved cure of the infection from 50% to 87.5% cure.	(Martinez et al., 2009)
Allergic disease	*L. reuteri ATCC 55730*	To investigate if probiotic could decrease the incidence of eczema of infants with a family history of allergic disease by giving mothers in their 36 gestational week and their babies the probiotic until 12 months old.	Infants had less IgE associated eczema at 2 years. Authors concluded that infants could possible have a reduced risk of developing respiratory allergic disease later on in life.	(Abrahamsson et al., 2007)

1.3.3 Antipathogenic activity of probiotics against potential pathogens

It is believed that the primary effect of probiotics in man is their ability to prevent pathogenic colonization (Fuller, 1991, Lu and Walker, 2001, Reid et al., 2001a). Certain features of probiotics have been shown to decrease pathogenic attack in man and these features include the ability to increase mucin production and reduce intestinal permeability (Caballero-Franco et al., 2007, Ewaschuk et al., 2008), strengthening the defence of man. Apart from these features, some probiotics are also thought to have some surface proteins that adhere onto the cell surfaces of its host and these prevent pathogenic bacteria from attaching to the same surfaces. For example, *Lactobacillus crispatus* has surface proteins (S-layer proteins) that are able to prevent the adhesion of *Escherichia coli* O157:H7 and Salmonella (Chen et al., 2007b).

Probiotics are also thought to compete for nutrients with pathogens and stimulate host's immunity. They are believed to produce antimicrobial compounds that are capable of inhibiting pathogens. The antimicrobial compounds include SCFA and organic acids, predominantly, acetate, butyrate, propionates and lactate. These generate an acidic environment that inhibits pathogens. They are also believed to synthesize specific proteins and peptides (bacteriocins) with inhibitory effect against some pathogens (Reid et al., 1990, Mishra and Lambert, 1996, Yildirim and Johnson, 1998). Table 1.3 shows the specific peptide and inhibitory spectrum respectively of some probiotics.

Table 1.3. A list of probiotics and the specific peptide (with antimicrobial properties) they produce

Probiotic	Peptide/ Protein	Properties/Inhibitory Spectrum	Reference
B. bifidum NCFB 1454	Bifidocin B	Active within pH 2 to 10. Stable at 121°C and -20°C. Sensitive to several proteolytic enzymes (protease IV, pronase E, protease XVII, proteinase K, trypsin, alpha-chymotrypsin, papain, and pepsin) but resistant to catalase, peroxidase, lipase, lysozyme, cellulase, ribonuclease A, and amylases. Resistant to organic solvents and heat. Active against *Listeria, Enterococcus, Bacillus, Lactobacillus, Leuconostoc,* and *Pediococcus* species.	(Yildirim and Johnson, 1998)
B. lactis BB-12	Bifilact BB-12	No activity at pH 3, -20°C or 121°C. Considerable biological activity retained at pH 4 and 7 but reduced at pH 9. Sensitive to pepsin and trypsin, but resistant to α-amylase or lipase. Inhibitory towards *Staphylococcus aureus, Salmonella typhimurium, Bacillus cereus* and *E. coli.*	(Abd El-Salam et al., 2004)
B. longum BB-46	Bifilong BB-46	Molecular weight of 25 to 127-kilodalton (kDa). No activity at pH 3, -20°C, 121°C. Considerable biological activity retained at pH 4 and 7 but reduced at pH 9. Sensitive to pepsin and trypsin, but resistant to α-amylase or lipase. Inhibitory towards *Staphylococcus aureus, Salmonella typhimurium, Bacillus cereus* and *E. coli.*	(Abd El-Salam et al., 2004)
L. sake MI401, *L. plantarum* ST31, *L. reuteri* LTH2584	Bavarin A, Plantaricn ST31, Reutericyclin	Optimum pH range 4-6. Resistant to heat and acidity, and inactivated by proteolytic enzymes, except for reutericyclin. Inhibit *Bacillus, Staphylococcus* and *Listeria* strains.	(Messens and De, 2002)
Lactobacillus gasseri SBT 2055	Gassericin T	Inhibits most Gram-positive food pathogens	(Kawai et al., 2000)

Table 1.3. A list of probiotics and the specific peptide (with antimicrobial properties) they produce (continued)

Probiotic	Peptide/ Protein	Properties	Reference
L. plantarum	Plantaricin 423	Resistant to treatment at 80°C, but loses 50% of its activity after 60 min at 100°C and 75% of its activity after 15 min at 121°C. Stable at pH 1-10 but inactivated when treated with pepsin, papain, a-chymotrypsin, trypsin and Proteinase K. It inhibits *Bacillus cereus, Clostridium sporogenes, Enterococcus faecalis, Listeria* spp. and *Staphylococcus* spp.	(van Reenen et al., 1998)
L. plantarum	Plantaricin TF 711	Stable to heat and to treatment with surfactants and organic solvents. Optimum antimicrobial activity is between pH 1 and 9. It inhibits *Bacillus cereus, Clostridium sporogenes and Staphylococcus aureus.*	(Hernandez et al., 2005)
Lactobacillus gasseri KT7	Gassericin KT7	Sensitive to proteolytic enzymes, resistant to heat, active over a wide range of pH. Active against food-borne pathogens including *Listeria, Clostridium* and *Enterococcus*.	(Zhu et al., 2000)
Lactobacillus gasseri LF221A	Acidocin LF221A/LF221 B	Molecular weight of 3.5 to 5-kDa. Hydrophobic and heat stable. Have inhibitory activity against *Lactococcus, Pediococcus, Staphylococcus, Enterococcus, Streptococcus, Listeria, Clostridium* and *Bacillus*.	(Majhenic et al., 2004)
Lactobacillus acidophilus LAP T1060	Acidophilucin A	Sensitive to proteolytic enzymes but stable at 120°C.	(Toba et al., 1991)
Lactococcus lactis subsp.*lactis* strains	Nisin	Molecular weight 3.5-kDa. Hydrophobic. Has inhibitory activity against a wide range of food-borne pathogens.	(Jack et al., 1995)

1.3.4 Probiotic market value

As the list of health benefits accredited to probiotics continues to increase, their industrial production and commercialization also continues to see a steep rise. It was estimated that the probiotic industry held about 10% share in the global functional food market, which represented €10 billion in 2008 (Champagne et al., 2011). The global probiotic supplement market was also said to be worth about $1.5 billion in 2008 (Champagne et al., 2011). The market is set to witness further notable growth as consumers become more mindful about their health and use probiotics for preventative measures and as newer types of probiotic products (for example biscuit, cereals, chewing gums, chocolates, honey, ice cream, juices, pizzas, aftershave, anti-aging serum, face and body lotion, toothpaste, tampons, shampoo, douche gel) find themselves on the shelves in supermarkets and pharmacies. Global probiotics market has been predicted to be worth $31.1 billion by 2015 and is forecasted to reach $48 billion in the next five years (Global Industry Analysis Report, Transparency Market Research, 2013). According to the report, Europe represents the largest and Japan the second largest market. Within Europe, United Kingdom and Germany represent the largest market with trade accounting for almost half of the total market in Europe.

With the increasing industrialization and commercialization of probiotics, more extensive research is needed to prove or support their health benefits; especially as new probiotic strains and new application of probiotics are being suggested.

1.3.5 Testing methods for establishing potential health benefits of probiotics

According to the WHO/FAO in its report on Expert Consultation on the Evaluation of Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria, tests for establishing health benefits of probiotics should first involve the use of an appropriate *in vitro* study before undertaking *in vivo* investigations. *In vitro* tests such as antimicrobial production, gastrointestinal tolerance and adherence ability of probiotic organisms to human intestinal cells predict functionality of probiotics (FAO/WHO, 2001) and are noted to be important for anticipated health benefits (Gismondo et al., 1999). The work in this thesis was focused on the antimicrobial production, gastric tolerance as well as the microbial stability of probiotics in mixed cultures.

1.3.5.1 Antimicrobial production

Traditionally, *in vitro* studies to determine the direct antagonistic activity of probiotics based on their antimicrobial substance production are done by two main methods even though there are adaptations to these methods. The first method is based on the observation of the growths of probiotics (or bioproducts from probiotics) and potential pathogenic organisms in a batch of medium or chemostats. Thus probiotics or their bioproducts are cultured together with pathogens of interest and changes in the microbial growth analyzed, usually by selective growth and colony counting (Apella et al., 1992, Drago et al., 1997, Annuk et al., 2003, Hutt et al., 2006), turbidity assay (Lee et al., 2003), biochemical methods or by molecular biological methods (Folkers et al., 2010, Tejero-Sarinena et al., 2013).

The second method, which is the most employed method for studying the antagonistic activity of probiotics is based on diffusion tests where potential probiotic is seeded on an agar plate together with a test bacterial organism (Annuk et al., 2003, Zhu et al., 2010) or agar plates spotted with probiotic organisms are overlaid with agar inoculated with pathogenic organisms (Barbosa et al., 2005, Chapman et al., 2012, Tejero-Sarinena et al., 2012). Alternatively bioproducts of potential probiotics are applied into punched wells of agar plates or impregnated into disks, which are placed on agar plates seeded with test bacterial organism (Bhunia et al., 1988, Naaber et al., 2004, Trejo et al., 2006, Olivares et al., 2006, Jara et al., 2011, Schoster et al., 2013). Diffusion of bioproducts produced by the probiotic or from the wells/disks into the medium leads to inhibition of bacterial growth in the vicinity of the probiotic or the well/disk and to the formation of clear zones without any bacterial lawn. Measurement of the zone of clearing around the probiotic species, well or disk is used as an indicator of antagonism.

1.3.5.2 Gastrointestinal tolerance

Conventionally, *in vitro* tests for studying the gastrointestinal tolerance of potential probiotics are also performed by culturing the probiotics in simulated gastric fluids or intestinal fluids (usually buffer, saline solution or growth media acidified to low pH value or with added bile salts/pepsin) and sampling the culture at different times to determine survival by plate counting (Cui et al., 2000, Sun and Griffiths, 2000,

Chandramouli et al., 2004, Iyer and Kailasapathy, 2005, Ding and Shah, 2009, Mokarram et al., 2009, Sahadeva et al., 2011, Cook et al., 2011).

1.3.5.3 Advantages and limitations of the conventional methods

As mentioned previously, these *in vitro* methods, which are also employed when testing other biological efficacy or assessing relative growth or potency in mixed cultures, are mostly based on culture techniques. Although these methods are well established and have several advantages including cost effectiveness, ease of use, and familiarity among users, their drawbacks are also well documented (Gracias and McKillip, 2004, Vine and Bishop, 2005, O'Mahony and Papkovsky, 2006, Sohier et al., 2014). These methods are widely known to be extremely laborious and time consuming (O'Mahony and Papkovsky, 2006, Schmidt et al., 2007, Sohier et al., 2014). For example, experiments based on mixed cultures (Apella et al., 1992, Drago et al., 1997, Annuk et al., 2003) demand selective or differential spread-plating to distinguish the different species in a culture and to determine their concentration. Such experiments comprise serial dilution followed by plating on agar with selective or differential media or agents, which permits the growth of only one of the species or differentiation of the bacterial species. This routine is labour involved and time consuming especially as the number of species or strains being assessed in mixed cultures increases (Spiegelman et al., 2005, Schmidt et al., 2007). In some instances, differential or selective plating is not an option since selective media might not be available for each species (Schmidt et al., 2007).

Also, measurements on agar using bioproducts produced by a species during growth, although easier alternatives to the broth culture assay, these assays are hinged on the diffusibility of the bioproducts (Linton, 1983, Rogers and Montville, 1991). Bioproducts that are large or interact with the agar may not readily diffuse (Hoover and Harlander, 1993). Zones of inhibition could also be influenced by factors such the nutritional status of the growth media (for example for fastidious bacteria) (Jorgensen and Ferraro, 2009), the incubation temperature and duration and inoculum size (Linton, 1983). For example, too dense an inoculum may result in no zone of inhibition because the high initial cell numbers may mask any subsequent zone formation (Hoover and Harlander, 1993). Also, large zones of inhibition could be formed when the bacteria are slow growing; making this method not very suitable for the accurate testing of fastidious or slow glowing bacteria (Piddock, 1990, Jorgensen and Ferraro, 2009) although generally,

for antimicrobial susceptibility testing, the diffusion method has been standardized by the Clinical and Laboratory Standards Institute (CLSI) for testing a number of species through the use of specialized media, inoculum size, incubation conditions, and specific zone size interpretive criteria (Clinical and Laboratory Standards Institute, 2009).

Growth on plate or colony-forming ability is influenced by morphological alterations and also by the plating procedure hence there is the possibility that viable cells could be underestimated during cell enumeration (Davey, 2011, Sohier et al., 2014). To give an example, stress during the serial dilution and spread plating can cause underestimation of viable organisms. Organisms may also sometimes occur in chains and/or clumps or be unevenly distributed on the plate and this could underestimate cell numbers. Furthermore, some cells may exist in dormant, cryptobiotic, moribund or latent states after a particular treatment hence may not form colonies on plates even though they may have other measurable activity (Davey, 2011). Also, the plate technique allows one to determine viability in a retrospective manner, and there is really no evidence that no growth on plate during sampling is an indication that the cell was dead during the time of sampling (Postgate, 1976, Davey, 2011). The use of turbidity assay for cell enumeration although relatively rapid, non-labour intensive and amenable to automation, also suffers from drawbacks such as inability to analyse heterogenous or complex samples and to distinguish viable cells from dead cells (Madigan et al., 1997, O'Mahony and Papkovsky, 2006).

As mentioned previously, there are a number of molecular and other rapid and automated methods, which are being applied in these assays for detection or enumeration of organisms (Ben-Amor et al., 2001, Grattepanche et al., 2005, Moreno et al., 2006, Masco et al., 2007, Rueger et al., 2012, Tejero-Sarinena et al., 2013, Sohier et al., 2014). For instance, microscopic techniques such as fluorescence microscopy has been employed by Moreno et al., (2006) for enumeration of viable lactic acid bacteria from commercial fermented milks. Flow cytometry has also been employed by Ben-Amor et al., (2001) to enumerate viable bifidobacteria after bile salt stress. However whilst some of these advanced techiques have enabled sensitive, more convenient and faster detection and enumeration, they may require special skills, reagents and the pretreatment of the culture before analysis, for example, fluorescently labeling of the organisms. Also in terms of enumeration of viable species in mixed cultures or determination of interspecies interactions, only few of the molecular techniques have been employed; most of them

have been used for characterization of diversity of samples rather than the determination of absolute cell numbers or viable cell numbers (Spiegelman et al., 2005, Schmidt et al., 2011, Rueger et al., 2012, Nagarajan and Loh, 2014). However, quantitative polymerase chain reaction (qPCR) has been employed by Grattepanche et al., (2005) for determining interaction between *Lactococcus cremoris*, *Lactobacillus rhamnosus* and *Lactococcus diacetylactis* during milk fermentation. Quantitative terminal restriction fragment length polymorphism analysis method (qT-RFLP) has been employed by Schmidt et al., (2007, 2011) for enumeration of mixed cultures of *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Burkholderia cepacia*. Flow cytometry has also been employed by Rueger et al., (2012) for enumeration of viable cells in mixed cultures of *S. aureus* and *B. cepacia.* Fluorescent in situ hybridization (FISH) has also been employed by DuTeau et al., (1998) for differential enumeration of *Pseudomonas putida*, *Burkholderia sp*. and *B. subtilis*.

As suggested earlier, a possibility to avoid some of the disadvantages of the conventional methods is also offered by isothermal microcalorimetry. It is simple, fast, sensitive, and most importantly, can allow the continuous detection of biological activity without sample pretreatment or destruction. It could also overcome the tedium of manually counting cells of a particular morphology, providing rapid acquisition of data in real-time and allowing analysis to be performed on heterogeneous systems although it has its own limitations. It could thus complement the conventional and other rapid methods for the routine evaluation of probiotics especially where mixed cultures are encountered.

1.4 Isothermal microcalorimetry as a potential tool for characterizing potential health benefits of probiotics

1.4.1 What is isothermal microcalorimetry?

Microcalorimetry (from *mikrós,* Greek, meaning small, *calor,* Latin, meaning heat and *metry*, Greek, meaning measurement) is a technique that operates on the basic principle of measurement of heat, as a means of measurement of change in a material. It traces its origin to Lavoisier and Laplace who introduced its concept between 1782-1783. With an ice calorimeter (schematic diagram shown in Figure 1.3) and a guinea pig, these two

realized that the mass of liquid water produced by the melting ice was directly proportional to the heat produced by the activities of the guinea pig. Because they knew the quantity of water produced and the latent enthalpy of fusion of water, they were able to calculate the total heat output for the process.
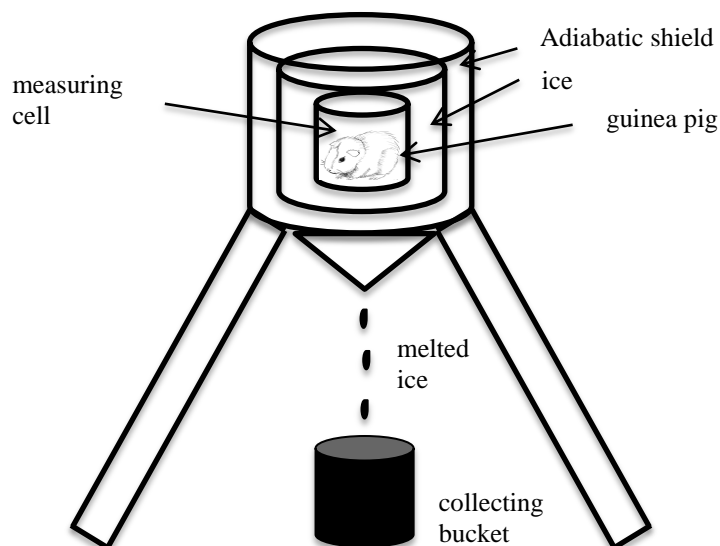
Figure 1.3. A schematic diagram of the ice calorimeter designed by Lavoisier and Laplace.

It is thought that when change (whether physical or chemical) occurs in a material, it invariably occurs with a change in heat content of the material, hence a calorimeter can detect and potentially quantify various changes in a wide variety of materials and processes including growth and metabolism of microorganisms (Gaisford and O'Neill, 2006, Braissant et al., 2010a). For example, microcalorimetry is known for applications such as determination of the stability of a product (Runge and Heger, 2000, Simoncic et al., 2007), quantification of amorphous content (Gaisford, 2012), determination of critical micelle concentrations (Bouchemal et al., 2009), dissolution studies (Ashby et al., 1989), etc. It is only imperative that the process being followed in a material produces a detectable amount of heat and that the material being studied fits in the calorimetric vessel.

The term "isothermal" is generally used to imply the constant temperature at which samples are held during an experiment. It must be noted that broadly, calorimeters can be operated isothermally or non-isothermally (Gaisford and O'Neill, 2006). Thus, either there is temperature change over time (either linear or modulated) for example in

Differential Scanning Calorimetry (DSC) or the sample is held at a constant temperature throughout the experiment. The isothermal technique is more suited for study of growth of bacteria. Isothermal microcalorimeters are instruments designed for use in the microwatt range; those that make measurement in the nanowatt range are referred to as isothermal nanocalorimeters (Wadso and Goldberg, 2001).

1.4.2 Isothermal microcalorimeters: principles and instrumentation

Isothermal microcalorimeters measure heat by three main principles:

- *Adiabatic*: where there is no heat exchange between the calorimetric vessel and its surrounding. An adiabatic shield is placed between the vessel and its surrounding to obtain the adiabatic condition. In such arrangement, a change in heat content of a system under investigation is equated to the change in temperature (either endothermic or exothermic) that has occurred in the vessel multiplied by an experimentally determined cell constant. In practice, true adiabatic conditions might be difficult to obtain due to heat-leak to the surroundings. However, if the instrument balances these heat leaks, then semi-adiabatic conditions are achieved and corrections are applied to the data (Wadso and Goldberg, 2001).

- *Power compensation*: where the heat gained or removed during an endothermic or exothermic process is balanced by an opposing power, supplied by an electrical element by using the Peltier principle (Wadso and Goldberg, 2001).

- *Heat conduction*: where heat produced or absorbed during a course of reaction in the calorimetric vessel is allowed to flow to or from a surrounding heat sink. The heat sink is maintained at a constant temperature (Wadso and Goldberg, 2001).

Most isothermal microcalorimeters are based on the principle of heat conduction. Isothermal condition is achieved by exchange of heat (q) produced or absorbed by the sample with the surrounding heat sink via thermopiles located between the two compartments (Figure 1.4).
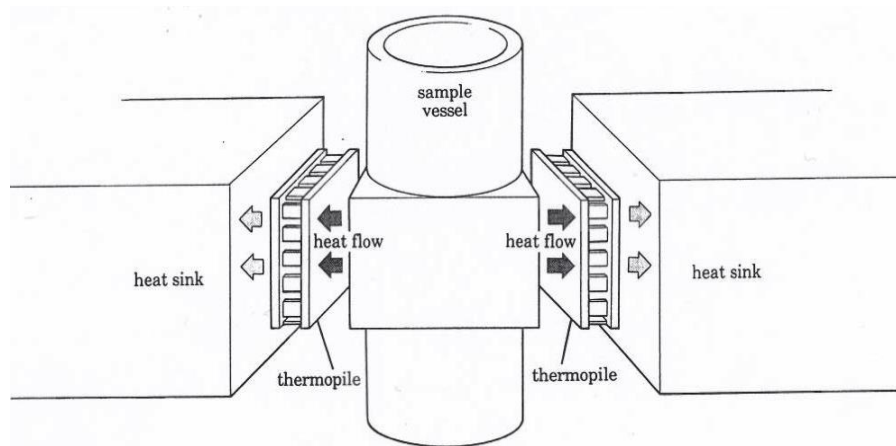
Figure 1.4. Schematic diagram of the heat-sink/thermopile arrangement in the Thermal Activity Monitor (TAM), courtesy of Thermometric
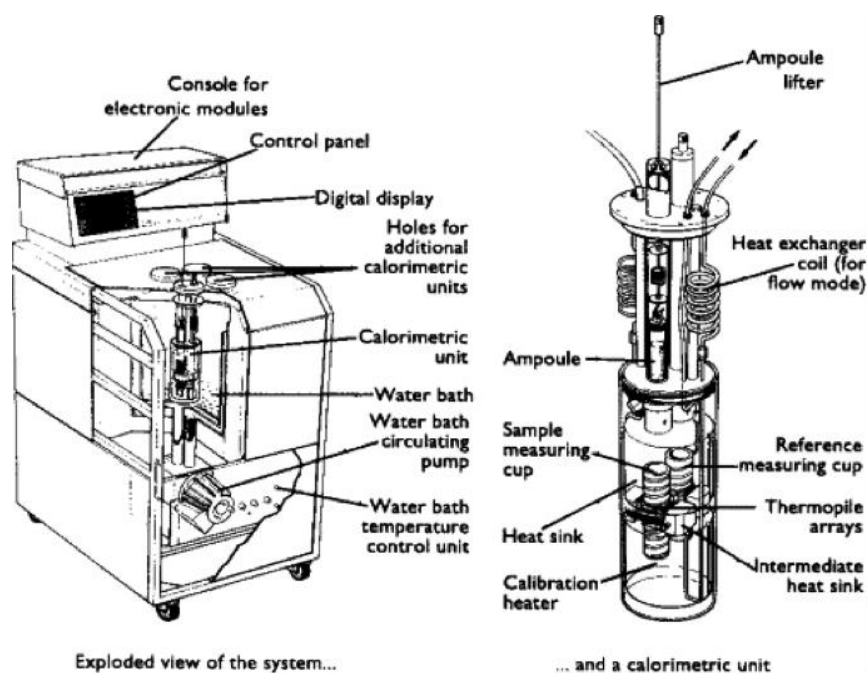
Figure 1.5 A diagram of the TAM (left) and one of its unit (right), courtesy of Thermometric

The thermopiles produce a voltage signal (U) proportional to the heat flowing across them anytime there is heat exchange between the vessel and the heat sink. This signal, when multiplied by a cell constant (ε) obtained through electrical calibration, produces a raw power signal (P_R) (Equation 1.1), which is plotted as a function of time (t) (Wadso and Goldberg, 2001, Gaisford and O'Neill, 2006). The total heat quantity (Q), released

by a sample, is given by the product of the electrical potential time integral and the cell constant (Equation 1.2).

$$P_R = dq/dt = \varepsilon U \hspace{4cm} \text{Equation 1.1}$$

$$Q = \varepsilon \int U dt \hspace{4cm} \text{Equation 1.2}$$

It must be noted that the isothermal microcalorimeter used for all the work reported in this thesis was a Thermal Activity Monitor 2277 (TAM, TA Instruments Ltd., UK). It has four separate microcalorimetric channels and capable of taking measurements from four experiments simultaneously, Figure 1.5. All four channels are immersed in a closed thermostatted water bath (the heat sink) that can be finely set within the range of 5°C and 80°C (Technical specifications, Thermometric). Each channel has both a sample and reference chamber connected in opposition. These chambers accept the sample and reference materials, which are often housed in 3-20 mL glass or stainless steel ampoules. The reference material is usually an inert material, which must be of similar heat capacity as the sample and is used in the same quantity as the sample. During measurement, the sample and reference, both generate an electrical potential, which is reported as a differential. This ensures that any noise affecting the sample chamber or both chambers is minimized, allowing only the thermal output of the reaction to be recorded (Wadso, 2010). The instrument is equipped with an amplifier module, which can be set to different ranges for example, 3, 10, 30, 100, 300, 1000 and 3000 μW depending on the expected thermal output from a reaction or the sensitivity required (Technical specifications, Thermometric). It is also linked with a computer, which captures data every 10 s using the software package, Digitam™.

Like most modern isothermal microcalorimeters, the TAM is very sensitive, has good reproducibility, precision and baseline stability. This is attributable to the efficiency with which isothermal conditions are achieved. To maintain such sensitivity and stability, they are often required to be housed in air-conditioned rooms that are maintained at a constant temperature of 20-30 $^{\circ}$C $\pm$ 0.1°C (Technical specifications, Thermometric).

1.4.2.1 Calibration

Electrical calibrations are usually performed before an instrument is used for the first time and also any time there is loss of power supply, change in amplifier range or change in experimental conditions (Technical specifications, Thermometric).

During calibration, the TAM is loaded with two reference samples (thus, both the reference chamber and sample chamber are loaded with the reference material in quantities equal to the one to be used for subsequent experiments). It must be noted that during experiments, the ampoules are first lowered to the equilibrium position (above the thermopile) for a period of 30 min in order for the ampoules and their contents to equilibrate with the temperature of the TAM. This reduces any thermal shock that would have otherwise been encountered. Once thermal equilibrium is attained, they are lowered into the measurement position carefully, with the aim of reducing frictional heat to the minimum. Since both the reference and sample chambers are loaded with the same inert material during calibration, it is expected that no power is produced and a baseline signal of zero should be attained. Practically, this may sometimes not occur because of noise. Therefore, when the baseline signal is stable for a period, it can be adjusted to zero via a potentiometer. Current is then passed through the calibration heater located at the base of the chamber for a given length of time, which should cause a deflection in the baseline signal to a constant set value. Usually during calibration, an amplitude range is set and the heater is expected to match this set limit. For example, if the amplifier is set to a range of 1000 μW, the heater is expected to produce a power of 997.2 μW (Technical specifications, Thermometric). Like the baseline signal, this value, can also be adjusted so that the output recorded by the instrument matches that delivered by the calibration heater. Current is then switched off and the signal should decay back to zero. During the calibration, the calibration constant or coefficient (ε), which is the proportionality constant between the measured signal (voltage) and the thermal power is obtained which is applied to the results (Wadso, 2010).

1.4.2.2 Instrumental designs

There are a lot of designs of the isothermal microcalorimeter due to its diverse applications: for example, there are ampoule microcalorimeters, flow microcalorimeters, gas perfusion microcalorimeters, isothermal tritration microcalorimeters, solution

microcalorimeters and so on. The common ones that have been explored to study microbial systems are the ampoule microcalorimeter and the flow microcalorimeter (Monk and Wadso, 1975, Hartung, 1986, Zhao et al., 2000, Gaisford et al., 2009, Lago Rivero et al., 2013, Bonkat et al., 2012). They all have their advantages that can be explored and limitations. The ampoule was used for the work reported in this thesis.

Ampoule microcalorimeters

These represent the closed batch culture experiments. They use removable glass or stainless steel ampoules, which are hermetically sealed. Samples (organism and reaction medium) are placed in these ampoules, sealed and placed in one of the measuring channel of the isothermal microcalorimeter. Heat flow measurements are recorded and can be evaluated as they occur. A major limitation as with any batch culture experiment is that growth is only possible for a finite period of time as energy sources are depleted with time. Again, because the ampoules are sealed, it is difficult to control the atmosphere inside of the ampoule (Winkelmann et al., 2004). Typically, the atmosphere within the ampoule is of limited oxygen supply (oxygen available is that dissolved in the medium and in the headspace), hence the study of aerobic microorganisms is difficult since aerobic respiration rapidly renders the medium anoxic (Braissant et al., 2010a). However, when it is ensured that the microorganisms have a direct air interface for example through use of solid media (Wadso et al., 2004), oxygen depletion could be avoided and the study of aerobic microorganisms in sealed ampoules is possible.

Another major limitation with the ampoule is that it does not allow the sampling of the system during investigation or the addition of substances during experimentation. Attempts to do that will upset the thermal equilibrium of the calorimeter. The ampoule technique does not also allow the stirring of samples during experiments. There is therefore the risk of sedimentation of cells, which could cause concentration gradients of pH, nutrients, etc (Beezer, 1980).

They also have associated benefits of batch culture experiments. For instance, they are simple, easily sterilized and also allow the study of samples without contact with the environment, supporting the study of high-risk organisms. The glass ampoules are disposable and prevent the risk of cross contamination during experiments.

In summary, isothermal microcalorimeters allow for monitoring of chemical, physical or biological processes of samples as they occur through the measurement of heat released or absorbed by the sample via a sample-thermopile arrangement. After the standardization of an instrument through calibration, heat flow measurement of a sample can be taken for as long as it is of interest. Most isothermal microcalorimeters like the TAM are designed as twin instruments, and it is thus a differential signal (between a sample and a "blank" reference) that is measured.

1.4.3 Data analysis- microbiological

Heat released or absorbed from the heat sink during the course of an experiment is expressed as power-time curves. The power-time curves are characteristic for a species if the inoculum is standardized and the experimental conditions are completely reproducible (explained in the later sections). Data therefore obtained from microcalorimetric experiments can be analysed in two ways: either qualitatively or quantitatively. Qualitative analysis is based on the description of changes that occurs in the power-time curves by the presence of a metabolic modifier, for example, a reduction in the power output by an antibiotic (Prat, 1953, Sedlacze.L et al., 1966, Said et al., 2014). Such descriptive analysis is more popular since the power-time curves obtained from a growing culture of bacteria are rather too complex to give quantitative information on the organisms and also vary with changes in the metabolic performance of inocula. Quantitative information can nonetheless be obtained when the bacteria are allowed only to respire and not to increase in biomass by the use of defined medium with limited energy source (Vine and Bishop, 2005). Such controlled processes could be described mathematically; for example, the kinetic profile of the respiring bacteria can be described by the Michaelis-Menten kinetics and hence quantitative information can be obtained (Vine and Bishop, 2005). Also, the power-time curve and its integral (heat curve) can be analysed to obtain information such as the growth rate (μ) and generation time ($t_{1/2}$), which are major growth indicators. These growth parameters could change depending on the particular treatment given to a culture, for example, whether a bacterial culture is growing with or without the presence of a metabolic modifier (Braissant et al., 2013).

1.4.4 Advantages and limitations of isothermal microcalorimetry

Isothermal microcalorimetry offers a lot of advantages compared to traditional batch culture or continuous culture experiments. The most important of all, are the real time information it provides, its simplicity, non-laborious and non-destructive/non-invasive nature of the experiments. For instance, Prat (1953) demonstrated that addition of streptomycin to a culture of *Esherichia coli* inhibited the species by decreasing the microcalorimetric output to one tenth of its original value but also noted that the inhibitory activity of the drug was greater when the culture was stirred. Thus the efficiency of the drug was limited by its rate of diffusion through the medium (Prat, 1953). If his experiment had been performed by conventional plate count, the same qualitative data would have been produced but in less detail since in such an assay, only end point data are obtained. Thus, the sample would have been taken at random time intervals and plated. Microcalorimetry does not also require the sample to be modified, for example, adding fluoresecent or radioactive labels nor does it alter samples during measurement, hence samples can be subjected to further assays after microcalorimetric measurements (Braissant et al., 2010c).

It is also a very sensitive technique. According to Braissant et al., (2010a), modern isothermal microcalorimeters can detect as low as 10^4 -10^5 per mL bacterial concentration when approximately 2 pW per cell is expected when the cell is active. However it is generally maintained that at least 10^6 microorganisms per mL is sufficient to produce detectable signals in most commercially used microcalorimeters (Gaisford et al., 2009). Lower cell density adds a time lag to the growth data until the cells have grown to this detectable concentration. Still this detectable concentration limit cannot be detected using conventional turbidity measuring techniques such as the McFarland standards which calibrate turbidity of bacterial concentrations to a minimum concentration of approximately 0.75 x 10^8 CFU/mL or spectrophotometry (when measuring optical density at 600 nm) (Vine and Bishop, 2005, Braissant et al., 2010a). The heat sensitivity of isothermal microcalorimeters also allows large changes in a sample to be measured apart from subtle ones (Braissant et al., 2010a). They also have good reproducibility, high precision, and are very simple and convenient to use; data acquisition is automated. They can also be operated isothermally at different temperatures, and this can be suited to the requirements of different reactions.

Like any technique, isothermal microcalorimetry is not without it problems. Some of the limitations have already been outlined based on the design of their use. But a major limitation of the use of isothermal microcalorimeters is the non-specificity of the data obtained. Heat flow signals obtained are a sum of all the biological, chemical or physical processes taking place in a sample (Beezer, 1980, Johansson and Wadso, 1999a). As a result of this, the total heat measured with this technique may be contributed by the process under investigation and other unknown or even unwanted phenomena. This makes data interpretation a real challenge. Careful planning of experimentation is therefore a necessity. Nevertheless, when the process under investigation is well described and conducted under controlled conditions, the process can be studied with a high accuracy (O'Neill and Gaisford, 2011).

Again, the use of the ampoule require that the ampoule together with samples contained in it are allowed to reach thermal equilibrium before any significant data can be obtained. As mentioned already, the ampoules are placed in an equilibration position for a set time, usually, 30 min before they are placed in the measuring position. The problem with this is some reactions can be initiated before measurements are captured hence it is often not possible to determine the calorimetric signal at time zero.

1.4.5 Isothermal microcalorimetry and its microbiological applications

Dubrunfaut, (1856) was the first person who reportedly used calorimetric measurement to study quantitatively, the heat production by a microbial fermentation system. Since his study, many scientists used the calorimeter to study the growth and metabolism of bacteria and other microorganisms. For example, Bouffard, (1985) measured the heat evolved by 1 litre of culture using Berthelot's instrument. Rubner, (1903, 1904, 1906, 1908) using a primitive calorimeter, also reported numerous studies on the heat evolvement of bacteria and yeast. So did Tangl, (1903), Terroine and Wurmser, (1922), Meyerhof, (1924), Shearer, (1921), Bayne-Jones, (1929), Prat et al., (1946), Battley, (1960), Forrest et al., (1961), Belaich, (1962 and 1963), Boivinet, (1964), Monk and Wadso, (1968) and many more. Although these pioneering microbiological studies were done in the early days of calorimetry, the use of calorimetry for microbiological studies waned in the 1970s (Gaisford and O'Neill, 2006). In the last few years, there has been the resurgence of calorimetry in microbiological studies and this has been attributed to

the development of more sensitive and high-throughput instruments (Gaisford and O'Neill, 2006, Braissant et al., 2010a, Wadso and Galindo, 2009) and the consideration by the pharmaceutical industry seeking more rapid and improve methods for investigating microorganisms (Vine and Bishop, 2005, Gaisford and O'Neill, 2006).

Microcalorimetry has applications in soil and environmental microbiology (Wadso, 2009, Braissant et al., 2010a, Maskow et al., 2010, Ning et al., 2013), pharmaceutical and medical microbiology (Vine and Bishop, 2005, Braissant et al., 2010a), food microbiology (Wadso and Galindo, 2009) including other biological applications in food science (Wadso and Galindo, 2009), biomedical science (Braissant et al., 2010c) and soil and environmental sciences (Rong et al., 2007, Russel et al., 2009). For instance, it has been used for monitoring and quantification of soil microbial activity and contamination (Bravo et al., 2011, Guo et al., 2012); detection of infection and contamination of clinical products and samples (Trampuz et al., 2007, von Ah et al., 2008, Braissant et al., 2010d); determination of effects of antimicrobial compounds (Li et al., 2000, O'Neill et al., 2003, Gaisford et al., 2009) including the mode of action of antimicrobial compounds-bacteriostatic or bacteriocidal (von Ah et al., 2009), viral infections and activities of antiviral compounds (Tan and Lu, 1999, Heng et al., 2005); spoilage of food (Alklint et al., 2005) etc. The detailed microbiological applications of microcalorimetry in the different scientific fields are reviewed in the references cited under the respective field. Most of the microbiological applications of microcalorimetry are in soil quality, food deterioration, infection detection and antibiotic testing.

1.4.6 Characterization of microorganisms

When placed in an appropriate medium, a bacterium will consume the nutrient provided for growth. In the isothermal microcalorimeter, the calorimetric output which is referred to as "power-time curve" is the net metabolic activity of the bacterium. A typical power-time curve shows an initial exponential increase in heat output followed by a series of peaks and troughs representative of the sequential utilization and exhaustion of different components of a given medium (Vine and Bishop, 2005).

Under strictly defined conditions, and reproducible experimental conditions, individual bacterial species are known to yield characteristic power-time curves. This principle was suggested as a premise to identify microorganisms since Boling et al., (1973) reported

that all Enterobacteriaceae tested, which included 17 species from 10 genera could be distinguished. Also, at least 200 clinically significant organisms representing 24 genera and 47 species could be distinguished by Russel et al., (1975) by means of their specific thermal pattern. Fujita et al., (1978) also identified 9 strains of lactic acid bacteria. Identification had also been implicit in earlier studies by Rubner (1906) and Prat et al., (1946) and studies by Staples et al., (1973) and Ljungholm (1976). But such universal identification received criticism on the notion that it was impossible to reproduce experimental conditions in reality and the fact that even small changes can affect data generated with isothermal calorimeters (Beezer et al., 1979, Newell, 1980). Also, there is variation between calorimetric instruments and procedures, which introduce discrepancies between experiments that are supposedly identical (Monk and Wadso, 1975). The biggest challenge is the standardization of an inoculum and its history. Beezer et al., (1976) addressed to some extent the standardization of an inoculum using freezing and storage protocols by freezing down and storing large volumes of a batch of an inoculum that allow an inoculum to be used as a "standard reagent". Hence when using the same medium, experimental condition and instrument, a batch of inoculum can be characterized. For instance, after the establishment of the power-time curve of a standardized organism using a specific medium, if a metabolic modifier is added to the medium, the power-time curve will change according to the modifier added, the concentration it was added and the time it was added. The change of the power-time curve due to the presence of metabolic modifiers has allowed the study of wide-ranging processes, for example, the effects of antibiotics and antimicrobial agents (Zhao et al., 2000, Li et al., 2002, O'Neill et al., 2003, Gaisford et al., 2009, Howell et al., 2012). Microcalorimetry has also been applied in the differentiation of strains and species. For example, methicillin-susceptible *Staphylococcus aureus* has been differentiated from methicillin-resistant *S. aureus* from clinical samples (von Ah et al., 2008, Baldoni et al., 2009). Baker's, brewer's and distiller's yeast have been differentiated in the microcalorimeter by Newell, (1975). Lago Rivero et al., (2013) have also differentiated between and characterised the growth of *S. aureus* and *Staphylococcus epidermis*. The growth of *S. aureus* and *E. coli* (Zaharia et al., 2013) and *E. coli*, *Proteus mirabilis* and *Klebsiella pneumoniae* (Lago Rivero et al., 2012) have also been characterised and compared.

However, to date there are very few reports of microcalorimetric measurements for detecting species in mixed cultures or monitoring interpecies interaction. One such

measurement was performed by Schaeffer et al., (2004) to determine the growth optima for a mixed culture of a probiotic species and an aroma-producing mesophilic microbe which the probiotics rely on to generate taste in a product. Their experiment showed that the two cultures did not impede each other in mixed cultures. With their observation, they were able to expound production procedures for probiotic butter cream and heat-resistant sour cream using the two cultures in a mixed product (Schaffer et al., 2004, Schaffer and Lorinczy, 2005). In another study, Chen et al., (2008) evaluated the toxic effect of cadmium on the growth of *Candida humicola* and *Bacillus subtilis* singularly or in mixed culture. Their result showed different growth rate and endurance for the two species and mixed culture. The sensitivity of *B. subtilis* to cadmium was higher than *C. humicola* but the mixed microorganisms had a higher endurance than single microorganisms. These authors however could not ascertain whether or not the two microorganisms have an interaction when put together. In a similar study, Wang et al., (2009) followed the toxic effect of a trivalent iron on single and mixed culture of *B. subtilis* and *C. humicola*. The results indicated that *C. humicola* had highest tolerance to iron overload while the mixed culture had moderate tolerance compared with the single species and *B. subtilis* followed. The authors speculated that the moderate tolerance of the mixed culture could be due to the interaction between the two species (Wang et al., 2009). Kong et al., (2012) also studied the effect of berberine on the growth of pure and mixed cultures of *E. coli* and *B. subtilis*. The authors demonstrated that the endurance of *E. coli* to berberine was lower than *B. subtilis* and also demonstrated that *E. coli* dominated the mixed culture and could reduce the endurance of *B. subtilis* in mixed culture (Kong et al., 2012). Recently, and very relevant, Vazquez et al., (2014) studied the interaction between *E. coli* and *P. mirabilis* by mixing them together at different proportions. Their result showed that *P. mirabilis* dominated in mixed cultures even at low proportions in a culture. Another study by Vazquez et al (2013) also demonstrated interaction between *Enterococcus faecalis* and *Klebsiela pneumoniae.*

Although the relevant application of microcalorimetry in detecting species in mixed cultures or studying interspecies interactions have only been recently explored, no documentation in literature has been made for its exploration in studying the antagonistic effect of probiotics against potential pathogens.

1.5 Aims and Objectives

The main aims and objectives of the research in this thesis were

- To explore the potential of isothermal microcalorimetry to detect bacteria in mixed cultures. This was to be applied:
  - To investigate the behaviour of *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Esherichia coli* in mixed cultures
  - To investigate whether commercial probiotics can inhibit *in vitro* potential pathogenic microorganisms particularly, the clinically important gut pathogen, *Clostridium difficile* in the microcalorimeter
  - To investigate whether commercial probiotics, which are packaged and sold as multistrain, are stable as a product
  - To investigate whether a potential prebiotic substrate would increase the growth of pure probiotic species or specific members of colonic bacteria in the microcalorimeter

Gastric tolerance assay of potential and commercial probiotics are routinely conducted in acidified saline solutions, buffers and growth media (Cui et al., 2000, Sun and Griffiths, 2000, Chandramouli et al., 2004, Iyer and Kailasapathy, 2005, Ding and Shah, 2009, Mokarram et al., 2009, Sahadeva et al., 2011, Cook et al., 2011); without consideration to other physiological factors such as transit time, gastric volume and presence of enzymatic and digestive materials. Also, the plate technique is conventionally used to determine survival. This research also aimed to:

- To develop a more realistic test for determining gastric tolerance of commercial probiotics

Chapter 2 Defined mixed culture of *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli*

2.1 Introduction

2.1.1 Defined mixed culture in the isothermal microcalorimeter

As reviewed in the previous chapter, microcalorimetry has had vast microbiological applications in various fields (section 1.4.5 and 1.4.6). Despite all the applications, only few studies have been conducted on defined mixed culture with the aim of investigating the relationship between two or more bacteria (Schaffer et al., 2004, Kong et al., 2012, Vazquez et al., 2014).

In this chapter, the growth and behaviour of *Pseudomonas aeruginosa*, *Staphyloccocus aureus* and *Escherichia coli* were studied as mixed culture to determine if the microcalorimeter could detect their relative growth. These 3 non-fastiduous species, previously characterised in the microcalorimeter (O'Neill et al., 2003, Gaisford et al., 2009, Said et al., 2014) were to be used in subsequent susceptibility test with some commercial probiotic strains.

P. aeruginosa is an opportunistic human pathogen that can cause various infections, for example infections of the ear, eyes, skin, urethra and respiratory tract in cystic fibrosis and patients with severe burns, as well as other immunocompromised and chronically debilitated individuals (Garrity et al., 2005). It is a very adaptable bacterium, known to colonize several environmental niches; one of the most common species in nature (Garrity et al., 2005, Khan et al., 2010). Its numbers are very low in the faeces but numbers can increase significantly after antibiotic treatment (Levison, 1977).

S. aureus forms part of the normal human microbiome, and colonizes the skin, skin glands, mucous membranes, especially anterior nares (Schleifer and Bell, 2009). It is an important cause of nosocomial infections and has caused considerable mobidity and mortality in hospitalised patients (Wisplinghoff et al., 2004, de Kraker et al., 2011). It is associated with surgical infections, lung infections, urogenital tract infections, skin infections, abscesses of the muscle and bacteremias. Its enterotoxin is involved in food poisoning (Schleifer and Bell, 2009).

E. coli is an essential part of the gut microbiota. Most strains are non-pathogenic but certain serotypes can cause diseases of the intestine and other parts of the body. They possess a variety of virulence factors responsible for their pathogenicity in healthy patients. For example, enteropathogenic and enterotoxigenic *E. coli* cause diarrhoea. Uropathogenic *E. coli*, neonetal meningitis *E. coli* and other types are described (Scheutz and Strockbine, 2005).

2.1.2 Standardization of inocula: cryopreservation

As a prerequisite for using bacteria as "standard reagents", ensuring that any difference between experiments is solely due to deliberate changes made, an objective was set to develop a cryopreservation protocol for the microorganisms, which, while maintaining uniformity of inocula, would give viable cell recoveries of at least 95%.

It must be noted that microbiological assays could be subject to inconsistencies between experiments because of variations in the metabolic performance of inocula used (Beezer et al., 1976). While this variation is tolerated in some assays because of other standardization protocol in place by a particular method, for quantitative characterization or identification by microcalorimetry (for example, antibiotic susceptibility testing) this may not be the case (Beezer et al., 1976). The source of variation of an inoculum can be credited to the volume or size, the phase of growth and particularly the history of the inoculum being used (Beezer et al., 1976). To avoid batch-to-batch variability, it is important that inocula are rigorously standardized. Standardization of inocula can be achieved through the development of standardized freezing and storage protocols for large volumes of inocula (Beezer et al., 1976, Gaisford et al., 2009). This will ensure that a consistent inoculum is used for each assay since the microorganisms would be sourced from the same batch per assay or would have gone through a similar cultivation process.

In the frozen state, the microorganisms are expected to remain viable, without any alterations in genotypic or phenotypic characteristics (Tedeschi and De Paoli, 2011). They should also be easily restored to the same condition they were before freezing or cryopreservation (Beezer et al., 1976). Conventionally, microbiological stocks are preserved by storing at -4°C to -80°C. Storage at these temperatures has however been shown to be less effective than at -160°C in liquid nitrogen vapour (McDaniel and Bailey, 1968) or liquid nitrogen storage at -196°C (Stapert et al., 1964, Sokolski et al.,

1964, Hwang, 1966, Cowman and Speck, 1965, Beezer et al., 1976, Gaisford et al., 2009). Freezing in liquid nitrogen at $-196^{o}C$ can store living microorganisms for several years while maintaining viability and reproducibility (Beezer et al., 1976, Gaisford et al., 2009, Tedeschi and De Paoli, 2011). For instance, some bacteria have been shown to store for up to 30 years without loss in viability (Gherna, 1981, Reimer and Carroll, 2004).

A number of factors are known to affect the effectiveness of cryopreservation of microorganisms, for example, the composition of the freezing medium, the storage temperature, the cooling rate and warming rate (Meryman, 1971, Beezer et al., 1976, Hubalek, 2003, Morgan et al., 2006, Tedeschi and De Paoli, 2011). The cooling rate during the freezing process is a critical factor for cell viability and recovery (Beezer et al., 1976). The optimal cooling rate for most bacteria has been suggested to be $1^{o}C$ per min (Mazur et al., 1972) even though some bacteria will withstand less than ideal cooling rates (Tedeschi and De Paoli, 2011). Very slow cooling rate results in death of cells caused by changes in osmotic pressure whereas very fast freezing rate can cause cell death from mechanical damage of cell membranes caused by recrystallization of intracellular ice (Meryman, 1971). Another critical aspect in freezing is the efficiency of a cryoprotective agent in protecting cells from injury and death (Meryman, 1971). As already pointed out, during freezing and defrosting processes, cells could suffer severe osmotic stress and ice crystal damage. A way of circumventing these possible lethal effects is to include a cryoprotectant prior to freezing. Addition of cryoprotectants has been shown to improve survivability of cells (Harrison, 1956, Postgate and Hunter, 1961, Nash et al., 1963, Baumann and Reinbold, 1966, Gibson et al., 1966, Green and Woodford, 1992, Vekeman et al., 2013).

There are different types of cryoprotectants and classifications: extensively reviewed by Meryman, (1971) and Hubalek, (2003). Briefly, there are those that penetrate the cells: methanol, ethanol, ethylene glycol, propylene glycol, dimethylformamide, dimethyl sulfoxide (DMSO), methylacetamide and glycerol. There are others that do not penetrate the cells: mannitol, sorbitol, dextran, methyl cellulose, albumin, gelatin, other proteins, polyvinylpyrrolidone (PVP) etc., (Meryman, 1971). The non-penetrating ones provide only extracellular protection whilst the penetrating ones can also delay intracellular freezing and provide both intracellular and extracellular protection (Meryman, 1971, Hubalek, 2003).

For the cryopreservation of the organisms, the effectiveness of different cryoprotective agents was tested in order to select the best cryoprotectant for the organisms to give recoveries of 95% or more. Storage of the stock was to be in liquid nitrogen because of the highlighted advantages from previous experiences (Beezer et al., 1976, Gaisford et al., 2009).

2.2 Objectives

The objectives of this chapter were:

- To develop a cryopreservation protocol for the microorganisms with a suitable cryoprotectant which will ensure the standardization of the inocula, hence, the uniformity in the metabolic performance of all inocula
- To study the growth of the microorganisms as pure culture in the microcalorimeter
- To study the growth of mixed cultures of the microorganisms in the microcalorimeter

2.3 Materials and Methods

2.3.1 Microorganisms

The strains used were *Staphylococcus aureus* NCIMB 9518, *Pseudomonas aeruginosa* NCIMB 8626 and *Escherichia coli* ATCC 25922.

Staphylococcus aureus NCIMB 9518 and *Pseudomonas aeruginosa* NCIMB 8626 were obtained from ConvaTec LTD., *Escherichia coli* ATCC 25922 was obtained from laboratory 208 (Department of Pharmaceutical and Biological Chemistry, UCLSOP), originally purchased from American Type Culture Collection, USA.

2.3.2 Microbiological media

Nutrient broth (NB), agar powder, iso-sensitest agar (ISA), tryptone soya broth (TSB), cooked meat medium (CMM), macconkey agar (MCA), cetrimide agar, mannitol salt agar (MSA) and all other microbiological media were from Oxoid Ltd, Basingstoke, UK and purchased from Fisher Scientific, UK.

2.3.3 Chemicals and reagents

Ringer's solution tablets and phosphate buffered saline (PBS) tablets were purchased from Sigma-Aldrich, UK. Absolute ethanol (99%), dimethyl sulfoxide and glycerol (analytical grade) were purchased from Fisher Scientific, UK. Polyvinylpyrrolidone (PVP) was purchased from Sigma-Aldrich, UK. All other reagents used were of analytical grade. Distilled water was used throughout.

2.3.4 Sterilization of culture media, chemicals and solutions

All culture media used were sterilized by autoclaving (21001 Classic Prestige Medical, England) for 15 min at 121°C prior to inoculation. Chemicals and solutions were sterilized by filtration using a membrane filter of 0.22 μm pore size (Sartorius AG, Germany) and a syringe. All procedures were carried out aseptically.

2.3.5 Stock culture maintenance and purity

To maintain a collection of all bacterial stock cultures:

Staphylococcus aureus NCIMB 9518, *Pseudomonas aeruginosa* NCIMB 8626 and *Escherichia coli* ATCC 25922 were obtained on agar slopes. Colonies were picked off the agar slopes using a sterile loop and streaked onto iso-sensitest agar (ISA) plate. This was incubated at 37°C ($\pm$1°C) for 24 h. Cultures were checked for purity by observing colony types and absence of contaminants. Colonies of the respective bacterial strains were picked off with a sterile cotton swab and used to make a lawn on a fresh agar plate. After incubation of the respective bacterial lawns at 37°C for 24 h, the respective

colonies were picked off with a sterile cotton swab and inoculated into 15% v/v glycerol in tryptone soya broth (TSB) to make a thick suspension of the culture which was divided into multiple aliquots and stored at -80°C until further use.

2.3.6 Growth curves

In order to determine the correct harvesting points for the bacterial cells during cryopreservation, growth curves of the bacteria were determined. Turbidity or optical density measurements, where an instrument measures the amount of light scattered by a suspension of cells was employed. Bacteria are known to scatter light in proportion to their numbers hence the turbidity or optical density (OD) of a cell suspension is directly related to the cell mass. Turbidity measurement does not however distinguish between viable and non-viable cells. Cell numbers or colony forming units (CFU) can be obtained by plate count.

The CFU for a given range of OD values were determined to establish the number of viable cells in a cell suspension. Once this was established, the OD was used as an approximate measure of the CFU in a given sample.

P. aeruginosa, S. aureus and *E. coli* were streaked on ISA plates and incubated (37°C for 24 h). Starter cultures of the bacteria were prepared by picking off a few bacterial colonies with a sterile loop from the respective plates and inoculating into 7 mL bijous containing 5 mL of NB. The bijous together with their sterile controls were then incubated respectively as described above.

100 mL of sterile NB prepared previously was pre-warmed to 37°C and inoculated with organism from the starter culture in a 1:100 dilution. The culture was placed in a shaking incubator (Innova 4080, New Brunswick Scientific, UK) at a speed of 274 rpm at 37°C. Changes in cell density were monitored with a spectrophotometer (Heλiosα, Thermo Scientific) at 600 nm. The OD reading was taken before the sample was placed in the shaking incubator representing the "0" time reading and every 30 min after, until the exponential growth phase ended. An uninoculated control was incubated under the same conditions. The OD readings were plotted with Origin Pro 8.6 (Microcal Software Inc.) to give the growth curve. Plate counts were done by taking 50 μL of the test culture at each time point and inoculating into 450 μL of PBS. Serial dilutions (1:10) were carried

out until 1:10^7 dilutions. Thus, 450 μL of PBS was placed into bijous labelled 1 to 7. At each time point, 50 μL of the test culture was inoculated into a labelled bijou 1 (for a 1 in a 10 dilution). Bijou 1 was vortexed for 30 s and 50 μL of it was transferred into bijou 2 and repeated for all the bijous. 50 μL of the test bacterial suspension from the bijous were plated out using sterile glass spreader onto ISA agar. The plates were incubated and colonies counted after incubation at 37°C over 24 h.

2.3.7 Cryopreservation

Cryopreservation and recovery of the bacterial stocks used was based on Beezer et al., (1976) method of cryopreservation of *Saccharomyces cerevisiae.* Freezing was carried out in a modified bath containing liquid nitrogen (Figure 2.1).
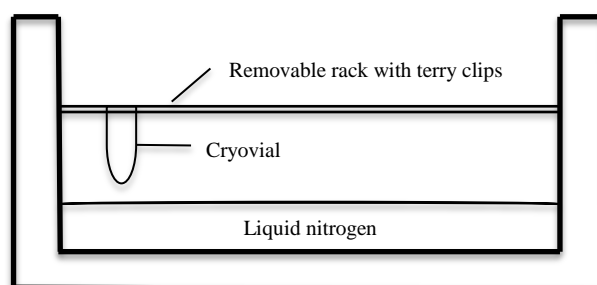
Figure 2.1. Schematic representation of the modified bath used for cryopreservation of bacteria in liquid nitrogen at -196°C. Cryovials were held in terry clips of racks, which were held above the surface of the liquid nitrogen

Starter cultures of relevant bacteria were made by picking off a few colonies of bacteria from plates, inoculating into broth and incubating at 37°C appropriately. The starter was inoculated into a freshly made relevant broth (200 mL) in a 1:100 dilution. The culture was then incubated in a shaking incubator (Innova 4080, New Brunswick Scientific, UK) until it reached a population density ca. 1x10^8 CFU/mL (from corresponding optical density reading). The cultures were then harvested by centrifuging at 2500 *g* for 10 min at 4°C using the Heraues Stratosbiofuge (Thermo Scientific, UK). The supernatant was carefully discarded and the cells washed twice in PBS by centrifuging at the same conditions. The washed cells were resuspended in ¼ strength Ringer's solution supplemented with a cryoprotectant in a reservoir. 1.8 mL of the cell suspension were dispensed rapidly while shaking the reservoir continuously using a stirrer to ensure uniform dispensing of the suspension into 2 mL labelled sterile cryovials and sealed.

A modified bath (the schematic diagram of the bath is shown above) was filled with liquid nitrogen to an appropriate level. The sealed cryovials were clipped onto terry clips of racks that gripped the caps of the vials. The loaded racks were then placed into the relevant fixture of the modified bath containing liquid nitrogen. The level of liquid nitrogen was measured and topped up to 7 cm below the level of the racks that the cryovials were attached to making sure the vials were not touching any liquid nitrogen. An alcohol thermometer was placed in an empty cryovial to monitor the temperature of freezing. The cryovials were cooled gradually in the nitrogen vapour. The cooling temperature was monitored and recorded every minute. Once the temperature reached -90°C, the vials were released from the terry clips and dropped into liquid nitrogen. The vials were removed from the bath using pre-cooled forceps and placed into a storage container of liquid nitrogen until needed.

2.3.7.1 Selection of a cryoprotectant for liquid nitrogen storage of the bacterial cells

Glycerol, DMSO and PVP were tested in order to select the best cryoprotectant for freezing of the microorganisms. *E. coli* was used as the test organism. The cryoprotectants were added to ¼ strength Ringer's solution at a concentration of 10% v/v for DMSO and PVP and 15% v/v for glycerol. Previous experience indicated that ¼ strength Ringer's solution was a good freezing and storage medium for the microorganisms due to its isotonicity. A control, without cryoprotectant, was also tested. Plate count of the organism was done pre and post freezing by serial dilution described in section 2.3.6. Cell recovery after 1 day, 1 month and 6 months was monitored to assess the effect of storage time (in liquid nitrogen) on the viability of the culture.

2.3.8 Defrosting of frozen bacteria

Vials of the frozen bacteria were removed from storage container of liquid nitrogen when needed. They were held in a floater (which kept their screwed caps above the surface of water) and placed in a water bath with temperature set at 40°C for 3 min. They were vortexed for 1 min before use.

2.3.9 Microcalorimetric experiments

A Thermometric Thermal Activity Monitor 2277 (TAM 2277) (TA Instruments Ltd., UK) was used for all the microcalorimetric experimentations reported in this thesis. The temperature of the instrument was set at 37°C ($\pm$ 0.1°C). All loaded samples were allowed to equilibrate thermally at the intermediate position for 30 min before measurement. Data were collected every 10 s, with an amplifier range of 1000 µW using the software package, Digitam 4.1 and analysed using Origin Pro 8.6 (Microcal Software Inc.). The reference ampoule was loaded with 3 mL of sterile media respective of the experiment being carried out. The instrument was calibrated at regular intervals.

Experiments were repeated at least in triplicate. Plate counts and pH measurements (pHenomenal®, UK) were done post calorimetric experiments.

2.3.9.1 Pure culture experiments

A vial was removed from the storage liquid nitrogen container, thawed at 40°C for 3 min and vortexed for 1 min. All stocks were frozen down to a population density of ca. 1×10^8 CFU/mL. 30 µL of the thawed culture was inoculated into 2970 µL of pre-warmed broth (NB or CMM) in a sterile 3 mL calorimetric glass ampoule giving a population density of 10^6 CFU/mL. The ampoules were sealed with crimped caps and vortexed for 10 s. They were placed in the TAM and data recorded.

All thawed aliquots were subcultured and checked for purity and uniformity of the stock.

2.3.9.2 Mixed culture experiments

One species was inoculated at a constant density of cell numbers (10^6 CFU/mL) and mixed with different densities of the other (10^3 - 10^6 CFU/mL) in a 3 mL calorimetric ampoule. For example, 30 µL (representing 10^6 CFU/mL) of thawed culture of one species and 30 µL (10^6 CFU/mL); 3 µL (10^5 CFU/mL); 0.3 µL (10^4 CFU/mL) etc. of the other organism were added to pre-warmed culture media to make a volume of 3 mL in a sterile calorimetric ampoule. The ampoule was treated as above.

Two or three species were also inoculated at the same population density: 10^6 CFU/mL,

10^5 CFU/mL and 10^4 CFU/mL.

2.3.10 Statistical Analysis

Results were expressed as mean and standard deviation throughout this thesis. Statistical analysis was performed in Origin Pro 8.6 (Microcal Software Inc.). T-test or Analysis of Variance (ANOVA) with Turkey post-hoc analysis for means comparison was used where appropriate. P values less than 0.05 were regarded as significant difference between means.

2.4 Results and Discussion

2.4.1 Growth curves

Given an appropriate medium, a bacterium will grow until all the nutrients in the medium are used up or the medium condition is no longer conducive to growth due to for example, toxic waste accumulation. Bacteria grow characteristically through self-replication. This implies that a bacterium is able to produce completely identical daughter cells, which also have the potential to divide again. Hence cell numbers will increase exponentially as a function of time in an appropriate medium. With time however, the nutrient is exhausted and growth is considerably slower or ceases when the rate of consumption of nutrients exceeds the rate of supply.

All the species studied, showed a typical bacterial growth pattern as shown in Figure 2.2. There was a lag period (A), during which the bacteria adapted their physiology to that required for growth on the available nutrients. This was followed by an active growth where the increase in bacterial numbers was exponential (B). As growth proceeded, nutrients were consumed and waste materials accumulated. This reduced the rate of growth as numbers of new cells formed was equal to those that were dying; the stationary phase (C).

The reason for studying the growth curves of these organisms was to determine their point for harvesting. Morgan et al., (2006), has proposed that the optimal phase for

harvesting of cells depends on the organism in question. However, other authors have suggested that for all microorganisms, cells grown and harvested at the late logarithmic phase or early stationary phase are most stable and viable (Mary et al., 1986, Corcoran et al., 2004). Others also claim that cells need to be grown to an optimal population density for harvest. Historically a population of 1×10^8 cells mL^{-1} or more has been proposed as ideal (Morgan et al., 2006, Prakash et al., 2013) on the basis that the higher the initial cell concentration, the greater the recovery of viable cells within a sample (Heckly, 1978, Alexander et al., 1980, Simione and Brown, 1991). For this study, the time required for the organisms to reach a population density of 10^8 CFU/mL was determined by OD measurement and plate count. For *S. aureus* NCIMB 9518 the time required for this population was at ca. 4 h; 6 h and 40 min for *P. aeruginosa* NCIMB 8626 and 3 h and 30 min for *E. coli* ATCC 25922 with OD readings at ca. 0.31, 0.32 and 0.44 respectively. The absorbance reading was used as a reference point for harvesting the cells in subsequent experiments.
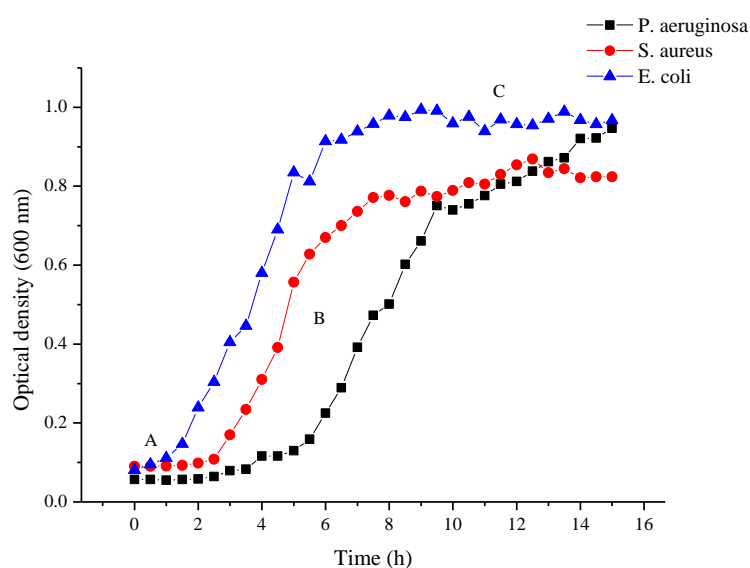
Figure 2.2. Growth curves of *P. aeruginosa*, *S. aureus* and *E. coli* in Nutrient broth

2.4.2 Selection of a cryoprotectant for liquid nitrogen storage of the bacterial cells

The percentage viable cells (if the total number of cells before freezing is taken as 100%) of freshly harvested and frozen samples of *E. coli* suspended in different

cryoprotective agents and a control without cryoprotectant (only ¼ Ringer's solution) is shown in Figure 2.3. The result showed that cell recoveries were better when the cryoprotectants were added to the freezing medium.
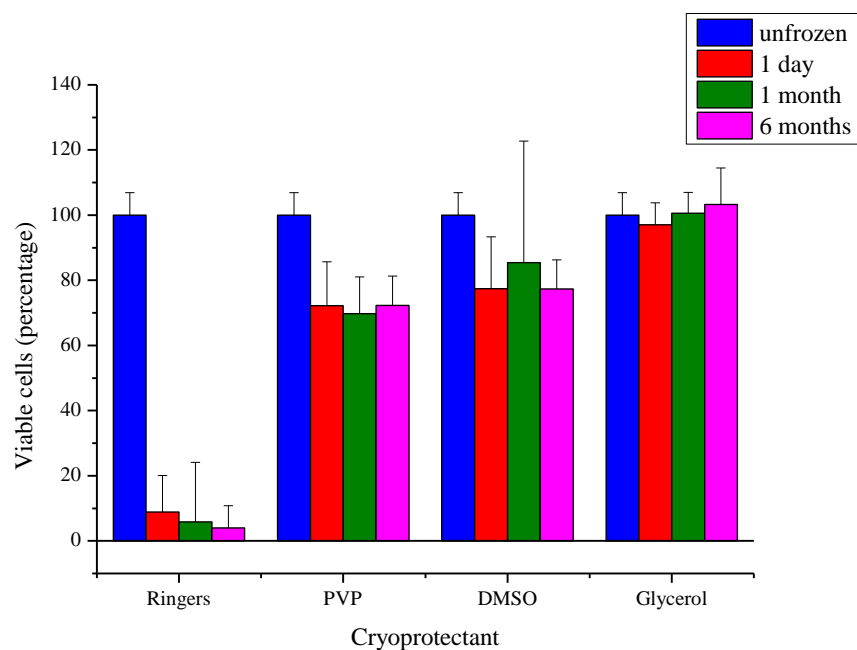
Figure 2.3. Comparison of the percentage viability of *E. coli* after freezing and storage using different cryoprotective agents. Bars indicate standard deviations from the mean of triplicate experiments

As noted earlier, during freezing of bacterial cells for long-term storage, certain variables need to be considered. Among the variables, the composition of the freezing medium, the storage temperature and the cooling rate are critical and these were considered in this research work. For the work conducted, the cooling rate recorded during cryopreservation was between -4°C and -7°C per min. The cells were stored in liquid nitrogen; it was observed that overall, cell numbers were not significantly affected ($P>0.05$) by the time of storage in liquid nitrogen for PVP, DMSO and glycerol but was significantly affected ($P<0.05$) for unsupplemented ¼ Ringer's solution.

The addition of a cryoprotectant to the freezing medium, certainly improved the recovery of the cells over the time period tested. For the cryoprotectants tested, DMSO and glycerol showed the better protection of the cells. This does not come as a surprise because DMSO and glycerol are both penetrating agents (although DMSO has better penetrating ability than glycerol; Hubalek, 2003, Prakash et al., 2013) capable of

protecting the cells from both intracellular and extracellular damage. PVP is nonpenetrating (Hubalek, 2003) and mainly protects against extracellular ice formation by increasing the viscosity of the medium and keeping the structure of ice amorphous at the surface of the cell. But DMSO and glycerol can also reduce the amount of frozen water, by decreasing the freezing-point of water and by so doing, lessening the concentration of salt dissolved in solution which effectively inhibit osmotic shock (Lovelock, 1953, Lovelock and Bishop, 1959). For example, addition of glycerol to water can decrease the freezing point of water to a minimum of -46°C whilst addition of DMSO can decrease it to -73°C (Hubalek, 2003). They also prevent the formation of large ice crystals within the cells but stimulate the formation of fine ice crystals (quasiamorphous) within the cells (Hubalek, 2003).

The selection of the cryoprotectants tested was based on their simplicity, universality and availability. The selected cryoprotectants, particularly DMSO and glycerol are widely used for cryopreservation of microbial cells. They are often used at median concentrations of 10-15% v/v and have been shown to be safe for cells (Hubalek, 2003). At higher concentrations, they have been reported to be toxic to some cells. For example, growth inhibitory activity of DMSO has been observed and reported when used at concentrations of 10% v/v for *Staphylococcus*, *Micrococcus*, *Pseudomonas*, *Streptococcus*, *Lactococcus*, *Corynebacterium*, and *E. coli* (Fomin et al., 1973) and toxicity has been shown for some cells at 40% v/v (Tsutsaeva and Vysekantsev, 1983) although many bacteria have also been shown to tolerate very high concentration of DMSO without toxic effects (Fedorka-Cray et al., 1998). Toxicity to DMSO is generally higher when the cells are kept at higher temperatures (Hubalek, 2003) due to the association of temperature to the permeability into the cells. Glycerol has also shown toxicity to a number of bacterial species: *Chlamydia* spp., *Staphylococcus*, *Micrococcus*, *Lactococcus*, *Streptococcus*, *Pseudomonas*, *Corynebacterium diphtheriae* and *E. coli* (Fomin et al., 1973) but PVP has the advantage of very low toxicity in majority of microorganisms even at higher temperatures for which glycerol and DMSO were shown to be toxic (Osborne and Lee, 1975, Eidtmann and Schauz, 1992). In our selection of cryoprotectants, complex cryoprotectants such as yeast extract, malt extract and culture media, peptides and proteins and sugars were not considered because they could introduce complications into the microcalorimetric experiments, being possibly metabolised by the cells from the moment they were thawed which could cause deviations from heat output.

In the present study and other studies using different organisms, (results not shown), glycerol consistently gave better recovery than DMSO and PVP with recoveries ≥ 95%. Glycerol was used for cryopreservation of all the organisms studied.

2.4.3 Microcalorimetric experiments

2.4.3.1 Pure culture experiments

As stated in Chapter 1, when a culture of bacteria in an appropriate growth medium is placed into a microcalorimeter, a power-time curve is generated (Figure 2.4 black curve) which shows the heat produced by the growing bacteria. This power-time curve shows periods of rises and decline. When integrated (Figure 2.4 red curve), it shows characteristics comparable to the conventional turbidimetric growth curve of bacteria: an initial lag phase, followed by an exponential phase, and a plateau (stationary phase). And has been shown to be directly proportional to biomass measured by optical density, plate counts or protein concentration (Braissant et al., 2010a).
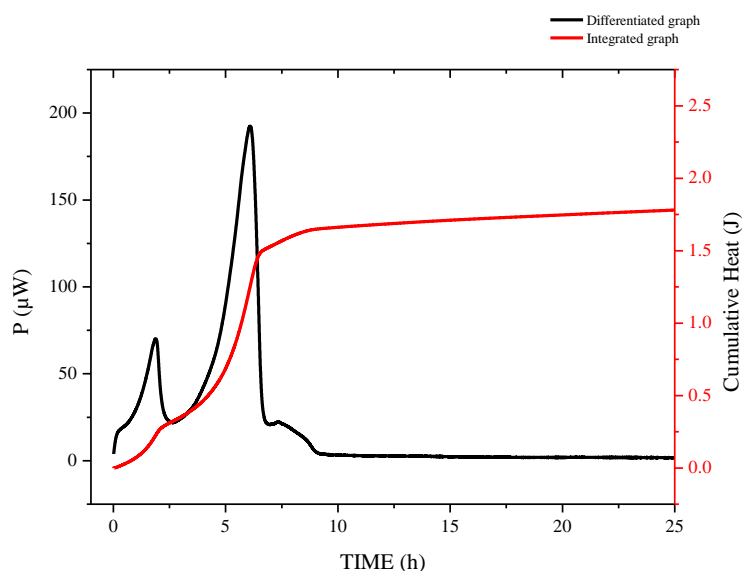
Figure 2.4. The relationship between microcalorimetric measurement and microbial activity. The typical power-time curve of *P. aeruginosa* (black curve) is integrated (red curve) to give a curve which resembles a classical growth curve obtained from optical density measurements

The power-time curves for each of the species studied are shown below. The power-time curves are complex and characteristic of organisms growing in complex media with restricted oxygen (the ampoule is sealed and the oxygen level is limited to that dissolved in the medium and present in the headspace).

2.4.3.1.1 *P. aeruginosa*

Figure 2.5 shows the power-time curves of 4 repeats of *P. aeruginosa* inoculated to a population density of 10^6 CFU/mL and studied over 20 h duration. The power-time curves are reproducible. There is an initial exponential increase in heat output with peak at ca. 2 h, which is followed by a second peak at ca. 5 h. The decline in heat flow between the first and second peaks does not necessarily mean a major decline in cell numbers. It could represent the diauxic growth of the organism in the environment created (complex media and aerobic/anaerobic atmosphere) (Schaarschmidt and Lamprecht, 1978, Von Stockar and Birou, 1989).
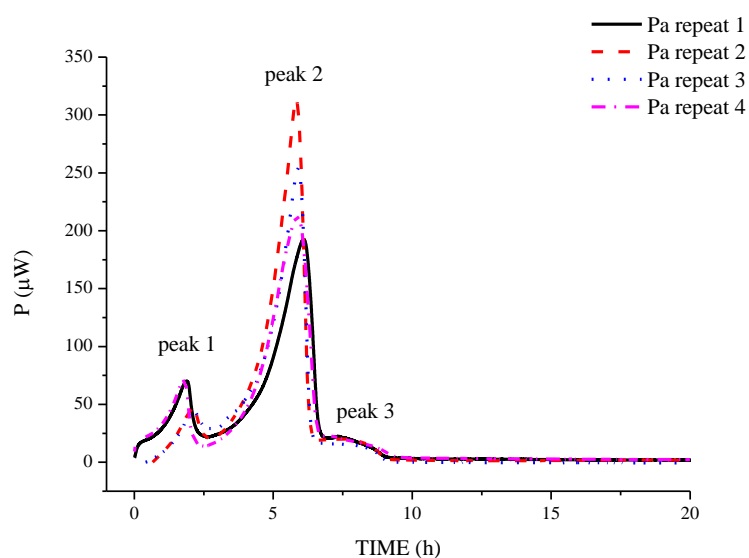
Figure 2.5. Power-time curves of four repeats of *P. aeruginosa* inoculated to a cell density of 10^6 CFU/mL in NB. Pa represents *P. aeruginosa*

When bacteria are presented with multiple energy substrates, they would consume these substrates sequentially resulting in diauxic growth (Monod, 1949). They would utilize first the substrate that is easier to metabolize which leads to rapid growth, followed by a

lag phase (heat flow decrease), where the bacteria have to synthesize new enzymes to metabolize the second substrate. Thus, the change from one energy substrate to another often requires the synthesis of new enzymes that can be subjected to catabolite repression. This diauxic growth has been demonstrated by Schaarschmidt and Lamprecht, (1978) when studying the anaerobic growth of *Saccharomyces cerevisiae* on defined multiple carbohydrate sources in the microcalorimeter. These authors demonstrated that on sole glucose or mannose, *Saccharomyces cerevisiae* had a single exponential peak in an anaerobic environment but could also potentially produce a second growth phase from accumulated ethanol (from metabolism) when the culture had sufficient supply of oxygen. They also demonstrated that glucose-galactose, glucose-sucrose, fructose-maltose and sucrose-maltose combination all showed diauxy in the microcalorimeter. They illuminated that the two pairs of carbohydrates are taken up and degraded by different enzyme systems. For example, galactose metabolism involves inducible transport in addition to inducible enzyme for degradation, requiring time for the induction; maltose also requires an induction time for transport and hydrolysis whereas glucose, fructose and mannose use the same constitutive transport system (Schaarschmidt and Lamprecht, 1978). The biphasic or diauxic growth has also been reported to be observed when bacteria and yeast are switching from aerobic metabolism to anaerobic metabolism in the microcalorimeter (Beezer, 1980, Von Stockar and Birou, 1989, Braissant et al., 2013). Some authors have suggested that for *P. aeruginosa*, the decline in heat flow between the first peak and second peak is the transition from aerobic growth to anaerobic growth (O'Neill et al., 2003, Gaisford et al., 2009). To test this, the experiment was conducted in an anaerobic environment by filling and sealing the ampoule in a Don Whitley DG250 Scientific Anaerobic workstation, UK. The organism was also inoculated to the same density (10^6 CFU/mL) in 1 mL of broth in the same 3 mL capacity ampoule, creating a larger headspace. Monophasic growth was observed (Figure 2.6) with a larger heat intensity and longer duration for a 1 mL normal atmospheric fill. When the ampoule was filled and closed in an anaerobic environment, for a 1 mL fill, only one peak again was observed, with a peak maximum occurring at approximately the same time as the second peak for the 3 mL fill done under normal atmospheric condition suggesting that possibly the diauxic growth observed in a normal fill was due to the transition from aerobic to anaerobic metabolism. The integrated plot (Figure 2.6 [B]) of these power-time data suggested that the preferred or primary metabolic pathway for *P. aeruginosa* was aerobic as the anaerobically filled samples returned infinitesimal cumulative heat.

For the standard *P. aeruginosa* growth curves (Figure 2.5), after the second peak, there was a final exothermic activity (peak 3), which could be attributed to the anaerobic utilization of an unconsumed energy substrate typical with complex growth media (Gaisford et al., 2009) before the return to baseline; shown to represent stationary phase (Fan et al., 2008, Kabanova et al., 2009, Braissant et al., 2013). The total area under the curve (AUC) for a *P. aeruginosa* culture over 20 h, representing the total heat output was 1.82 ± 0.12 J.
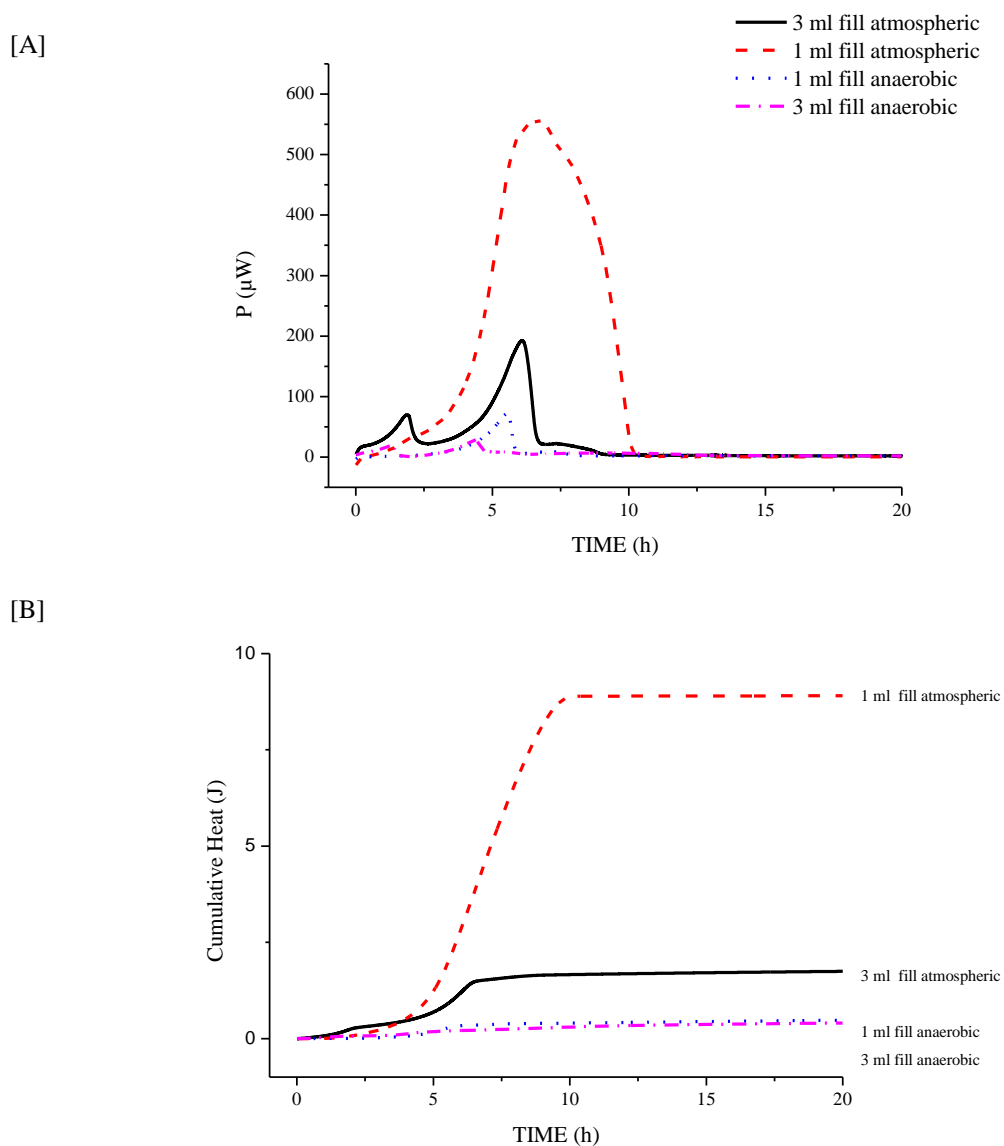
Figure 2.6. Power and heat curves of *P. aeruginosa* filled and sealed under aerobic and anaerobic environments. [B] is the integrated plots of [A]

2.4.3.1.2 *S. aureus*

Figure 2.7 shows the power-time curves of 4 repeats of *S. aureus* with good reproducibility. The area under the curves, representing the total heat output was 3.91 ± 0.83 J. The power-time curves show two peaks within the first 5 h. There is then a transition period (between ca. 5 and 11 h) and this is followed (at ca. 12.5 h) by a third peak, before the growth curve returned to the baseline. The first peak, which has been previously assumed to represent aerobic metabolism of the organism in the medium (O'Neill et al., 2003), is still present, though in lower intensity when the ampoule was filled and sealed under anaerobic condition (Figure 2.8). The second and third peaks, also previously assumed to be associated with sequential anaerobic metabolism of major carbohydrates (O'Neill et al., 2003) are also present when the oxygen headspace is increased. The third peak was however lost when the ampoule was sealed under anaerobic condition.
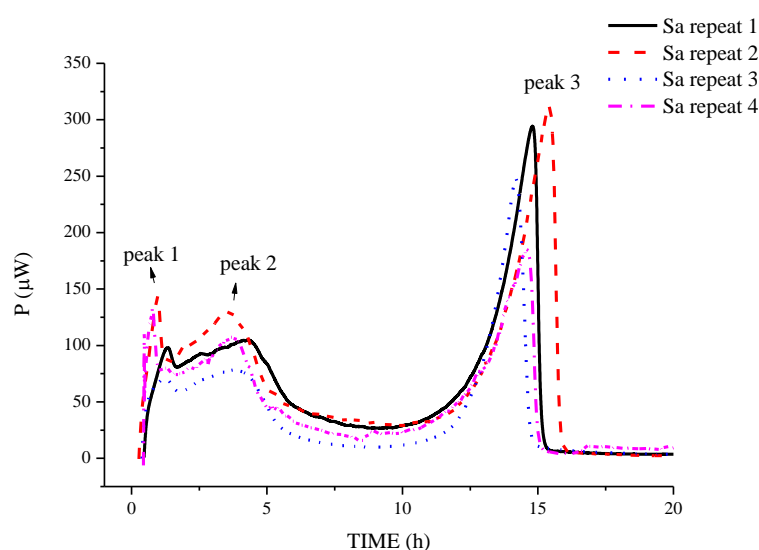
Figure 2.7. Power-time curves of *S. aureus* inoculated to a cell density of 10^6 CFU/mL in NB. Sa represents *S. aureus*

For an atmospheric fill, increasing the headspace meant a longer aerobic phase; hence it could be reasonably assumed that the peak that was amplified when the headspace was increased was the aerobic phase of metabolism. This peak (peak 3) was also absent in the anaerobic cultures. It is plausible that the third peak could be associated with aerobic growth. The initial exponential peaks could be associated with micro-aerophilic growth.
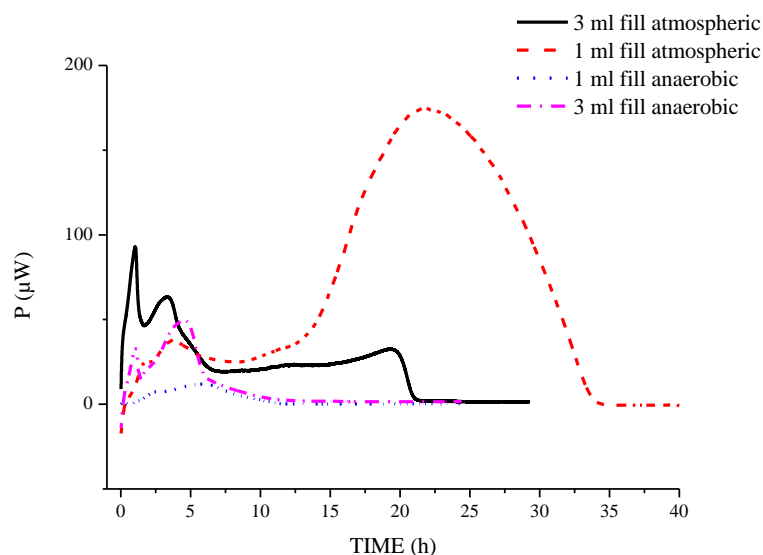
Figure 2.8. Power-time curves of *S. aureus* filled and sealed under aerobic and anaerobic environments

2.4.3.1.3 *E. coli*

The power-time curves of 4 repeats of *E. coli* in NB are shown in Figure 2.9 and they show exponential phases (occurring within 7.5 h) and a decline phase (after ca. 9 h). The area under the curve, representing the total heat output was 3.07 ± 0.44 J.
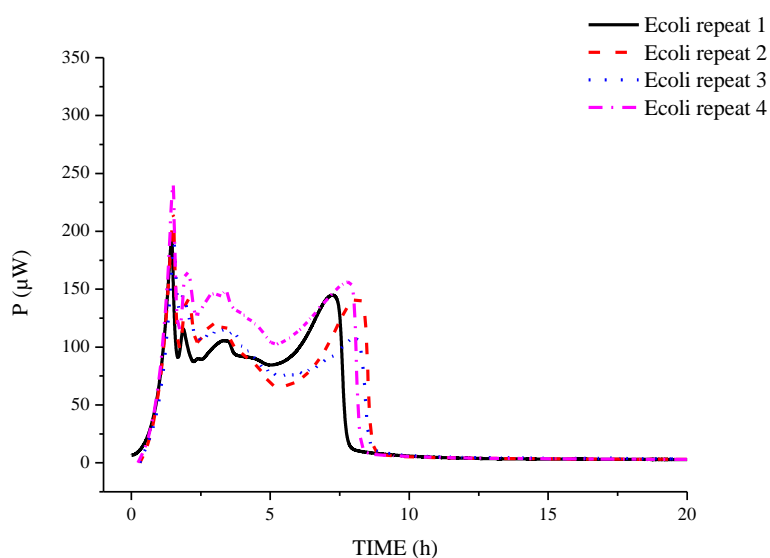
Figure 2.9. Power-time curves of *E. coli* inoculated to a cell density of 10^6 CFU/mL in NB

Overall, the three species studied had very characteristic power-time curves in NB. The curves were very reproducible and could be used for identifying them. Between the three species, *E. coli* showed more peaks and troughs in the growth curve probably because it can utilize more substrates in the environment created than the other species (Scheutz and Strockbine, 2005). As mentioned and shown with the experiments, the gaseous environment within the ampoules was of limited oxygen supply and it was expected that the organisms would switch from aerobic metabolism to anaerobic. Both the aerobic and anaerobic growths are demonstrated by exponential increase in heat output followed by a decline. Also, a switch from one energy substrate to another will result in the decline of an exponential growth and a rise of another (Schaarschmidt and Lamprecht, 1978, Beezer, 1980). Therefore the complexity of the power-time data can be attributed to the gaseous environment in the ampoule as well as the complexity of the medium used and an organism's ability to utilize the multiple energy substrates (including its metabolites (Schaarschmidt and Lamprecht, 1978) in the complex medium. For example, *E. coli* an *Enterobacteriaceae*, is known to ferment glucose and other carbohydrates to pyruvate, which is further converted to lactic acid, acetic and formic acids with part of the formic acids degraded into CO_2 and H_2 (Scheutz and Strockbine, 2005). A sequential degradation and uptake of the carbohydrates present in the medium (given the medium condition) and its metabolites (Narang et al., 1997) could have contributed to its complex curve.

It can be expected that if an anaerobic environment is generated within the ampoule, the peaks and troughs of the power-time curves will be reduced, unless the species being studied utilizes a number of different fermentation pathways given a complex medium. This was observed when the ampoule was filled and sealed anaerobically and also when a reduced medium was used in Chapter 3.

Among the three species, *S. aureus* showed metabolism for the longest time, producing metabolic heat for periods in excess 15 h. The heat curves and total heat output of the three species studied are compared in Figure 2.10 [A] and [B] respectively. The heat curve shows that the growth profile of the species within the first 2 h were similar but *S. aureus* and *E. coli* kept similar growth profile for a further 5 h whilst *P. aeruginosa* showed a biphasic log (diauxie) in the heat curve during that period. Also, *P. aeruginosa* recorded the least heat output whilst *S. aureus* recorded the highest inferring that *S.*

aureus metabolized the medium more energetically in the environment created than the others, the reason for this is not yet known.

[A]
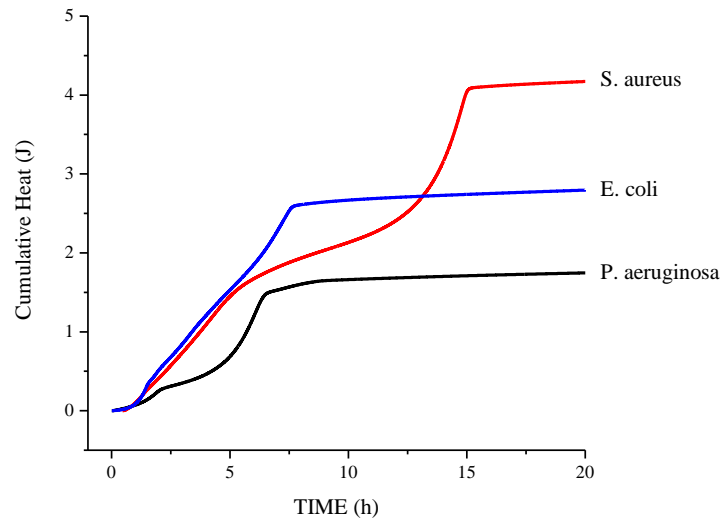
[B]
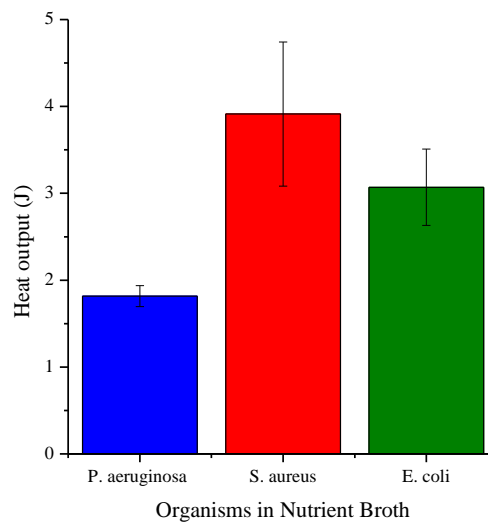
Figure 2.10. Comparison of [A] cumulative heat curves and [B] mean heat output of *P. aeruginosa*, *S. aureus* and *E. coli* inoculated to 10^6 CFU/mL in NB. The heat outputs were calculated from the AUC of four repeats.

2.4.3.2 Mixed culture experiments

These experiments were undertaken to study how a species of bacteria behaved metabolically in the presence of other species in the microcalorimeter. They were also done to establish whether heat outputs were proportional to cell numbers of microorganisms in mixed cultures and whether the microcalorimeter could show the growth of the species concurrently.

2.4.3.2.1 Mixed culture of *P. aeruginosa* and *S. aureus*

The power-time curves and heat outputs of *P. aeruginosa* and *S. aureus* and a 1:1 mixed culture of them are compared in Figures 2.11 and 2.12 respectively.

When *P. aeruginosa* and *S. aureus* were mixed in the same ratio in NB and placed in the TAM, the resulting power-time curve that was generated showed similarities to the power-time curve of *P. aeruginosa* alone in NB (Figure 2.11) even though the first exponential peak could also represent both species since they both had similar growth pattern over the first few hours. However the characteristic transitional phase between the second and third exponential phase of *S. aureus* was lost in the mixed culture power-time curve. The heat output generated by the mixed culture was 2.10 ± 0.41 J. This heat output was significantly different ($P<0.05$) from heat output generated by a pure culture of *S. aureus* but did not significantly differ ($P>0.05$) from the heat output generated by a pure culture of *P. aeruginosa* (Figure 2.12). The results suggest that a mixed culture of *P. aeruginosa* and *S. aureus* behaves metabolically like *P. aeruginosa* alone, inferring that either *P. aeruginosa* suppressed the growth of *S. aureus* because the medium was nutritionally more favourable to the growth and metabolism of *P. aeruginosa* than *S. aureus* or that *P. aeruginosa* could adapt and use the medium faster in a mixed culture situation. Another explanation that can be given to the observed occurrence is the possibility that *P. aeruginosa* produced inhibitory factors that suppressed or inhibited the growth of *S. aureus*. These inhibitory factors however could not be acidic; this is because when the two species were inoculated into NB with an initial pH of 7.15 ± 0.1, the final pH of the ampoule after the calorimetric experiment revealed a significantly lower pH for *S. aureus* ($P<0.05$), 6.31 ± 0.2, whilst the pH after growth of *P. aeruginosa* (6.77 ± 0.2) was not significantly different ($P>0.05$) from the uninoculated medium.
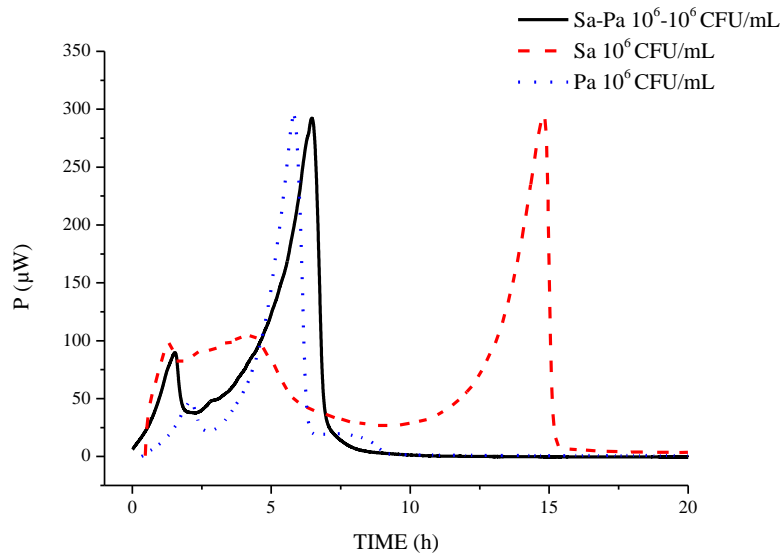
Figure 2.11. Comparison of the power-time curves of *P. aeruginosa* (blue), *S. aureus* (red) and a mixed culture of them (black) in NB. Sa represents pure culture of *S. aureus*. Pa represents pure culture of *P. aeruginosa.* Sa-Pa represents co-culture of *S. aureus* and *P. aeruginosa*. The species were each inoculated to 10^6 CFU/mL. The final density in the mixed culture was 2 x10^6 CFU/mL
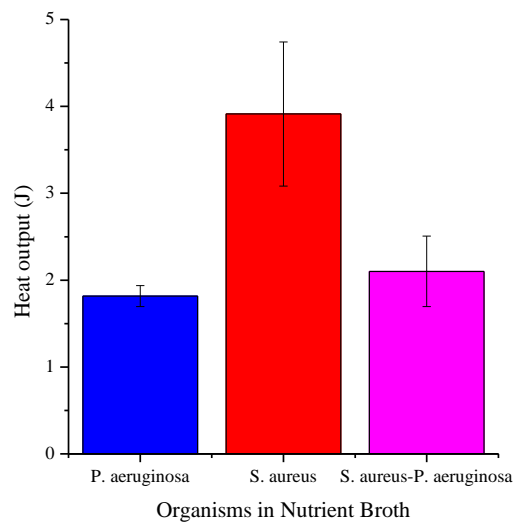
Figure 2.12. A bar chart comparing the mean heat outputs (n=4) of *P. aeruginosa, S. aureus* and a mixed culture of them in NB. All species were inoculated to a final density of 10^6 CFU/mL. In a mixed culture, the individual species were each inoculated to 10^6 CFU/mL, therefore, the final density in the mixed culture was 2 x10^6 CFU/mL

P. aeruginosa, a Gram-negative organism like *E. coli* is known to produce allelopathic substances (Riley and Gordon, 1992, Michel-Briand and Baysse, 2002, Zhou et al.,

2011) that target and kill other organisms. It is known to produce antistaphylococcal substances: 4-hydroxy-2-heptylquinoline-N-oxide (HQNO), hydrogen cyanide and pyocyanin that suppress the growth of *S. aureus* and many other Gram-positive bacteria (Machan et al., 1992, Biswas et al., 2009). This suppression has been observed by a number of authors. For instance, Kluge et al., (2012), during a batch cultivation of mixed culture consisting of *Burkholderia cepacia*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* with and without a cephalosporin antibiotic, ceftazidime, observed the dominancy of *P. aeruginosa* in the mixed culture whilst *S. aureus* was inhibited in growth (Kluge et al., 2012). Inhibition of *S. aureus* by *P. aeruginosa* has also been reported by Perestelo et al., (1985), Hoffman et al., (2006), Biwas et al., (2009) and Baldan et al., (2014).

This inhibition or suppression has been associated with the fact that in co-infections with *S. aureus* and *P. aeruginosa*, *S. aureus* is frequently less cultured although both could also be coisolated in infected cultures (Hoffman et al., 2006). For instance, for chronic lung infection of patients with cystic fibrosis, *S. aureus* is the pathogen most commonly cultured from the respiratory cultures of young children with the disease but as the patients age, the infection is known to be predominated by *P. aeruginosa* (Cystic Fibrosis Foundation 2008, Baldan et al., 2014). This predominance has been associated with this arsenal of compounds produced by *P. aeruginosa* in mixed cultures, which inhibits *S. aureus* (Biswas et al., 2009, Baldan et al., 2014).

It is possible that the microcalorimeter was able to detect this inhibition or suppression. However, the inhibition was not absolute; a post microcalorimetric plate count of the mixed culture (Table 2.1) still showed the presence of viable *S. aureus*, although decreased. Also, the power-time curve of the mixed culture was not an exact replica of that of the pure culture of *P. aeruginosa*. To confirm this inhibition in the microcalorimeter, a study was carried out where the population density of *S. aureus* was kept constant (at 10^6 CFU/mL) and that of *P. aeruginosa* decreased from 10^6 to 10^3 CFU/mL. The resulting power-time curves are compared in Figure 2.13. This figure shows that the power-time curve of a mixed culture of *P. aeruginosa* and *S. aureus* reverts gradually from the dominating characteristic curve of *P. aeruginosa* to curves that show recuperation of *S. aureus* as the population of *P. aeruginosa* was decreased in the mixed culture.
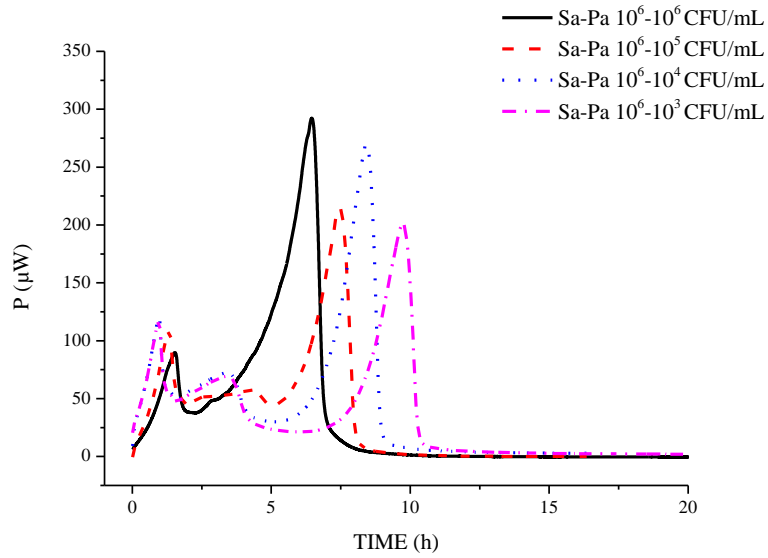
Figure 2.13. Comparison of power-time curves of mixed culture of *P. aeruginosa* and *S. aureus* when the density of *S. aureus* was kept constant at 10^6 CFU/mL and that of *P. aeruginosa* was decreased from 10^6 to 10^3 CFU/mL. Sa-Pa 10^6-10^6 represents co-culture of *S. aureus* at a density of 10^6 CFU/mL and *P. aeruginosa* at 10^6 CFU/mL. The first value is representative of the preceding organism and the later for the later organism. Sa-Pa $10^6 10^5$ represents co-culture of *S. aureus* at a concentration of 10^6 CFU/mL and *P. aeruginosa* at 10^5 CFU/mL. The same trend applies for all cases of co-culture
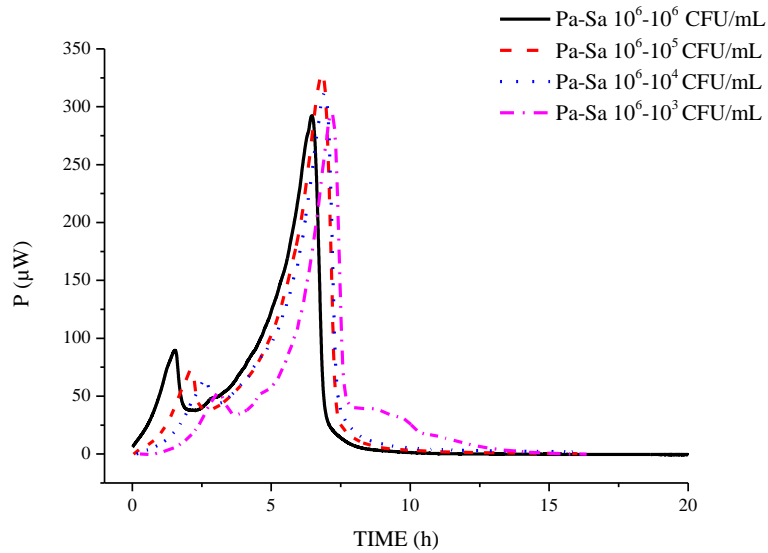
Figure 2.14. Comparison of power-time curves of mixed culture of *P. aeruginosa* and *S. aureus* when the density of *P. aeruginosa* was kept constant at 10^6 and that of *S. aureus* was decreased from 10^6 to 10^3 CFU/mL

87

When plate counts of the different mixed culture of *P. aeruginosa* and *S. aureus* were done after the microcalorimetric experiment, it further confirmed inhibition (Table 2.1), showing that anytime the population of *P. aeruginosa* was decreased relative to *S. aureus* in a mixed culture, the growth of *S. aureus* was enhanced. When the population density of *P. aeruginosa* was kept constant (at 10^6 CFU/mL) and that of *S. aureus* decreased from 10^6 to 10^3 CFU/mL, the power-time curves suggested dominance of *P. aeruginosa* in all cases (see Figure 2.14). However, a time lag was added to the power-time curve as the density decreased exponentially. To verify that the time lag was as a result of the decreasing volume of the inocula, both *P. aeruginosa* and *S. aureus* were inoculated in the same ratio but decreasing inoculum densities ($10^6:10^6$; $10^5:10^5$; $10^4:10^4$ CFU/mL) the power-time curves generated (Figure 2.15) were reproducible at these concentrations (and more identifiable with *P. aeruginosa*) and showed a clear time lag seen upon decreasing inoculum density (Figure 2.16) of a culture.
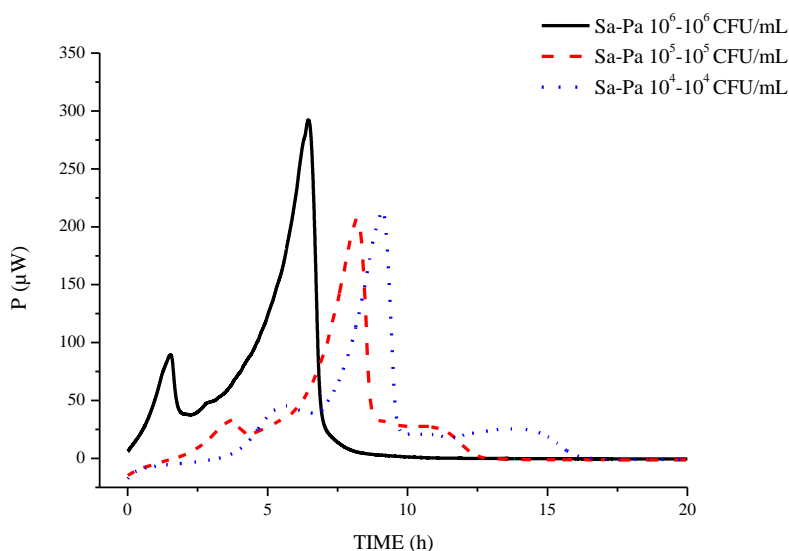
Figure 2.15. Comparison of the power-time curves of mixed cultures of *P. aeruginosa* and *S. aureus* in NB when inoculated in the same ratio (1:1) but different CFU (10^6, 10^5, 10^4)
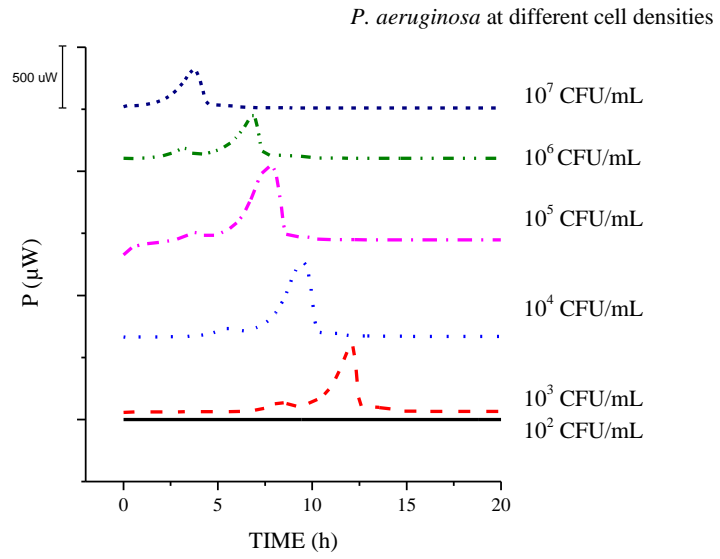
Figure 2.16. Power-time curves of decreasing inoculum density of *P. aeruginosa* showing linear time lags as the densities are decreased exponentially from 10^7 CFU/mL

Table 2.1. A table comparing the pH and bacterial plate counts of the species in NB post TAM experimentation. – no growth observed

Post incubation analysis of cultures		
Pure Culture	**pH**	**Plate count (log CFU/mL)**	
P. aeruginosa	6.8 ± 0.2	7.8 ± 0.1	
S. aureus	6.3 ± 0.2	8.1 ± 0.1	
E. coli	5.2 ± 0.3	8.2 ± 0.2	
Mixed Culture (CFU/mL)		***P. aeruginosa***	***S. aureus***
P. aeruginosa : *S. aureus*: $10^6 10^6$	6.3 ± 0.3	7.4 ± 0.2	5.7 ± 0.3
P. aeruginosa : *S. aureus*: $10^6 10^5$	6.4 ± 0.1	7.4 ± 0.3	6.5 ± 0.1
P. aeruginosa : *S. aureus*: $10^6 10^4$	6.4 ± 0.2	7.2 ± 0.4	6.0 ± 0.5
P. aeruginosa : *S. aureus*: $10^6 10^3$	6.5 ± 0.2	7.6 ± 0.2	3.1 ± 0.2
P. aeruginosa : *S. aureus*: $10^3 10^6$	6.3 ± 0.1	7.1 ± 0.2	7.2 ± 0.4
P. aeruginosa : *S. aureus*: $10^4 10^6$	6.4 ± 0.2	7.4 ± 0.3	7.4 ± 0.2
P. aeruginosa : *S. aureus*: $10^5 10^6$	6.4 ± 0.1	7.4 ± 0.2	7.1 ± 0.2
		P. aeruginosa	***E. coli***
P. aeruginosa : *E. coli*: $10^6 10^6$	5.4 ± 0.1	6.9 ± 0.5	8.1 ± 0.1
P. aeruginosa : *E. coli*: $10^6 10^5$	5.5 ± 0.2	6.8 ± 0.7	8.2 ± 0.2
P. aeruginosa : *E. coli*: $10^6 10^4$	5.8 ± 0.3	6.7 ± 0.1	8.2 ± 0.2
P. aeruginosa : *E. coli*: $10^6 10^3$	5.9 ± 0.2	7.4 ± 0.2	8.2 ± 0.1
P. aeruginosa : *E. coli*: $10^3 10^6$	5.6 ± 0.2	4.5 ± 0.3	8.2 ± 0.1
P. aeruginosa : *E. coli*: $10^4 10^6$	5.5 ± 0.2	4.7 ± 0.1	8.2 ± 0.2
P. aeruginosa : *E. coli*: $10^5 10^6$	5.4 ± 0.1	5.0 ± 0.3	8.3 ± 0.2
		S. aureus	***E. coli***
S. aureus : *E. coli* : $10^6 10^6$	5.6 ± 0.1	5.4 ± 0.5	7.9 ± 0.2
S. aureus : *E. coli* : $10^6 10^5$	5.9 ± 0.1	6.1 ± 0.3	7.7 ± 0.2
S. aureus : *E. coli* : $10^6 10^4$	5.9 ± 0.3	6.6 ± 0.1	7.3 ± 0.6
S. aureus : *E. coli* : $10^6 10^3$	5.9 ± 0.1	6.8 ± 0.1	6.4 ± 0.4
S. aureus : *E. coli* : $10^3 10^6$	5.4 ± 0.2	-	7.9 ± 0.2
S. aureus : *E. coli* : $10^4 10^6$	5.3 ± 0.1	-	8.1 ± 0.1
S. aureus : *E. coli* : $10^5 10^6$	5.3 ± 0.2	5.8 ± 0.2	8.1 ± 0.1

2.4.3.2.2 Mixed culture of *P. aeruginosa* and *E. coli*

When *P. aeruginosa* and *E. coli* were mixed in the same ratio in NB and placed in the TAM, the power-time curve that was generated could represent a power-time curve with the two species dominating equally (Figure 2.17). The power-time curve had some of the characteristic peaks of *E. coli* within the first two and half hours but the subsequent peak after that was identifiable with *P. aeruginosa*. The heat output generated by the mixed culture was 2.74 ± 0.52 J. This heat output was not significantly different (P>0.05) (Figure 2.18) from the heat output generated by a pure culture of *E. coli* (although decreased) suggesting that heat output was not necessarily proportional to cell numbers in mixed cultures but probably related to the interspecies effect.
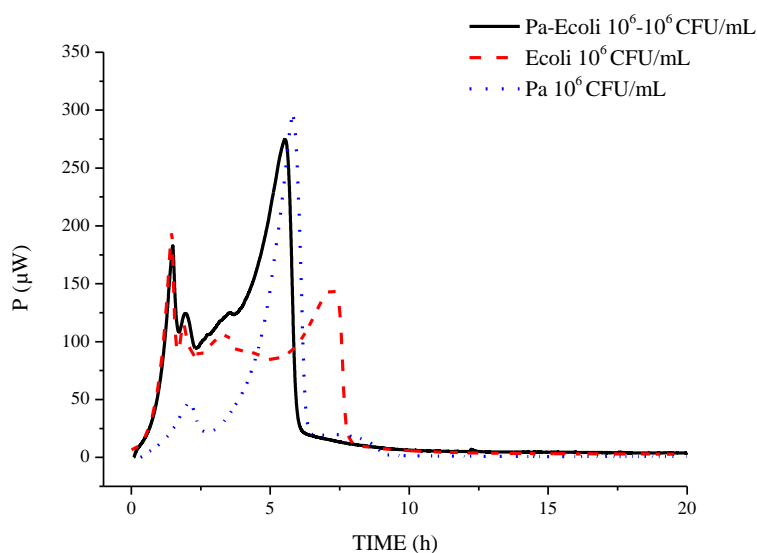
Figure 2.17. Comparison of the power-time curves of *P. aeruginosa* (blue), *E. coli* (red) and a mixed culture of them (black) in NB. Pa-Ecoli represents co-culture of *P. aeruginosa* and *E. coli*. The species were each inoculated to a density of 10^6 CFU/mL
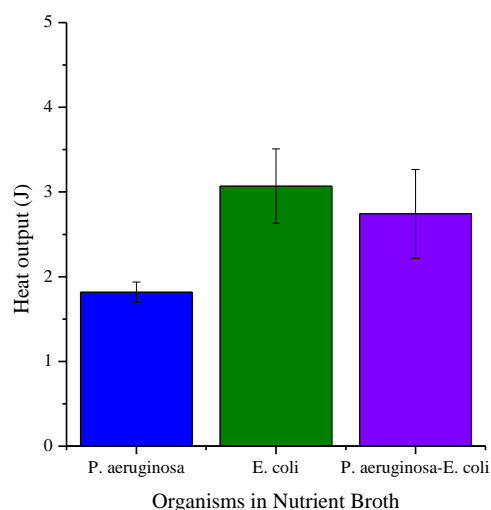
Figure 2.18. A bar chart comparing the mean heat outputs (n=4) of *P. aeruginosa, E. coli* and a mixed culture of them in NB. Each species was inoculated to a cell density of 10^6 CFU/mL

Both *P. aeruginosa* and *E. coli* produce allelopathic substances. More than 90% of both environmental and clinical isolates of *P. aeruginosa* are known to produce bacteriocins (Michel-Briand and Baysse, 2002) and 10 to 80% of *E. coli* strains (Gordon and O'Brien, 2006) produce bacteriocins: microcin and colicin (Gillor et al., 2008). In a mixed culture of them in a complex medium, *E. coli,* an extensive fermenter of carbohydrates, producing organic acids as products of fermentation, significantly decreased (P<0.05) the pH of the medium (from a starting pH of 7.15 ± 0.1 to 5.4 ± 0.1), which could have also inhibited *P. aeruginosa*. It is likely they may have both dominated in a mixed culture.

To further understand what was happening, experimentation was carried out like *P. aeruginosa – S. aureus* where the population density of *P. aeruginosa* was kept constant (at 10^6 CFU/mL) and that of *E. coli* decreased from 10^6 to 10^3 CFU/mL. Again, the resulting power-time curves are compared in Figure 2.19. This figure shows that the power-time curve of mixed culture of *P. aeruginosa* and *E. coli* reverts gradually to *P. aeruginosa* as the population density of *E. coli* is decreased in the co-culture. When the population density of *E. coli* was kept constant (at 10^6 CFU/mL) and that of *P. aeruginosa* decreased from 10^6 to 10^3 CFU/mL, the power-time curve also reverted to that of *E. coli* (Figure 2.20).
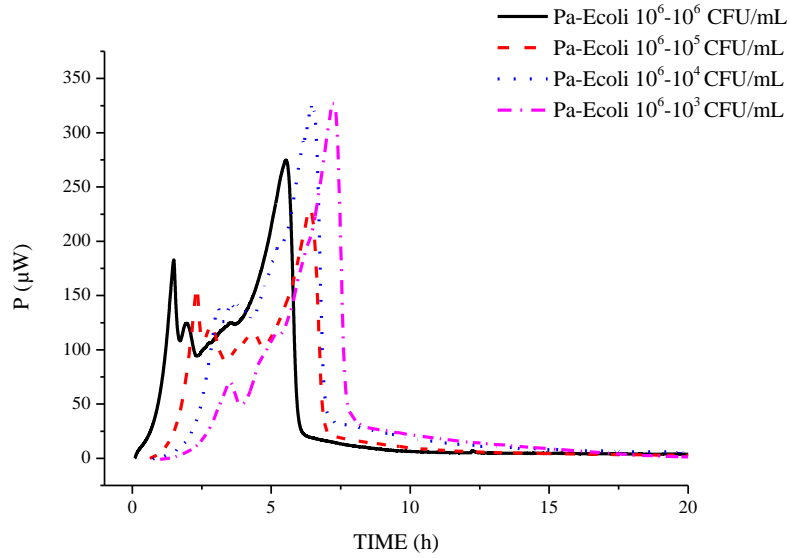
Figure 2.19. Comparison of the power-time curves of mixed culture of *P. aeruginosa* and *E. coli* when the density of *P. aeruginosa* was kept constant at 10^6 CFU/mL and that of *E. coli* was varied between 10^6 and 10^3 CFU/mL
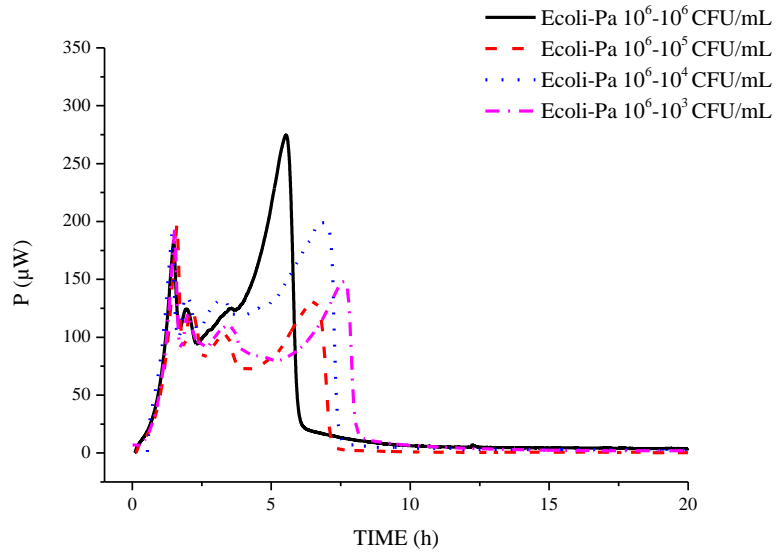
Figure 2.20. Comparison of the power-time curves of mixed culture of *P. aeruginosa* and *E. coli* when the density of *E. coli* was kept constant at 10^6 CFU/mL and that of *P. aeruginosa* was varied between 10^6 and 10^3 CFU/mL

93

2.4.3.2.3 Mixed culture of *S. aureus* and *E. coli*

The same trend of observation in mixed culture of *P. aeruginosa* and *S. aureus* was seen in mixed culture of *S. aureus* and *E. coli* (Figures 2.21, 2.22, 2.23 and 2.24). *E. coli* clearly dominated in its co-culture with *S. aureus*. Again, there is the possibility that *E. coli* inhibited the growth of *S. aureus* through the release of specific growth inhibitory substances. *E. coli* dominated irrespective of the population of *S. aureus*. *S. aureus* only began to persist when the population of *E. coli* began to decrease. Plate count done post microcalorimetry showed decreased presence of *S. aureus* in co-culture combinations especially when its inoculum density was less than 10^6 CFU/mL.
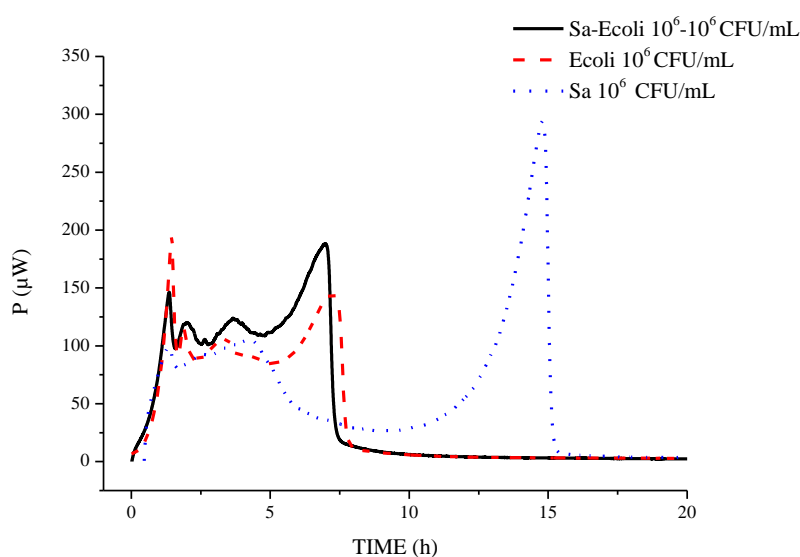
Figure 2.21. Comparison of the power-time curves of *S. aureus* (blue), *E. coli* (red) and a mixed culture of them (black) in NB. Each species was inoculated to a cell density of 10^6 CFU/mL
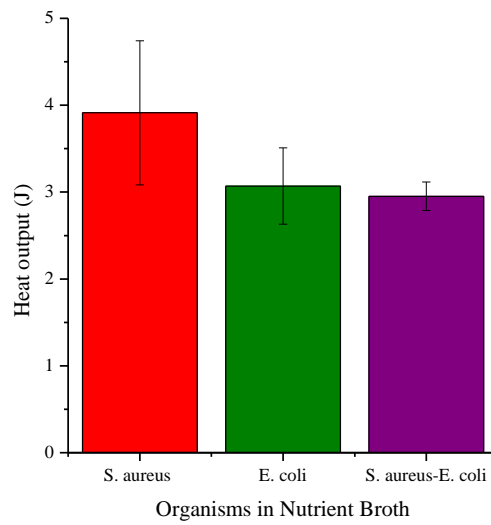
Figure 2.22. A bar chart comparing the mean heat outputs (n=4) of *S. aureus, E. coli* and a mixed culture of them in NB. Each species was inoculated to a cell density of 10^6 CFU/mL. The error bars are SD from the mean
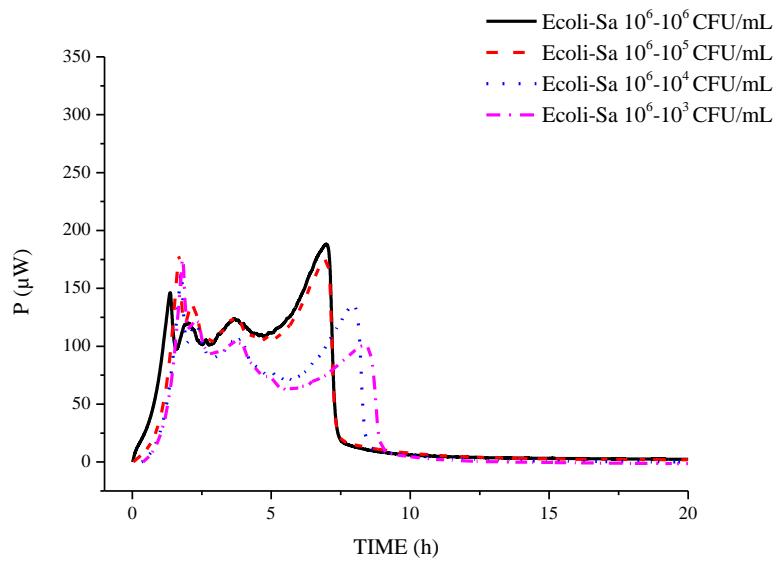
Figure 2.23. Comparison of the power-time curves of mixed culture of *S. aureus* and *E. coli* when the density of *E. coli* was kept constant at 10^6 CFU/mL and that of *S. aureus* was varied between 10^6 and 10^3 CFU/mL
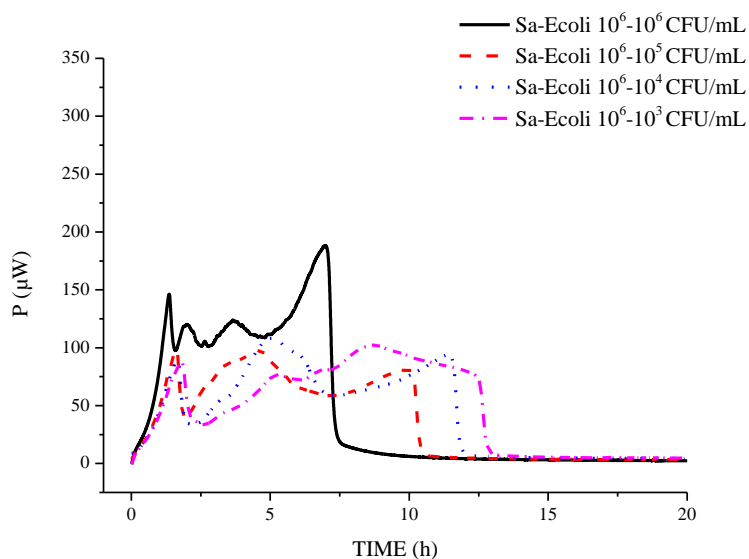
Figure 2.24. Comparison of the power-time curves of mixed culture of *S. aureus* and *E. coli* when the density of *S. aureus* was kept constant at 10^6 CFU/mL and that of *E. coli* was varied between 10^6 and 10^3 CFU/mL

2.4.3.2.4 Mixed culture of *P. aeruginosa*, *S. aureus* and *E. coli*

The power-time curves and heat outputs of *P. aeruginosa, S. aureus, E. coli* and a mixed culture of all three species NB are compared in Figures 2.25 and 2.26. The power-time curve and heat output (2.66 ± 0.1 J) suggest that a mixed culture of the three species behaves like a co-culture of *E. coli* and *P. aeruginosa.* One could thus argue that both *E. coli* and *P. aeruginosa* dominated in the mixed culture. *S. aureus* could be said to be the weakest species of the three. Even though *S. aureus* is also a virulent species capable of producing exotoxins (Belay and Rasooly, 2002), both Peterson et al., (1962) and Noleto et al., (1987) have also shown it to be a poor competitor which grows slowly in the presence of other microorganisms. Using conventional plating approach, they were able to demonstrate that *S. aureus* was outgrown in the mixed cultures and produced no enterotoxins in the mixed cultures unless the inoculum density of *S. aureus* was greater or outnumbered the other species (Noleto et al., 1987). It is interesting that microcalorimetry also detects these important interactions occurring in these staphylococci mixed cultures.
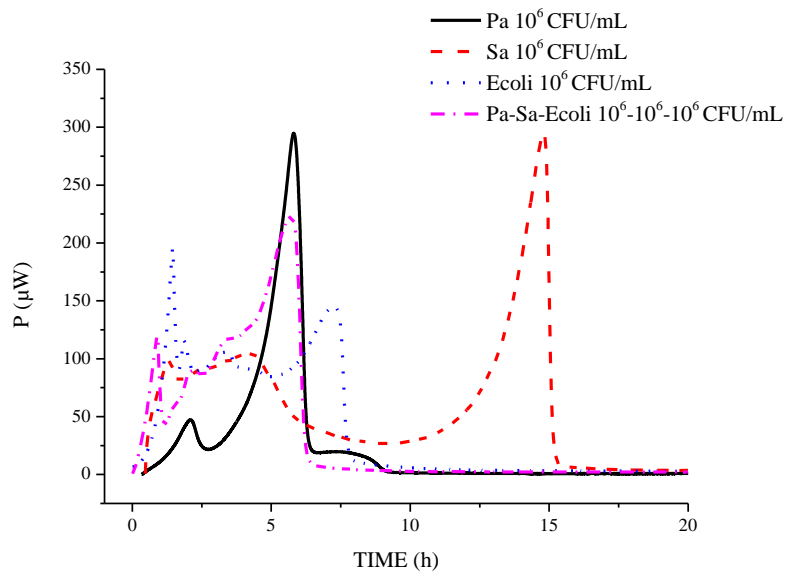
Figure 2.25. Comparison of the power-time curves of *P. aeruginosa* (black), *S. aureus* (red), *E. coli* (blue) and a mixed culture of them (magenta) in NB. Each species was inoculated to a cell density of 10^6 CFU/mL. In a mixed culture, the individual species were each inoculated to 10^6 CFU/mL
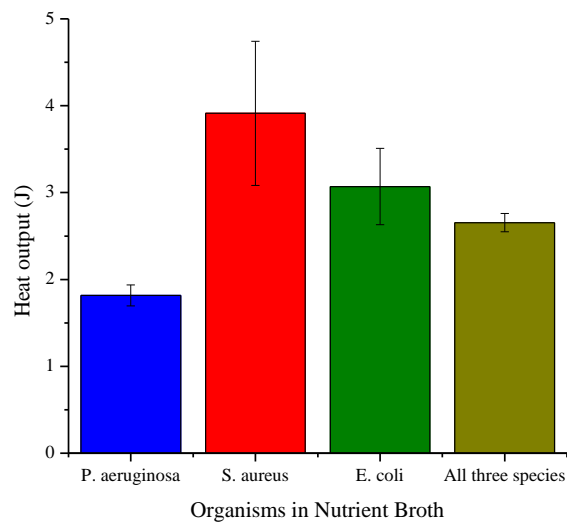
Figure 2.26. A bar chart comparing the heat outputs of *P. aeruginosa, S. aureus, E. coli* and a mixed culture of them in NB. All species were inoculated to a final density of 10^6 CFU/mL. In a mixed culture, the individual species were each inoculated to 10^6 CFU/mL, therefore, the final density in the three species mixed culture was 3×10^6 CFU/mL

2.4.3.2.5 Changing the medium

As already indicated, a central part of the work in this thesis was the study of the antipathogenic activity of probiotics. The probiotics were therefore to be co-cultured with these non-fastidious species. Probiotics are fastidious organisms that grow in anaerobic (for example *Bifidobacterium*) or microaerophilic (*Lactobacillus*) conditions with complex nutritional requirement. It was important that a medium was chosen that supported the growth of all these species in the microcalorimeter. CMM, an excellent medium for the primary growth and maintenance of aerobic, microaerophilic and anaerobic organisms supporting aerobic, microaerophilic and anaerobic organisms was chosen for the culture. The use of CMM for the growth of a variety of fastidious species has been demonstrated by Scythes et al., (1996) who found out that CMM was one of the three best media, that was able to support growth of the most variety of organisms at the lowest inoculum when they compared the nutritive capacities of 10 broths which included brain heart infusion (BHI), BHI supplemented with BIO-X, a supplement for fastidious organisms, BHI supplemented with 5% sheep blood, thioglycolate medium, enriched thioglycolate broth, fastidious anaerobe broth, Wilkins-Chalgren anaerobe broth, cooked meat enriched broth and Schaedler broth. CMM is composed of minced meat, which naturally contains reducing substances, which produce and maintain anaerobic conditions in the medium (Harrigan, 1998) when freshly prepared and cultured in sealed containers.

Figures 2.27, 2.28 and 2.29 compares the power-time curves of the three species in NB and CMM.

Except for *E. coli,* which some differences can be observed in the power-time curves in both media, the power-time curves for *P. aeruginosa,* and *S. aureus* in CMM showed similarities to the power-time curves in NB. It was easily recognizable which species was metabolizing by observing the power-time curves in both media. The aerobic metabolic peaks of the curves were however decreased and this is due to the fact that the medium condition was quite anaerobic. The heat output generated in CMM was also identifiable with the species (Figure 2.30) except for *S. aureus*, which produced significantly less heat ($P<0.05$) in CMM than in NB.
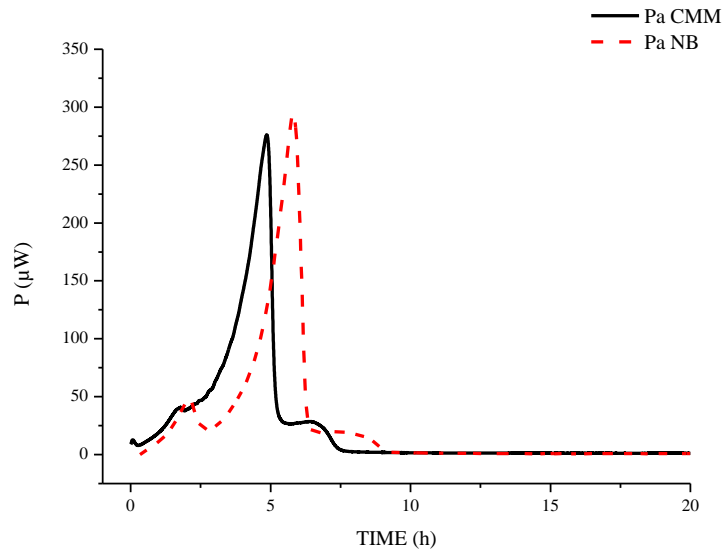
Figure 2.27. Comparison of the power-time curve of *P. aeruginosa,* in different culture media: NB and CMM. The density of both cultures was 10^6 CFU/mL
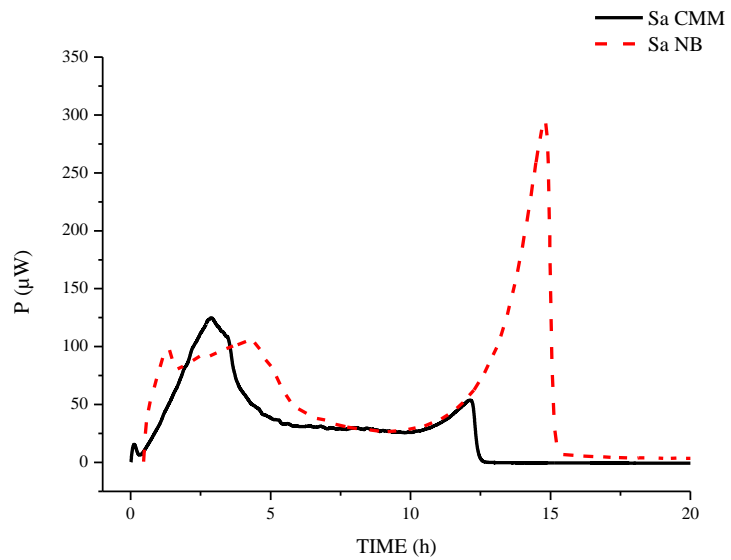
Figure 2.28. Comparison of the power-time curve of *S. aureus* in different culture media: NB and CMM. The density of both cultures was 10^6 CFU/mL
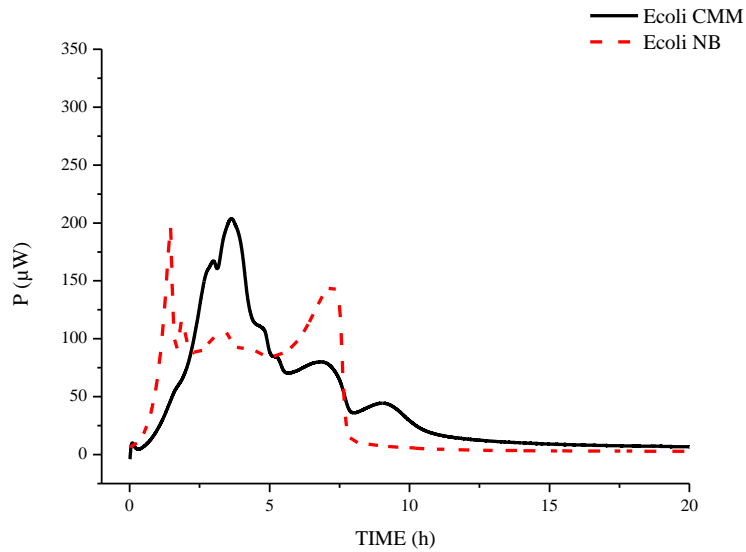
Figure 2.29. Comparison of the power-time curve of *E. coli* in different culture media: NB and CMM. The density of both cultures was 10^6 CFU/mL
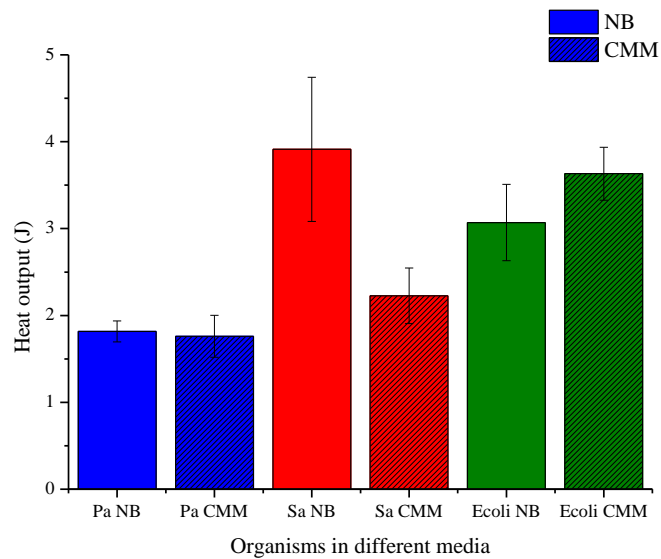
Figure 2.30. A bar chart comparing the mean heat outputs (n=4) of *P. aeruginosa, S. aureus* and *E. coli* in the different culture media: NB and CMM. All species were inoculated to a final density of 10^6 CFU/mL

It has already been demonstrated that the power-time curve for a particular organism changes respective of the medium and medium condition (Beezer, 1980). For this experiment, the power-time curves of the species in the different media still showed

some similarities because these two media share some similarities in composition.

The power-time curves of the three species and their mixed culture in CMM are compared in Figure 2.31. The initial exponential phases (ca. between 0-5 h) of the curve of the mixed culture is quite identifiable with *E. coli* and the later exponential phase (ca. between 5-10 h), identifiable with *P. aeruginosa* (Figure 2.31). Again, it is likely that both *E. coli* and *P. aeruginosa* dominated the mixed culture.
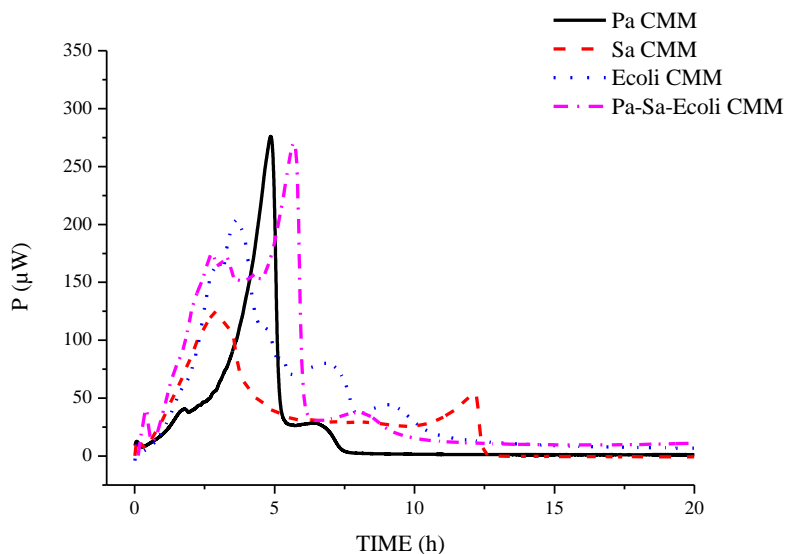
Figure 2.31. Comparison of the power-time curves of mixed cultures of *P. aeruginosa, S. aureus* and *E. coli* in CMM and that of pure culture of the species in CMM. Each species was inoculated to a density of 10^6 CFU/mL, hence a mixed culture of all three species had a final density of 3 x10^6 CFU/mL

2.5 Chapter summary

The work in this chapter was a first step towards understanding of the behaviour of microorganisms in mixed cultures in the microcalorimeter. In this chapter, it was shown that *P. aeruginosa, S. aureus* and *E. coli* exhibit characteristic growth curves in a given medium; this was used for subsequent identification/detection of the species in mixed culture. In mixed cultures, it was shown that the growth profile of *P. aeruginosa* and *E. coli* dominated *S. aureus*. Plate counts done after microcalorimetric measurements correlated with the observation in the microcalorimeter. It is likely the dominance of *P. aeruginosa* and *E. coli* could have been due to inhibitory substances produced by these species to which *S. aureus* may have been susceptible. As intimated, this dominancy has been noted to occur in some infected environments. For instance, in cystic fibrosis

infections, it was previously observed that the presence of *P. aeruginosa* in the sputum of infected patients significantly correlated with the absence of *S. aureus* (Machan et al., 1992) but in the recent past, it has also been recognized that *P. aeruginosa*, through its inhibition could cause the selection for a different variant of *S. aureus* hence both could be co-isolated from an infection (Hoffman et al., 2006, Biswas et al., 2009). It has also been previously demonstrated that the production of pyocyanin (which targets the electron transport chain of *S. aureus*) coincides exactly with the time *S. aureus* concentration decreases in co-cultures with *P. aeruginosa* when both were previously growing parallel together for approximately 8 h (Biwas et al., 2009). Thus the experiments and results presented in this chapter concurs with previous studies and show that microcalorimetry could be a valuable tool for studying behaviour of mixed cultures and could potentially be used for studying clinically relevant interaction as it opens up a lot of possibilities.

Chapter 3 Antagonistic activity of probiotics against *P. aeruginosa*, *S. aureus* and *E. coli*

3.1 Introduction

Even though probiotics have been associated with a number of health benefits, as discussed in Chapter 1, the mechanism of probiosis has not been fully elucidated. It is however believed that this is achieved by modulation of the immune system, enhancement of the epithelial barrier, competition for available nutrients or attachment sites on intestinal epithelial cells or the direct antagonism of pathogens (O'Toole and Cooney, 2008, Bermudez-Brito et al., 2012). Direct antagonism through specific inhibitory susbstance production (discussed in Chapter 1) is one of the most important functional properties which is often assessed *in vitro* for a strain if a potential probiotic effect is expected (Gismondo et al., 1999, FAO/WHO 2001, Jensen et al., 2012). This is often routinely tested using conventional techniques by culturing the probiotics or their bioproducts together with targeted pathogens and observing relative growth of the species or by the use of agar diffusion tests (Apella et al., 1992, Drago et al., 1997, Annuk et al., 2003, Naaber et al., 2004, Hutt et al., 2006, Trejo et al., 2006, Olivares et al., 2006, Jara et al., 2011, Chapman et al., 2012, Tejero-Sarinena et al., 2012, Schoster et al., 2013).

In the work reported in this chapter, the microcalorimeter was explored to study the antimicrobial or antagonistic activity of some commercial probiotic strains using the probiotics in mixed culture with *P. aeruginosa*, *S. aureus* and *E. coli*.

3.2 Objectives

The objective of this chapter was:

- To study the growth of commercial probiotic strains with *P. aeruginosa*, *S. aureus* and *E. coli* in the microcalorimeter

3.3 Materials and Methods

3.3.1 Microorganisms

Bifidobacterium bifidum ATCC 11863 was purchased from American Type Culture Collection, USA. Pure cultures of *Lactobacillus acidophilus* LA-5® and *Bifidobacterium lactis* BB-12® were obtained from Chr. Hansen's Culture Collection (Reading, UK). *Staphylococcus aureus* NCIMB 9518, *Pseudomonas aeruginosa* NCIMB 8626 and *Escherichia coli* ATCC 25922 were obtained as previously reported in section 2.3.1 of Chapter 2.

3.3.2 Microbiological media and chemicals

Cooked meat medium (CMM), macconkey agar (MCA), cetrimide agar, mannitol salt agar (MSA), de man rogosa sharpe (MRS) broth and agar, brain heart infusion (BHI) broth and agar, agar powder, reinforced clostridial medium (RCM), nutrient broth (NB), iso-sensitest agar (ISA) and all other microbiological media were from Oxoid Ltd, Basingstoke, UK and purchased from Fisher Scientific, UK. L- cysteine hydrochloride was purchased from Fisher Scientific, UK. D-(+)-glucose was purchased from Sigma-Adrich, UK.

3.3.3 Stock culture maintenance and purity

B. bifidum ATCC 11863, *L. acidophilus* LA-5® and *B. lactis* BB-12® were obtained as freeze-dried cultures. These were stored at -20°C (±2°C) before use. *B. bifidum* was rehydrated with 5 mL RCM. To test for viability and purity, the suspension was sub-cultured onto RCM agar and BHI agar and incubated aerobically (for aerobic contamination check) and anaerobically in an anaerobic jar using AnaeroGen (Oxoid) to generate the anaerobic environment at 37°C for 48 h. The plates were checked for contaminants. Single colonies from the RCM plate incubated anaerobically were picked using a sterile cotton swab and used to make a lawn of growth on a fresh agar plate. The plate was incubated anaerobically for 48 h at 37°C after which the colonies were picked from the plate and inoculated into RCM supplemented with 15% v/v glycerol to make a

thick suspension of the cells and stored at -80°C until further use.

L. acidophilus, LA-5$^{\circledR}$ and *B. lactis,* BB-12$^{\circledR}$ were rehydrated with MRS broth and MRS broth supplemented with 0.05% w/v L-cysteine hydrochloride "MRSc" respectively. The broth suspensions were sub-cultured onto MRSc agar to obtain single colonies, which were used to make respective lawns of the strains. Thick suspensions of the cells in MRSc supplemented with 15% v/v glycerol were made from the lawn and this was stored at -80°C until further use. To test for the absence of aerial contaminants, they were streaked onto BHI agar and incubated aerobically at 37°C for 48 h and observed for colony type.

3.3.4 Cryopreservation and defrosting of bacteria

Cryopreservation of the organisms was done according to section 2.3.7. Parent stocks of *B. bifidum,* BB-12$^{\circledR}$ and LA-5$^{\circledR}$ were streaked onto respective agar plates, RCM (for *B. bifidum*) and MRSc (for BB-12$^{\circledR}$ and LA-5$^{\circledR}$). Pure colonies obtained were inoculated into 7 mL RCM or MRSc broth and incubated anaerobically at 37°C for 24 h to make starter cultures of the organisms. A 1 in 100 dilution of the starter cultures were made in fresh media and these were incubated anaerobically and harvested by centrifugation at 3500 *g* for 10 min at 4°C when the cells reached their stationary phase of growth. The cells were washed twice with PBS, resuspended with ¼ Ringer's solution supplemented with 15% v/v glycerol to a concentration of 10^{8} CFU/mL and dispensed into multiple aliquots in cryovials. Cryopreservation procedure, recovery determination, and defrosting procedures were carried out as previously described for the other species.

3.3.5 Pure and mixed culture of probiotic strains and *P. aeruginosa*, *S. aureus* and *E. coli* in the isothermal microcalorimeter

This was done according to the method in section 2.3.9 of the previous chapter. A dilution of a thawed culture of the species was made in CMM or CMM supplemented with 2% w/v glucose ("CMMg") to give a pure culture density of 10^{6} CFU/mL in a sterile 3 mL calorimetric glass ampoule. The ampoules were sealed with crimped caps and vortexed for 10 s. They were placed in the intermediate position of a TAM 2277 for thermal equilibration. The loaded ampoules were allowed to equilibrate at 37°C at the

intermediate position for 30 min before being lowered into the measurement position. Probiotic species: *B. bifidum*, LA-5® and BB-12® were mixed with *S. aureus* or *P. aeruginosa* or *E. coli*, to produce a 10^6 CFU/mL of each species in 3 mL CMMg in the calorimetric ampoules. The ampoules were treated as above.

3.3.6 Preparation of cell-free culture supernatants from probiotic cultures

The culture supernatants of the probiotics were prepared by cultivating the respective probiotic species in CMMg over 48 h anaerobically. The cells and debris were removed by centrifuging at 3500 *g* for 10 min at 4°C. The supernatant was collected and filter-sterilized to remove cells using a 0.22 μm membrane syringe filter (Sartorius AG, Germany). The pHs of the supernatants were examined and equal aliquots modified by: adjusting the pH to neutral (pH 7.0) with 1M NaOH and heat treatment (heating the supernatant at 100°C for 60 min).

3.3.7 Agar well diffusion assay

Cell-free culture supernatants (CFS) of *B. bifidum*, LA-5® and BB-12® were examined for their antimicrobial activity by the agar well diffusion assay as described by Jara et al., (2011) with some modifications. After the treatment, the supernatants were filter sterilized through a 0.22 μm membrane syringe filter. 10^6 CFU/mL of *S. aureus*, *P. aeruginosa* and *E. coli* were spread onto ISA agar plates with a sterile cotton swab to give a lawn of confluent growth of cells. Wells of 9 mm diameter were made with a sterile borer and filled with 100 μL of unmodified, neutralised and heat-treated CFS. The plates were kept on the bench for 2 h for diffusion of the CFS from the wells into the agar and incubated after at 37°C. The zones of inhibition were measured after 24 h of incubation.

3.3.8 Co-incubation of *P. aeruginosa*, *S. aureus* and *E. coli* with probiotic culture supernatants in the microcalorimeter

1.5 mL of unmodified CFS, (repeated for neutralised and heat treated CFS), as obtained in section 3.3.6 for *B. bifidum*, LA-5® and BB-12® was added to 1.5 mL double strength

CMMg "dsCMMg". The mixture was vortexed for 10 s. The species: *S. aureus*, *P. aeruginosa* and *E. coli* were individually inoculated into the respective CFS-broth mixture to a population density of 10^6 CFU/mL and placed in the TAM. Power-time measurements were taken for as long as the sample was generating a signal in the TAM. A control experiment was done by replacing the CFS with sterile distilled water. Plate counts of the species were done after the TAM experiments.

3.3.9 Statistical Analysis

Statistical analysis was performed in Origin Pro 8.6 (Microcal Software Inc.). T-test or Analysis of Variance (ANOVA) with Turkey post-hoc analysis for means comparison was used where appropriate. P values less than 0.05 were regarded as significant difference between means.

3.4 Results and Discussion

3.4.1 Pure culture of the different species in CMMg

The probiotic species: *B. bifidum*, BB-12® and LA-5® showed poor growth in CMM but very good growth in CMMg. Figure 3.1 shows the power-time curves of 3 repeats of LA-5® inoculated to a density of 10^6 CFU/mL in CMM unsupplemented. The power-time curves of 10^6 CFU/mL repeats of pure cultures of the probiotic species and the other species inoculated into CMMg are shown in Figures 3.2, 3.3, 3.4, 3.5, 3.6 and 3.7. The power-time curves of the different species were characteristic in CMMg even though the curves lacked the complexity/structure demonstrated in CMM or NB in Chapter 2. The cumulative heat outputs associated with the growth of LA-5® and most of the species were significantly greater (P<0.05) in CMMg than in unsupplemented CMM.
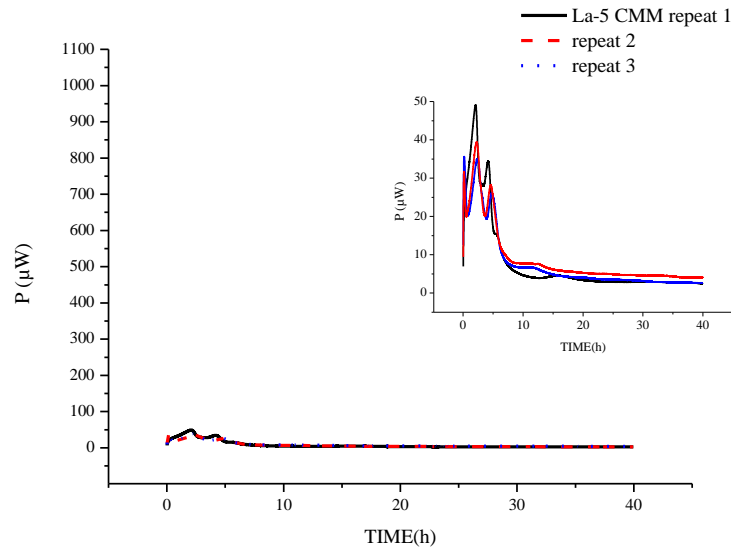
Figure 3.1. Power-time curves of 3 repeats of *L. acidophilus,* LA-5® in CMM inoculated to 10^6 CFU/mL
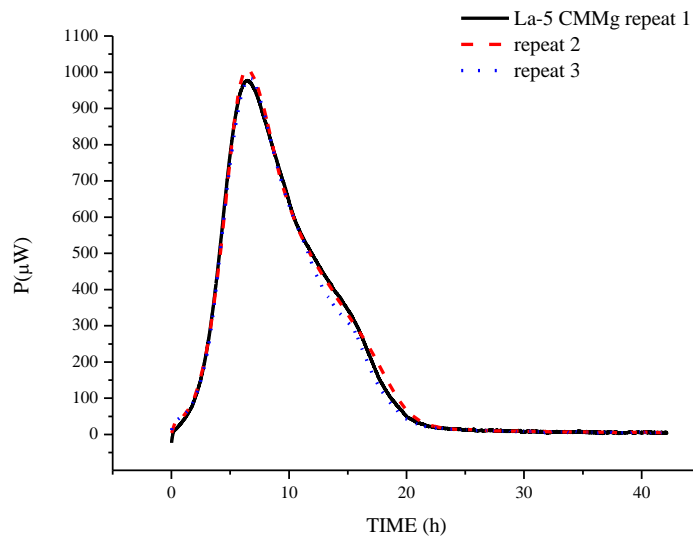
Figure 3.2. Power-time curves of 3 repeats of *L. acidophilus* LA-5® in CMMg inoculated to 10^6 CFU/mL
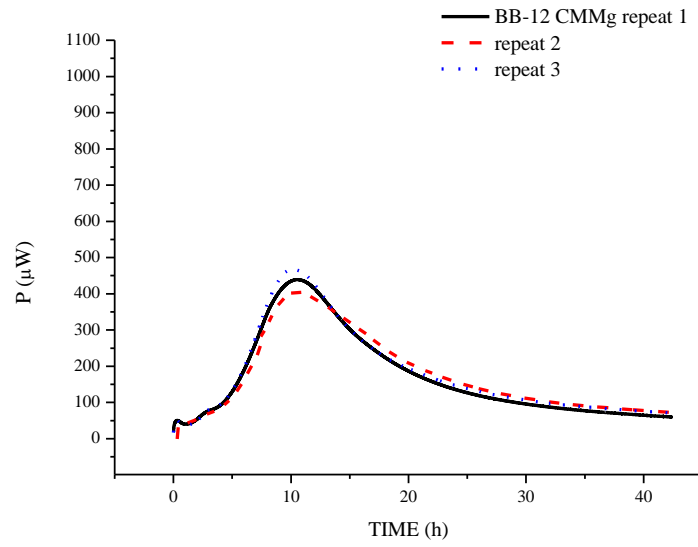
Figure 3.3. Power-time curves of 3 repeats of *B. lactis* BB-12® in CMMg inoculated to 10^6 CFU/mL
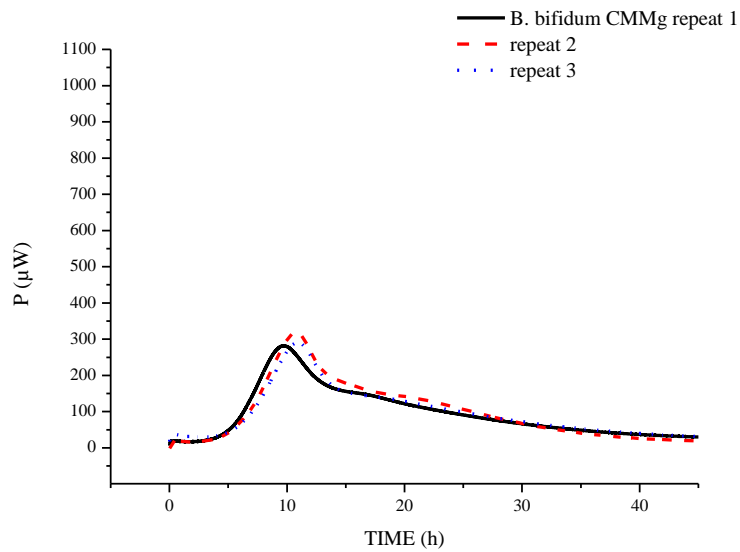
Figure 3.4. Power-time curves of 3 repeats of *B. bifidum* in CMMg inoculated to density of 10^6 CFU/mL
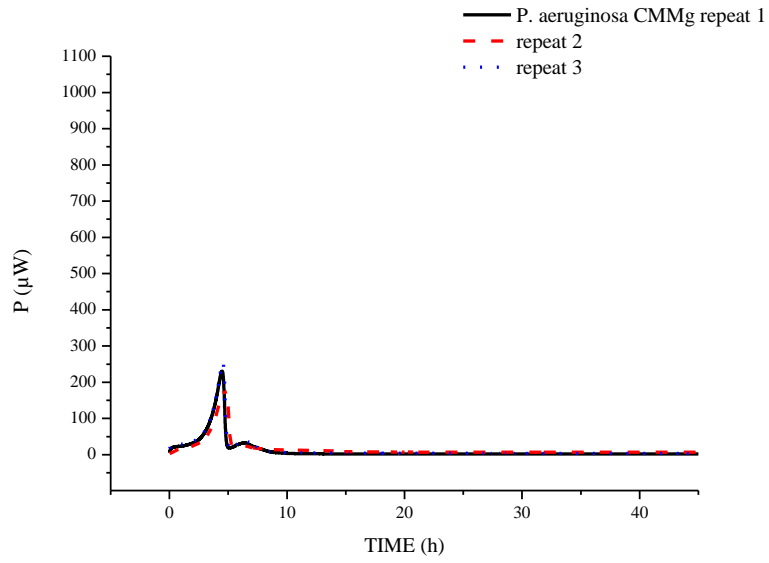
Figure 3.5. Power-time curves of 3 repeats of *P. aeruginosa* in CMMg inoculated to density of 10^6 CFU/mL
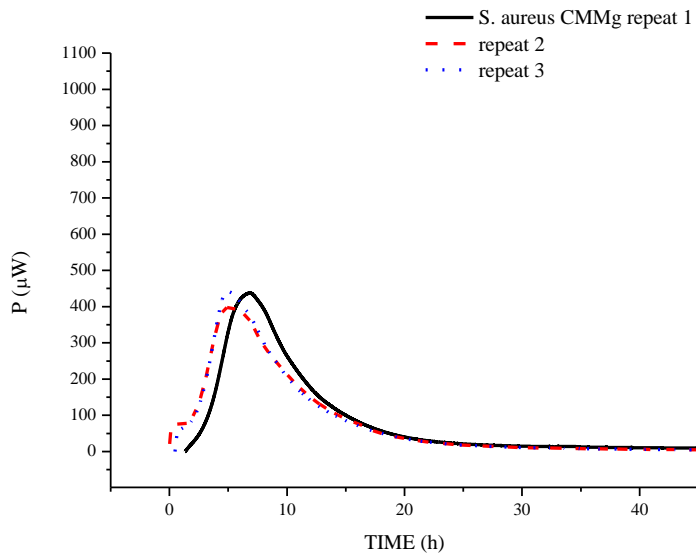
Figure 3.6. Power-time curves of 3 repeats of *S. aureus* in CMMg inoculated to density of 10^6 CFU/mL
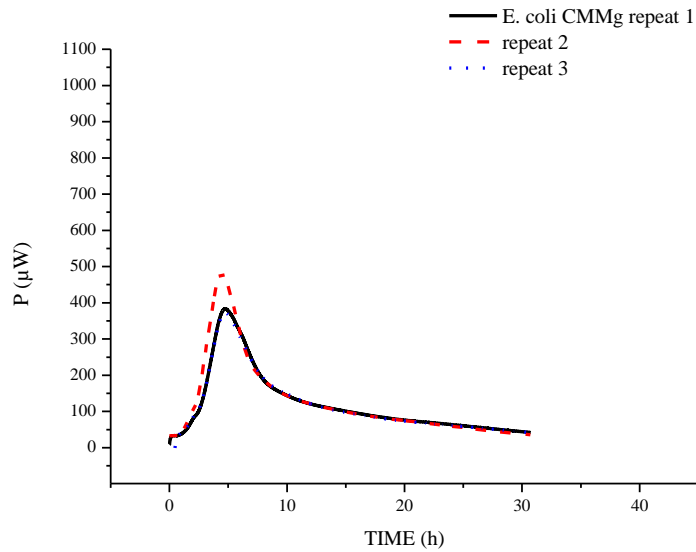
Figure 3.7. Power-time curves of 3 repeats of *E. coli* in CMMg inoculated to density of 10^6 CFU/mL

It should be noted that most of the members of the *Lactobacillus* genus are facultative anaerobic organisms (Schleifer, 2009). Optimal growth is however achieved in microaerophilic or anaerobic conditions for some members (Schleifer, 2009). Members of the *Bifidobacterium* genus are however obligate anaerobes. *S. aureus* and *E. coli* on the other hand are facultative anaerobes (Schleifer and Bell, 2009, Scheutz and Strockbine, 2005) and *P. aeruginosa,* an aerobe, but sometimes considered a facultative anaerobe because it is known to survive in anaerobic environments using nitrate or nitrite if available as terminal electron carriers or arginine in the absence of nitrate or nitrite (Eschbach et al., 2004, Garrity et al., 2005). Growth is however generally favoured in aerobic environment for *P. aeruginosa* (Garrity et al., 2005) also demonstrated by the results in Chapter 2. Thus the probiotic species, in particular, the bifidobacteria species can only be grown in oxygen-free environments which is achieved by the utilisation of natural medium containing reducing substances or by the addition of reducing substances for example glucose, sodium thioglycollate, ascorbic acid, cysteine hydrochloride etc. to some media (Charteris et al., 1997, Champagne et al., 2011). They are easily killed when exposed to oxygen because of their lack or limited activity of superoxide dismutase and or catalase (Brioukhanov et al., 2002). On the other hand, the other species in particular *P. aeruginosa*, require oxygen for growth and has limited growth in anaerobic environment, hence the ampoule oxygenation or deoxygenation was very important in the study of the growth of the species in mixed cultures. Also,

111

compared to the other species, the probiotic species are fastidious and metabolise by fermentation. For instance, LA-5®, which grew optimally in microaerophilic or anaerobic environment is extremely fastidious and is adapted to complex organic substrates. Apart from requiring carbohydrates as energy and carbon sources, these species also require nucleotides, amino acids, and vitamins (Schleifer, 2009) for growth. The various requirements for essential nutrients of these species are however normally met when the media contains fermentable carbohydrate, peptone, meat and yeast extracts (Harrigan, 1998).

CMM consists of minced meat in nutrient broth (Harrigan, 1998). The minced meat naturally contains reducing substances, which produce and maintain anaerobic conditions in the medium (Harrigan, 1998). As has been stated in Chapter 2 of this thesis, CMM is a useful media for the culture of both aerobic and anaerobic organisms. By heating the medium, dissolved oxygen is removed and anaerobic environment can be maintained by incubation in an oxygen-free environment. Similarly, aerobic environment can be achieved when the medium is incubated uncapped or with loosed cap. Aerobic organisms grow at the top of the medium whilst anaerobic grow deeper in the medium. The addition of 2% w/v glucose to CMM to reduce the medium further for optimum growth of the probiotic species notably changed the power-time curves of the other species as well. Its addition in a naturally reduced medium kept the concentration of oxygen extremely low in the sealed ampoules. Hence, the characteristic aerobic peaks of all the facultative species were completely lost. Presumably, only anaerobic or also possibly microaerophilic growth occurred in the CMMg filled calorimetric ampoules. The species also metabolised more energetically and for longer time likely because of the substrate potential of the additional glucose. This is well illustrated by LA-5®, *S. aureus* and *E. coli*, which produced mean heat outputs of 34.52 ± 1.2 J in CMMg compared to 0.70 ± 0.03 J in unsupplemented CMM; 13.63 ± 0.66 J compared to 2.23 ± 0.32 J and 14.34 ± 1.08 J compared to 3.63 ± 0.30 J respectively.

Comparing all of the species in CMMg (Figure 3.8), the power-time and heat curves suggest that BB-12® and *B. bifidum* are slower growing species relative to the other species. The peak of the growth curves occurred at ca. 10 h for BB-12® and 11 h for *B. bifidum*; whilst it occurred at 7 h for LA-5®; 4.5 h for *P. aeruginosa*; 6.5 h for *S. aureus* and 5 h for *E. coli*. Amongst the species, LA-5® showed maximum growth in the

medium generating the greatest heat outputs and reducing the pH of the medium the most (from 7.20 ± 0.2 to 4.0 ± 0.13).

[A]
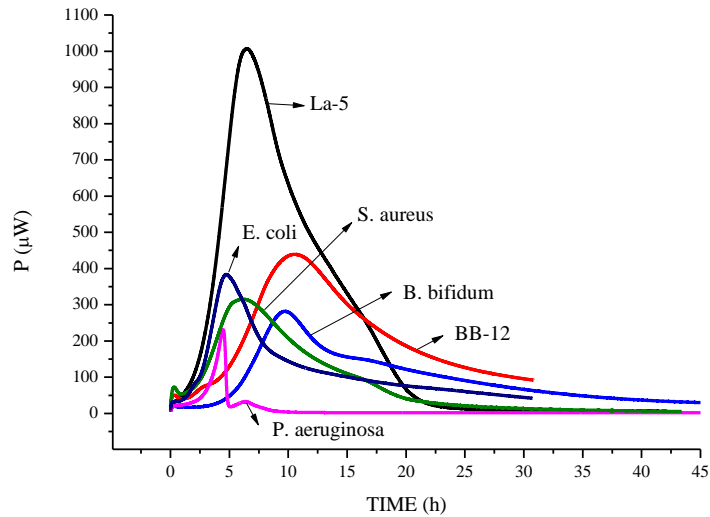
[B]
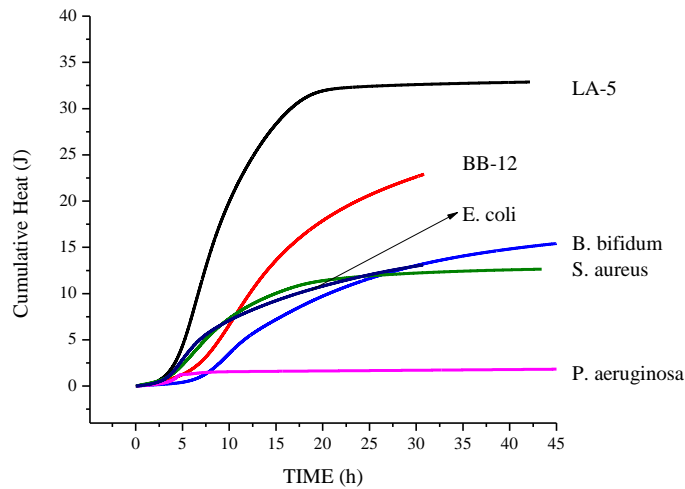
Figure 3.8. Comparison of the power-time curves [A] and heat curves [B] of pure cultures of LA-5[®], BB-12[®], *B. bifidum*, *P. aeruginosa*, *S. aureus* and *E. coli* in CMMg at densities of 10^6 CFU/mL of each. The curves show differences in the growth peaks and lag time duration respectively for the different species

P. aeruginosa on the contrary showed the least growth, with heat output of 2.17 ± 0.31 J and changing the pH of the medium only slightly. The superior growth of LA-5[®] in the medium relative to the other species could be probably because the environment

113

generated in the ampoule suited its growth the most. *P. aeruginosa* on the other hand showed least growth in this reduced medium probably due to its inability to ferment available substrates in the medium for growth like the others. To illuminate further, it has been previously shown by Eschbach et al., (2004) that apart from denitrification and arginine fermentation, *P. aeruginosa* is also capable of fermenting pyruvate; but while this fermentation has been shown to afford it a capacity for long-term survival in anaerobic environment, it has been shown not to sustain significant anaerobic growth (Eschbach et al., 2004).

3.4.2 Mixed culture of the species in CMMg

The power-time curves for the mixed cultures of the probiotic species: LA-5®, BB-12® and *B. bifidum* with *P. aeruginosa*, *S. aureus* and *E. coli* are shown in Figures 3.9, 3.10 and 3.11. The power-time curves suggest the likely growth of both the probiotic and the other species in mixed culture.
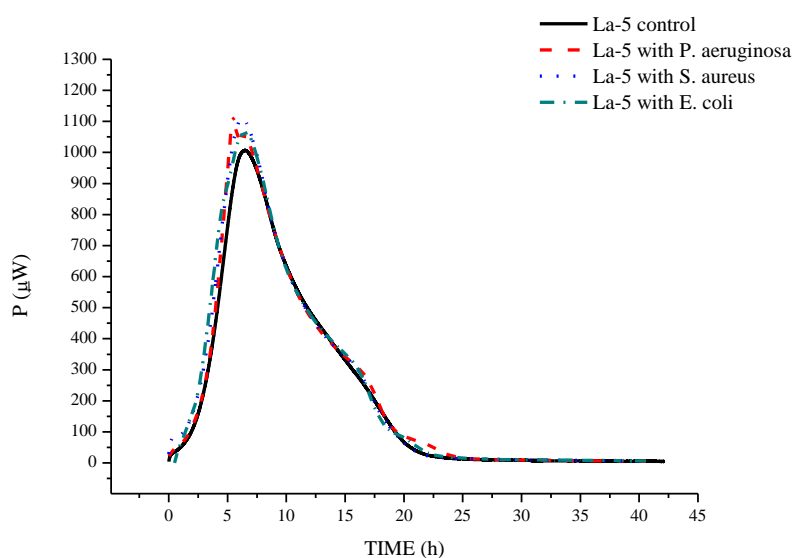
Figure 3.9. Comparison of the power-time curves of LA-5® (black) and as mixed culture with *P. aeruginosa* (red), *S. aureus* (blue) and *E. coli* (green) in CMMg. All species were inoculated to 10^6 CFU/mL
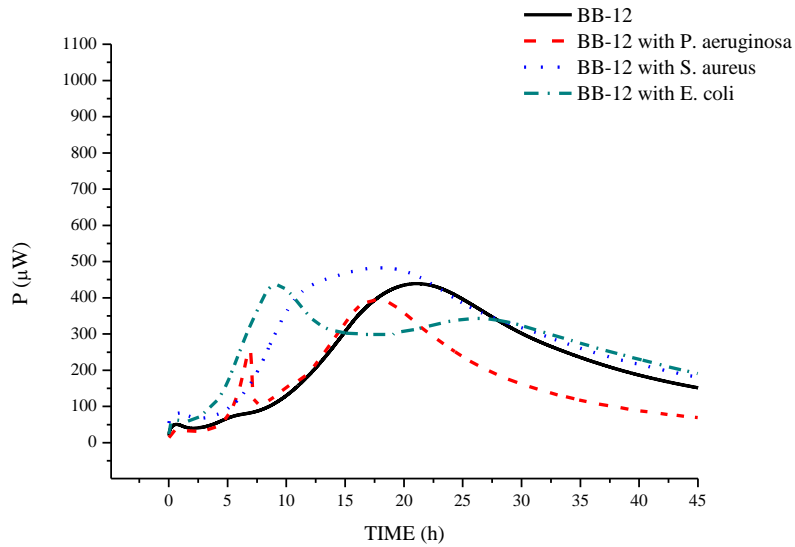
Figure 3.10. Comparison of the power-time curves of BB-12® (black) and as mixed culture with *P. aeruginosa* (red), *S. aureus* (blue) and *E. coli* (green) in CMMg. Each species was inoculated to a density of 10^6 CFU/mL
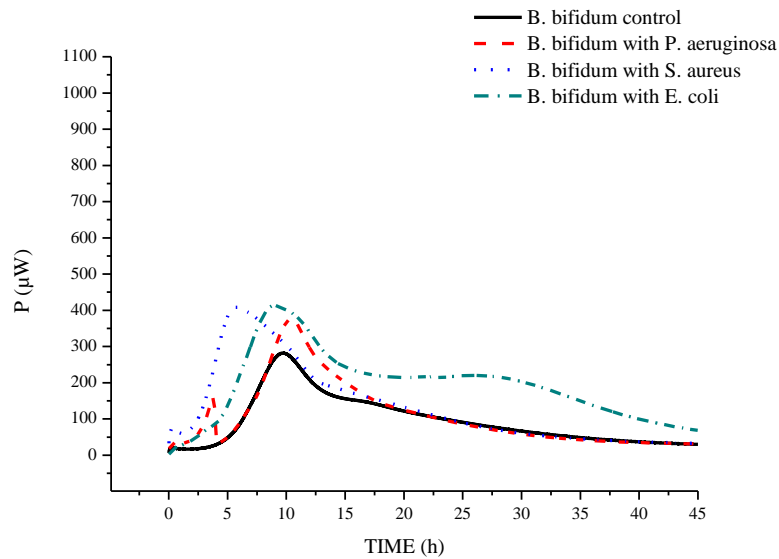
Figure 3.11. Comparison of the power-time curves of *B. bifidum* (black) and as mixed culture with *P. aeruginosa* (red), *S. aureus* (blue) and *E. coli* (green) in CMMg. Each species was inoculated to a density of 10^6 CFU/mL

It should be noted that lactic acid bacteria are known to produce two main varieties of antimicrobial compounds which may affect both the lactic acid bacteria themselves as well as undesirable or pathogenic strains (Piard and Desmazeaud, 1991, Piard and

115

Desmazeaud, 1992). They produce low molecular weight compounds such as organic acids (acetic, formic and lactic), metabolites of oxygen (H_2O_2 and free radicals), which may have bacteriocidal or bacteriostatic effects on themselves or other microorganism. They also produce proteinaceous compounds called bacteriocins, which may have a narrow spectrum of activity (Barefoot and Klaenhammer 1983, Anand et al., 1984, Anand et al., 1985, Kang et al., 1989).

In these experiments, it was expected that the probiotic species would inhibit the other species through release of these antimicrobial compounds. However the other species likely contributed to growth curves/heat outputs when co-cultured with the probiotic species. In co-culture with LA-5®, *P. aeruginosa* may have contributed a peak at 4.5 h (Figure 3.9). The total area under the control curve (total heat output) of LA-5® which was reproducible to 3.5% (n=3) was increased by almost 10% by the presence of *P. aeruginosa*. It was quite inconclusive whether *S. aureus* and *E. coli* also contributed to the growth curve in their co-culture with LA-5® although an increase in heat output of ca. 10% was also observed. Co-cultures of the species with BB-12® and *B. bifidum* clearly showed the presence of the pathogenic species in all the co-cultures, Figures 3.10 and 3.11 respectively. This synchronous or preceding growth of the other species with the probiotic species may have occurred because the probiotic species were slower growing species as evident by the late occurrence of the peak of the curves or the longer lag phase (Figure 3.8) compared to the other species hence it is likely that *P. aeruginosa*, *S. aureus* and *E. coli* could have attained exponential phase of growth before the probiotics could begin exponential growth. For instance, comparing a co-culture of BB-12® with *P. aeruginosa* (Figure 3.12a), it is very likely that *P. aeruginosa* contributed the first peak and the second growth peak contributed by BB-12®. The curve for BB-12® however commenced just before *P. aeruginosa* attained stationary growth phase. The peaks also occurred later for *P. aeruginosa* (ca. 6 h) and earlier for BB-12® in comparison to their control curves. It could therefore be reasoned that perchance BB-12® to some extent inhibited *P. aeruginosa* since a change in inoculum density results in a linear change in heat output (ie. a time lag is added to the curve). To that effect even though both species were inoculated at 10^6 CFU/mL, the presence of BB-12® possibly influenced *P. aeruginosa* (by competing for nutrients) such that it was detected when its population had increased from 10^6 CFU/mL.
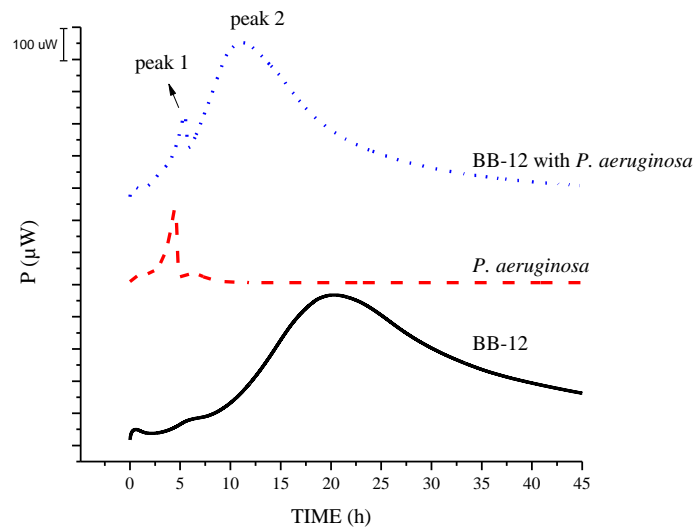
Figure 3.12a. Comparison of the power-time curves of BB-12® (black) *P. aeruginosa* (red) and their mixed culture (blue) in CMMg
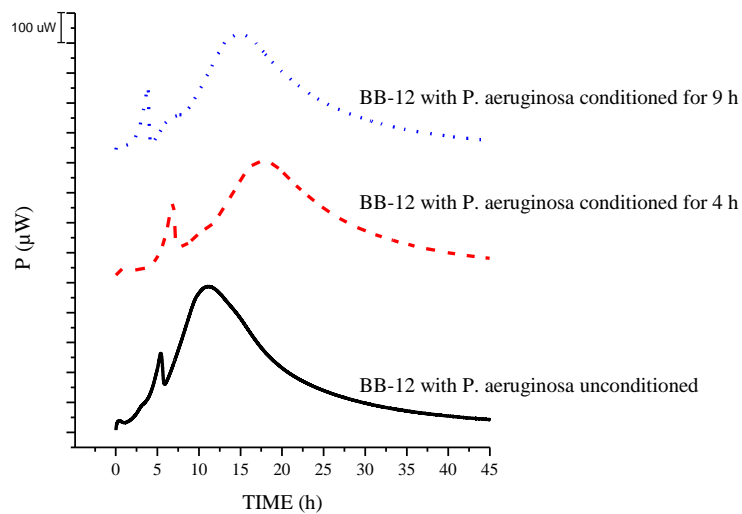
Figure 3.12b. Comparison of the power-time curves of BB-12® with *P. aeruginosa*: both unconditioned (black), conditioned for 4 h (red) and conditioned for 9 h (blue) in CMMg

It had been a concern that, when inoculated into medium immediately after being thawed from liquid nitrogen storage, organisms will adapt differently in the medium (some recovering earlier than others). BB-12® and *P. aeruginosa* were therefore inoculated into media and left to adapt ("conditioned") for 4 h and 9 h before being co-cultured into

117

fresh medium and placed in the TAM. The resulting curves are compared in Figure 3.12b. Conditioning of the organisms for 4 h added a time lag to the curve of *P. aeruginosa* with peak occurring at ca. 7 h. When the culture was conditioned for 9 h, the peak occurred earlier, ca. 3 h 45 min. Conditioning the culture for 9 h meant *P. aeruginosa* was well into its exponential phase of growth hence the volume of culture inoculated into fresh medium contained more organisms per mL than the culture conditioned for only 4 h (which could have been a mere dilution of the initial inocula of *P. aeruginosa*). On the other hand, conditioning of BB-12® for any of the time used was simply a dilution of the initial population.

For all mixed cultures of probiotics with the other species, inhibition was only detected by plating out at the end of incubation (after TAM measurement). Plating out of all co-cultures showed total inhibition of *P. aeruginosa*, *S. aureus* and *E. coli* (see Table 3.2 for plate count post TAM). Plating out during the first 5 h of co-cultures of the probiotics with *E. coli* (Table 3.1) showed growth of both the probiotics and *E. coli*.

Table 3.1. Plate count of BB-12®- *E. coli* co-culture and LA-5®- *E. coli* co-culture within first 5.5 h of microcalorimetric incubation

Time (h)	Plate count of mixed culture of BB-12® and *E. coli* in CMMg (CFU/mL)		Plate count of mixed culture of LA-5® and *E. coli* in CMMg (CFU/mL)	
	BB-12®	*E. coli*	LA-5®	*E. coli*
0.5	9.5×10^5	1.2×10^6	2.4×10^6	9.5×10^5
1	8.8×10^5	1.1×10^6	2.1×10^6	9.5×10^5
1.5	1.1×10^6	1.2×10^6	2.4×10^6	9.3×10^5
2	9.5×10^5	1.4×10^6	2.4×10^6	1.2×10^6
2.5	2.3×10^6	4.0×10^6	2.1×10^6	1.9×10^6
3	4.8×10^6	1.6×10^7	1.0×10^7	1.0×10^7
3.5	1.9×10^7	2.8×10^7	6.7×10^7	4.2×10^7
4.5	9.7×10^7	2.0×10^8	1.9×10^7	3.0×10^8
5.5	1.2×10^8	6.4×10^8	1.1×10^8	8.1×10^8

Table 3.2. A table comparing the post TAM profile of the pure culture and mixed culture of probiotics and *P. aeruginosa*, *S. aureus* and *E. coli*

Organism in CMMg	pH post TAM	Plate count (CFU/mL) post TAM	
Pure Culture			
L. acidophilus LA-5®	4.00 ± 0.13	3.6×10^8	
B. lactis BB-12®	4.55 ± 0.01	8.0×10^8	
B. bifidum	4.79 ± 0.01	4.6×10^7	
P. aeruginosa	6.78 ± 0.09	2.8×10^7	
S. aureus	5.05 ± 0.05	1.8×10^8	
E. coli	5.39 ± 0.01	3.9×10^8	
Mixed Culture		**Probiotic (LA-5® or BB-12® or *B. bifidum***	*P. aeruginosa* or *S. aureus* or *E. coli*
LA-5® and *P. aeruginosa*	4.30 ± 0.01	2.5×10^8	0
LA-5® and *S. aureus*	4.12 ± 0.02	4.5×10^8	0
LA-5® and *E. coli*	4.20 ± 0.05	2.0×10^8	0

Table 3.2. A table comparing the post TAM profile of the pure culture and mixed culture of probiotics and *P. aeruginosa*, *S. aureus* and *E. coli* (continued)

Organism in CMMg	pH post TAM	Plate count (CFU/mL) post TAM	
Mixed culture		**Probiotic (LA-5® or BB-12® or *B. bifidum***	*P. aeruginosa* or *S. aureus* or *E. coli*
BB-12® and *P. aeruginosa*	4.35 ± 0.02	6.1 x 10^8	0
BB-12® and *S. aureus*	4.30 ± 0.06	9.2 x 10^8	0
BB-12® and *E. coli*	4.45 ± 0.02	1.8 x 10^8	0
B. bifidum and *P. aeruginosa*	4.48 ± 0.01	8.6 x 10^8	0
B. bifidum and *S. aureus*	4.49 ± 0.08	2.7 x 10^8	0
B. bifidum and *E. coli*	4.48 ± 0.01	4.6 x 10^8	0

This implied that the probiotic species could have only inhibited the other species after an active or exponential growth. Thus they produced extracellular inhibitory susbstances after consumption of the nutrient substrates and accumulation of inhibitory substances; prior to that phase, they could only coexist with the other species. This observation agrees with a previous study by Apella et al., (1992) who showed that *Shigella sonnei* could grow together with *Lactobacillus casei* or *L. acidophilus* in mixed culture for the first few hours but noted that inhibition began at 6 h and death phase at 9 h of incubation. A study by Bendali et al., (2011) also demonstrated that co-cultures of *Lactobacillus paracasei* with either *E. coli* or *Salmonella typhimurium* showed inhibition at approximately 2 h and 6 h respectively when the growth kinetics of the mono-cultures of the pathogens were compared with the co-cultures. Another study by Kos et al., (2008) showed that *S. typhimurium* was immediately inhibited in growth when the species was added to a selection of previously growing probiotics but inhibition occurred

much later and also for *Listeria monocytogenes* when the probiotics were co-cultured together with the pathogenic species at the same time.

3.4.3 Co-incubation of probiotic supernatant with *P. aeruginosa*, *S. aureus* and *E. coli*

It was observed that there was significant acidification of the medium after incubation of the probiotic species (Table 3.2) from pH of 7.2 ± 0.2 of the medium to 4.0 ± 0.13 for LA-5® ($P<0.05$), 4.55 ± 0.01 ($P<0.05$) for BB-12® and 4.79 ± 0.01($P<0.05$) for *B. bifidum* in CMMg. This low pH (depicting the presence of organic acids) could have most likely produced the inhibitory effects of the probiotics towards the other species. To determine if there were other extracellular substances contributing to the antibacterial effects of the probiotic strains, the cell free culture supernatants (CFS) of the probiotics were evaluated after neutralisation of the pH and heat inactivation. Figure 3.13 compares the power-time curves of *P. aeruginosa* inoculated into a double strength CMMg "dsCMMg" diluted (1 in 2 times) with water and diluted with CFS (unmodified, neutralised and heat treated) of LA-5®. It also shows the power-time curves of *S. aureus* and *E. coli* inoculated into diluted CFS of LA-5®. The power-time curves demonstrate the inhibition of the species with the CFS of LA-5®. The inhibitory activity of LA-5® was however lost when neutralised.

The same inhibitory effect was demonstrated with the CFSs of BB-12® (Figure 3.14) and *B. bifidum* (Figure 3.15). Again, inhibitory activities were lost upon neutralisation (evident by the reappearance of the growth curve of *P. aeruginosa*) but retained after heat treatment. It was however observed that even though the power-time curve for *P. aeruginosa* reappeared after neutralisation of the CFS of BB-12® and *B. bifidum*, these growth curves peaked at a much later time and in lesser intensity compared with the control, suggesting that inhibitory activities of the CFSs of these species were not completely lost upon neutralisation. Plate count of the co-culture of the supernatants of the probiotics with the species only showed survival of the species when the CFSs were neutralised (Table 3.3). The plate count did not however suggest that the cells were inhibited in growth by the neutralised CFSs.
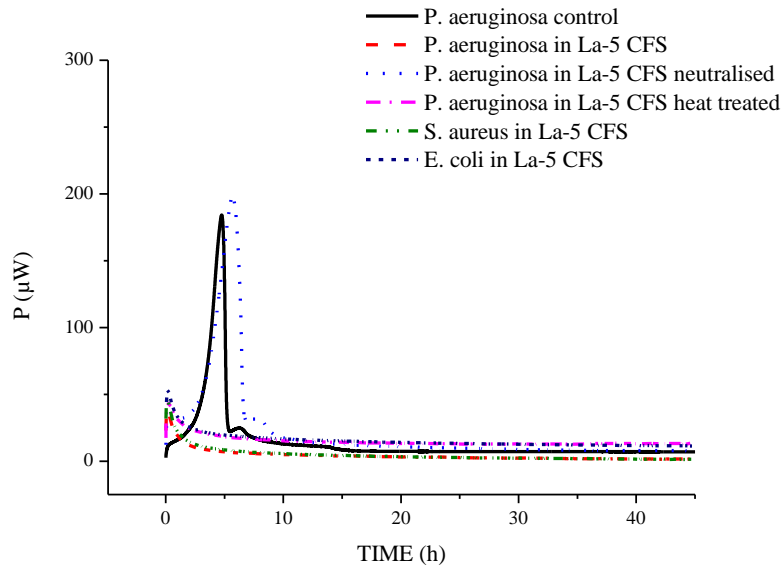
Figure 3.13. Comparison of the power-time curves of *P. aeruginosa* control (black) and *P. aeruginosa* with cell free supernatant (CFS) of LA-5® unmodified (red), neutralised (blue) and heat treated (magenta); *S. aureus* (green) and *E. coli* (violet) with CFS of LA-5®
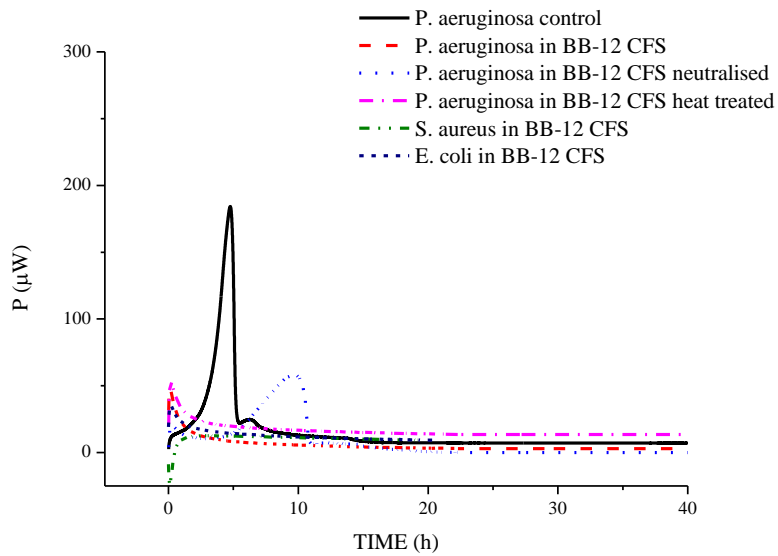
Figure 3.14. Comparison of the power-time curves of *P. aeruginosa* control (black) and *P. aeruginosa* with cell free supernatant (CFS) of BB-12® unmodified (red), neutralised (blue) and heat treated (magenta); *S. aureus* (green) and *E. coli* (violet) with CFS of BB-12®
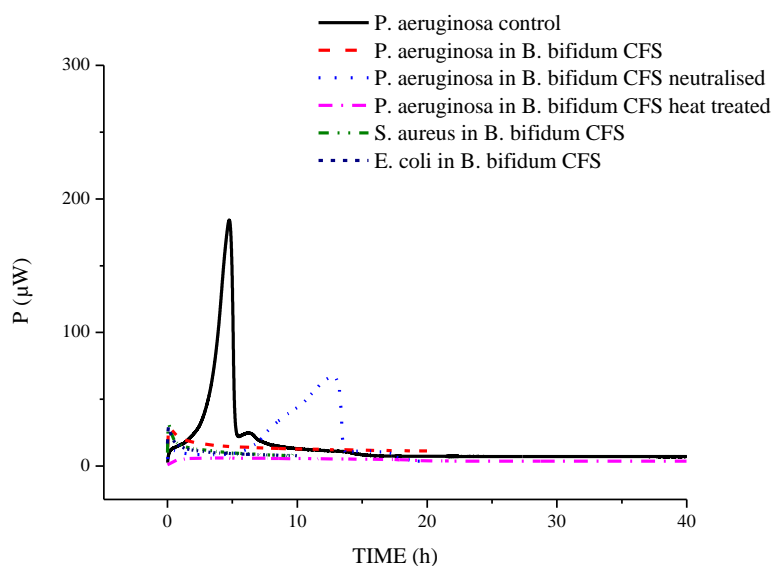
Figure 3.15. Comparison of the power-time curves of *P. aeruginosa* control (black) and *P. aeruginosa* with cell free supernatant (CFS) of *B. bifidum* unmodified (red), neutralised (blue) and heat treated (magenta) and *S. aureus* (green) and *E. coli* (violet) with CFS of *B. bifidum*

Table 3.3. Plate count demonstrating antagonistic activity of unmodified and modified cell free supernatant (CFS) of LA-5[®], BB-12[®] and *B. bifidum* against *P. aeruginosa*, *S. aureus* and *E. coli* after microcalorimetric co-incubation.

Supernatant	CFU of target bacteria (CFU/mL)		
	P. aeruginosa	*S. aureus*	*E. coli*
LA-5[®] CFS	0	0	0
LA-5[®] CFS neutralised	3.0×10^7	2.2×10^8	4.0×10^8
LA-5[®] CFS heat treated	0	0	0
BB-12[®] CFS	0	0	0
BB-12[®] CFS neutralised	2.3×10^7	8.9×10^7	1.7×10^8
BB-12[®] CFS heat treated	0	0	0
B. bifidum CFS	0	0	0
B. bifidum CFS neutralised	4.2×10^7	4.0×10^8	2.9×10^8
B. bifidum CFS heat treated	0	0	0

3.4.4 Agar well diffusion assay

The diameters of growth inhibition zones of *P. aeruginosa*, *S. aureus* and *E. coli* are shown in Table 3.4 and images of zones of inhibition of *P. aeruginosa* with the unmodified and modified CFS of LA-5® are shown in Figure 3.16. In the experiment, *P. aeruginosa* was most sensitive towards the CFS of the probiotics and *E. coli*, the least sensitive. Again, by this test, it was confirmed that inhibitory activities of the CFSs were lost after neutralisation but retained when heat-treated implying that these extracellular substances were heat stable and could probably be acids.

Table 3.4. Zones of inhibition of unmodified and modified cell free supernatant (CFS) of LA-5®, BB-12® and *B. bifidum* against *P. aeruginosa*, *S. aureus* and *E. coli*. The values are arithmetic means (n=4) ± SD of inhibition zones (mm)

Supernatant	Zone of inhibition of target bacteria (mm)		
	P. aeruginosa	*S. aureus*	*E. coli*
LA-5® CFS	9.0 ± 0.8	5.5 ± 1.2	2.5 ± 0.6
LA-5® CFS neutralised	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
LA-5® CFS heat treated	12.0 ± 0.8	13.5 ± 0.6	3.5 ± 2.1
BB-12® CFS	5.8 ± 1.5	4.8 ± 0.5	2.0 ± 0.0
BB-12® CFS neutralised	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
BB-12® CFS heat treated	7.5 ± 1.0	6.0 ± 0.0	2.3 ± 0.5
B. bifidum CFS	6.3 ± 1.0	5.8 ± 0.5	3.0 ± 1.4
B. bifidum CFS neutralised	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
B. bifidum CFS heat treated	9.3 ± 0.5	6.3 ± 1.0	3.8 ± 2.2

[A]   [B]   [C]
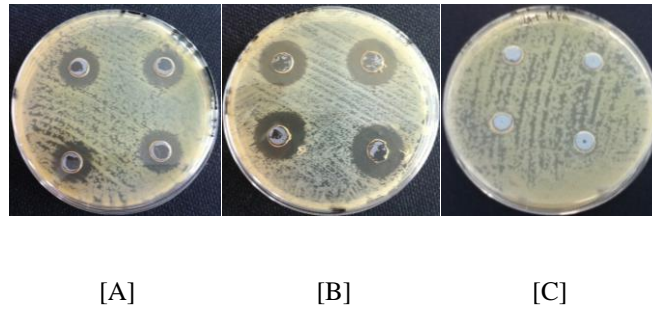
Figure 3.16. Images of zone of inhibition of *P. aeruginosa* by unmodified [A], heat treated [B] and neutralised [C] CFS of LA-5®

The zones of inhibition values were plotted against the change in pH of the different probiotic culture supernatants compared to the pH of the broth prior inoculation for each of the respective species to find out if there was a correlation between the amount of acid production by the probiotic species and their inhibitory effect. The plots are shown in Figure 3.17. A good correlation (R^2 value of 0.8135) was obtained for *P. aeruginosa* but very weak correlations (respective R^2 values of 0.0054 and 0.0878) were obtained for *S. aureus* and *E. coli*. The result suggests that the amount of acid production by the probiotics could have been a critical factor for the inhibition of *P. aeruginosa* but not *S. aureus* and *E. coli*. Between the three species, both *S. aureus* and *E. coli* could themselves produce acids; they both also significantly lowered ($P<0.05$) the pH of the broth (Table 3.2). It is likely these two species could have the ability to protect themselves from the self-produced acids and thereby may have developed the necessary factors for surviving acid stress (Cotter and Hill, 2003, Chung et al., 2006) relative to *P. aeruginosa*. These two species may have also been mainly inhibited by other non-acidic metabolites or may have been synergistically hindered by the acidic and non-acidic antimicrobial metabolites of the probiotic species. This is however not in total agreement with the other results, for instance, the co-incubation enumeration assay with the CFSs of the probiotics, which suggests that inhibition was acid dependent for all the species (Table 3.3). Inhibition zones were also not observed for the neutralised CFSs. This disparity in the results may be due to firstly, the possibility that the other non-acidic antimicrobial metabolites were produced in smaller quantities than the acids and were further diluted during the CFS co-culture assay or their activities depended on the acidity of the supernatant and were therefore hindered when neutralized. Oliveira et al., (2008) have shown the possibility of the latter situation. Messens and De, (2002) have also reported that most lactic acid bacteria and bacteriocins show maximum activity at lower pH.
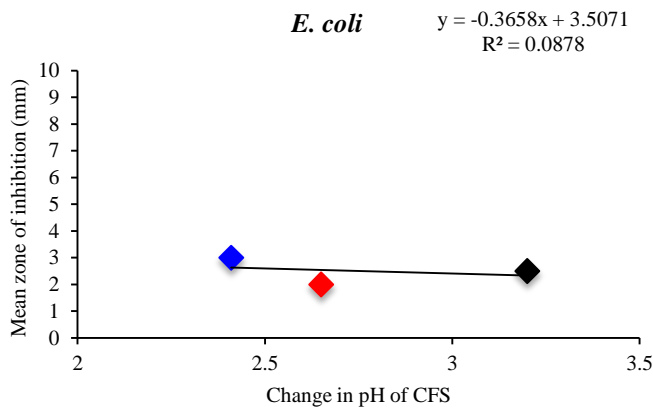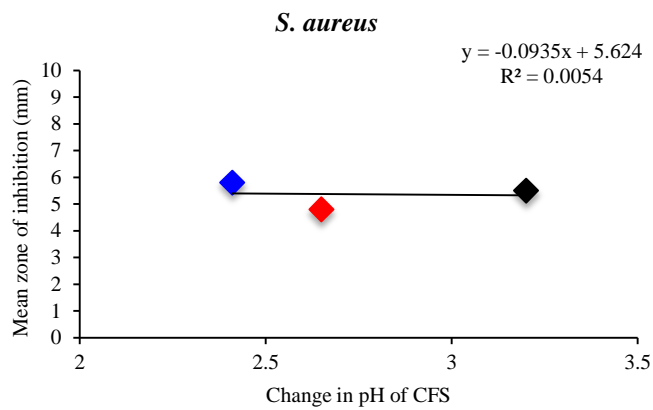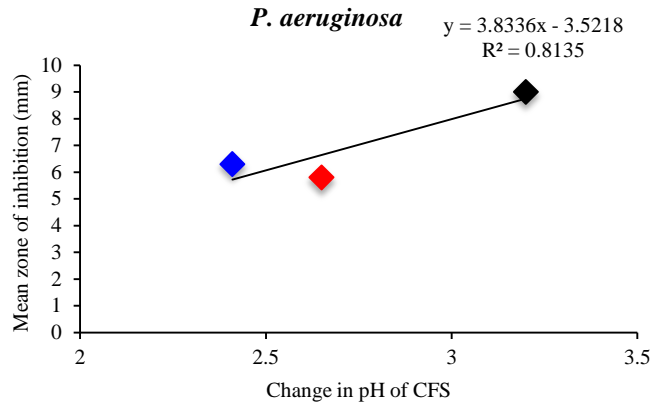
Figure 3.17. Zones of inhibition of the different species plotted against change in pH of the culture supernatant of the different probiotic species. (Black series, LA-5[®] CFS; red, BB-12[®] CFS and blue, *B. bifidum* CFS)

The results obtained show some consistency and constrast with results from previous studies. For instance, a study by Tirloni et al., (2014) reported that among food spoilage

or potential pathogenic species tested against *Lactobacillus animalis* and *L. paracasei*, *P. aeruginosa* was one of the least susceptible species although other Pseudomonas species were the most susceptible. The Enterobacteriaceae showed variable susceptibility but a strain of *E. coli* also showed least susceptibility and *S. aureus* showed moderate susceptibility. The authors also reported a lack of activity by the neutralized CFS. Sepova et al., (2011) also reported that among potential pathogens tested against a lactic acid bacterium E isolated from a lamb, when the species tested in the present study are considered, the inhibition potential of their lactic acid bacterium E against the potential pathogens decreased from *S. aureus, P. aeruginosa* to *E. coli.* Other authors have also reported different degrees of inhibition by different potential probiotic species and have shown either loss or retention of activity after neutralisation (attributable to presence or absence of other extracellular substances apart from acids) using either broth culture or agar diffusion assay (Vaughan et al., 1994, Drago et al., 1997, Annuk et al., 2003, Ammor et al., 2006). However in the present study, the data from the microcalorimeter indicates that there could be other extracellular susbstances in the CFS, which could have had an influence on the metabolic activity or growth of the species (especially CFS produced from BB-12® and *B. bifidum*); although data from the plate count and the agar diffusion assay do not give this indication.

In the present study, it was observed that heat-treated CFS of the various probiotics demonstrated significantly greater (P<0.05) inhibition zones against the species. During the heat treatment, 100°C for 60 min, it was likely that some water evaporated from the CFS resulting in the concentration of the CFS. This provides some evidence that the CFS contained other non-acidic inhibitory substances, which could have been produced in smaller quantities but concentrated during heat treatment. Smaller zones of inhibition were also noted for the CFSs when they were stored at 4°C for one week even though the pHs of the CFSs were not significantly affected during storage. These stored CFSs also did not demonstrate bactericidal activity against the species as cell counts of 10^{6}-10^{5} CFU/mL were obtained for the species when co-cultured in the unmodified CFSs. This suggests that the inhibitory substances were unstable in the supernatant which is in agreement with findings by Najim et al., (2012) who reported significant reduction in activity of CFS obtained from a *L. acidophilus* isolate containing a bacteriocin during storage at 4°C or room temperature for 21 days. They explained that the decreased activity during storage could be due to the action of proteolytic enzymes in the supernatant. Their CFS was also reported to be heat stable (80 and 60°C).

3.5 Chapter summary

In the work reported in this chapter, the potential of the isothermal microcalorimeter was explored to assess the antagonism of some commercial probiotics using the probiotic strains in mixed culture with *P. aeruginosa*, *S. aureus* and *E. coli*. It was shown that the microcalorimeter could differentiate between the species. However detailed structure (peaks and troughs) of the curves was lost in contrast to that obtained in Chapter 2. This, as explained in Chapter 2 could be due to the anaerobic environment generated in the ampoule as well as the saturation of the medium with one energy/carbon source (glucose).

The results demonstrated that the probiotic species could have co-existed with the pathogenic species in the microcalorimeter when co-cultured together but inhibited them after a particular duration. This implied that antagonism of the probiotics may have depended on their production and accumulation of extracellular inhibitory metabolic products, which could have occurred after they had fermented the nutrient sources but during which the other species may have grown because of their faster growth rate. This was in agreement with previous studies which have demonstrated inhibitory activity of potential probiotics occurring after active growth of the probiotics (Apella et al., 1992, Kos et al., 2008) or inhibition occurring after a particular duration when the kinetics of the growth of monoculture of potential pathogenic species were compared with co-cultures with potential probiotic species (Apella et al., 1992, Kos et al., 2008, Bendali et al., 2011). However, the antagonistic activity of the probiotics could not be observed with the live cells in the microcalorimeter due to exhaustion of nutrient sources as a result of the static batch culture model that was simulated in the microcalorimeter by the use of the ampoule. The exhaustion of nutrient sources in the ampoule made it difficult to distinguish between the dead or inactive pathogenic species and live stationary probiotics cells (determined during plate count and also when CFS produced by the probiotics were used instead of the live cells). This limitation could be circumvented with a flow microcalorimeter where growth media can be constantly pumped into the reaction vessel (Beezer et al., 1979, Morgan et al., 2001) or by titrating fresh media into the reaction vessel at determined intervals using a titration unit (Johansson and Wadso, 1999b). These are also not without their own limitations. For example, attaining thermal equilibrium of the continuously added medium could take a very long time if it is added in large volumes.

Inhibition of the pathogenic species by the probiotics was shown to be pH-dependent using the CFS produced by the probiotics. But the results from the microcalorimeter also indicated that other non-acidic metabolites could have contributed to the inhibition of the species. These non-acidic metabolites, likely bacteriocins (Barefoot and Klaenhammer 1983, Anand et al., 1984, Anand et al., 1985, Kang et al., 1989) could have been produced in small quantities relative to the acids. The inhibitory substances produced by the probiotics were also shown to be heat stable but unstable during storage, which correlated with a previous study by Najim et al., (2012).

Chapter 4 Probiotics for treatment/prevention of *Clostridium difficile* infection: Antagonistic activity of probiotics against *C. difficile*

4.1 Introduction

4.1.1 Pathogenesis of *Clostridium difficile* infection

As discussed in Chapter 1, the normal human gut microbiota suppresses the growth of opportunistic pathogens and prevents the invasion of potential pathogens (Gordon and Pesti, 1971, Stecher and Hardt, 2008, Walker and Lawley, 2013). This was in fact recognised in the 1950s when Bohnhoff et al., (1955) showed that when mice were treated with antibiotics, the dose of *Salmonella enterica* serovar Typhimurium needed to infect them decreased by 100000 fold relative to untreated group (Bohnhoff et al., 1955). So far, none of the elements of the mammalian immune system has been shown to provide such a degree of protection (Walker and Lawley, 2013). The protective effect of the resident microbiota, proposed to be brought about by competition, immune modulation, direct antagonism towards potential pathogens (O'Toole and Cooney, 2008, Bermudez-Brito et al., 2012) coupled with the hostile acidic environment of the stomach, peristalsis and intestinal washout are factors which prevent invading microorganisms from colonizing or suppresses the growth of pathogenic members of the gut (Stecher and Hardt, 2008, Walker and Lawley, 2013).

The protective function of the microbiota is however reduced or compromised when there is dysbiosis through host lifestyle (diet, stress), medical practices (the use or overuse of antibiotics, chemotherapy, vaccination, toxic compounds, hygiene), host genetics (Stecher and Hardt, 2008, Round and Mazmanian, 2009, Dore and Corthier, 2010). Following the disturbance of the resident microbiota through these practices or compounds, there is a consequential overgrowth of the opportunistic pathogens of the microbiota or colonization by invading ones (Figure 4.1).

Clostridium difficile is the pathogen most often associated with opportunistic proliferation after disruption of the normal microbiota through use of broad-spectrum antibiotics (Tedesco, 1981, Parkes et al., 2009, Rupnik et al., 2009).

1. Homeostasis of the gut microbiota with members of the microbiota consisting of beneficial bacteria (blue) and potential pathogens (red)

Dysbiosis through broad-spectrum antibiotics, chemotherapy etc.

2. Broad-spectrum antibiotics or other toxic substance disrupts the normal microbiota and breaks down colonization resistance

Ingestion of *C. difficile* spores through faecal-oral route

3. Overgrowth of *C. difficile*

Production of toxins by *C. difficile*

4. *C. difficile* overgrowth with associated toxins (dark red) damage epithelial cells and tight junctions, compromising the gut barrier. There is also decrease in SCFA production (hence reduced energy for colonocytes), inflammation, etc. manifests as pseudomembranous colitis, diarrhoea

Figure 4.1. Pathogenesis of *C. difficile* infection.

It is a fastidious, Gram-positive, anaerobic, spore-forming bacillus. During its overgrowth/infection, which occurs as a result of antibiotic usage and or through ingestion of spores of the organism, spread via the faecal-oral route (which germinate in the intestine), it produces two toxins, Toxins A (Tcd A) and Toxin B (Tcd B) which are responsible for its pathogenicity (Warny et al., 2005, Kelly and LaMont, 2008). When these toxins are produced in sufficient amounts, they bind and structurally damage epithelial cells and/or the tight junctions, and compromise the gut barrier, consequently leading to an inflammatory cascade also involving the nervous system (Hell et al., 2013). Specifically, Toxin A is proposed to increase intestinal permeability, inhibit protein synthesis, disrupt colonic mucosal cell adherence to colonic basement membrane and also damage villous tips (Poxton et al., 2001, Tonna and Welsby, 2005). Toxin B enters the cell by endocytosis and induces apoptosis; it is thought to induce its effect once the gut wall has been damaged (Poxton et al., 2001). Toxin B has been reported to be 1000 times more potent with 100-fold higher enzymatic activity than toxin A, this accounting for the difference in cytotoxic activity between the two (Poxton et al., 2001, Tonna and Welsby, 2005). Both toxins however stimulate monocytes and macrophages, which in turn release interleukin 8, resulting in tissue infiltration with neutrophils (Tonna and

Welsby, 2005). The consequent manifestation of their activities are diarrhoea, colitis, pseudomembranous colitis, toxic megacolon and in worst case, death (Mitty and LaMont, 1994, Hurley and Nguyen, 2002, Rupnik et al., 2009). However, the infection could also be asymptomatic in some people infected with toxigenic strains, which has implied that there could be other factors, including the environment within the gut which are important for disease pathogenesis (Haslam et al., 1986, Karlsson et al., 2003, Tonna and Welsby, 2005, Kuijper et al., 2006). Collectively, the manifestations of *C. difficile* infection are known as *Clostridium difficile* associated disease (CDAD).

As hinted, *C. difficile* is not uncommon in a healthy intestine. It can be cultured in the stools of 1-3% of healthy adults and up to 80% of healthy newborns and infants (Viscidi et al., 1981, Bartlett, 2002). Generally, contamination of hospital environments with *C. difficile* spores is a major factor associated with infection spread (Cloud and Kelly, 2007). When indigenous gut microbiota is disrupted with antibiotics, *C difficile* spores germinate, resulting in vegetative growth, and toxin production, which consequently leads to the clinical manifestations of the disease. It should be noted that there are some strains of *C. difficile* which are non-toxigenic i.e., they do not produce either toxin A or B hence do not cause *C. difficile* infection (Rupnik et al., 2009, Best et al., 2012), while there are also strains which produce toxin B but not A but these strains are also capable of causing *C. difficile* infection (Lyras et al., 2009, Hell et al., 2013). Strains which produce toxin A but not B may not necessarily be virulent (Drudy et al., 2007, Lyras et al., 2009). A third, so called *C. difficile* toxin (CDT) associated with epidemic strains has also been identified (Schwan et al., 2009, Kuehne et al., 2014).

4.1.2 History of *C. difficile* infection

C. difficile was first described by Hall and O'Toole in 1935 as a commensal organism, in the faecal flora of healthy newborn infants. They referred to it as *Bacillus difficilis;* its unusual name, *difficilis* because unlike other bacteria, they noticed it grew slowly and was difficult to isolate (Lamont, 2002). They also noticed and reported that *B. difficilis,* secreted toxins that were lethal to animals. But because it had been isolated from normal infants, and there was no indication that its presence had any adverse effects on newborns, so for the next forty years, *B. difficilis* or *C. difficile* was considered a part of the normal microbiota of infants and remained unimportant clinically until 1978 (Lyerly

et al., 1988).

In the 1960s and 1970s, pseudomembranous colitis (Figure 4.2), which had also been described as early as 1893, characterized by inflammation of the colon with the formation of plaques, or pseudomembranes (composed of fibrin, mucous, necrotic epithelial cells and leukocytes) (Lyerly et al., 1988), had become prevalent following the introduction into clinical practice, broad-spectrum antibiotics for the treatment of infections (Tedesco, 1981, Lyerly et al., 1988, Parkes et al., 2009). The incidence of pseudomembranous colitis was reported to be as high as 10% in some hospitals following use of the broadspectrum antibiotics, which in some patients presented as a sheath over the entire colonic mucosa at autopsy (Lyerly et al., 1988). Pseudomembranous colitis was therefore thought to be caused by broad-spectrum antibiotics that were commonly prescribed: clindamycin and lincomycin (Tedesco, 1981, Lyerly et al., 1988). The condition was then referred to as "clindamycin colitis" (Cohen et al., 1974) and was thought to have a high mortality rate but was later associated with other antibiotics (Lyerly et al., 1988). Although many aspects of research were ongoing during this era, it was the intensive research that was conducted during the late 1970s utilizing animal models that brought to light the association of clostridial species and later, the toxins reported by Hall and O'Toole to be implicated in the disease (Tedesco, 1981, Lyerly et al., 1988). Indeed it was the study of Bartlett and colleagues (1977) that demonstrated an overgrowth of a clostridial species to be associated with enterocolitis in clindamycin-treated hamsters (Bartlett et al., 1977). Larson et al., (1977) also discovered that cell free filtrates of stools of patients with pseudomembranous colitis had cytopathic activities on cell cultures and proposed that pseudomembranous colitis may be caused by a bacterial toxin (Larson et al., 1977). Studies conducted after these important findings showed that the cell free filtrate toxins found in the stool of patients with pseudomembranous colitis were produced by clostridial species (Rifkin et al., 1977, Bartlett et al., 1978, Tedesco, 1981). At first, the species was thought to be *Clostridium sordellii* since the cytopathic effects of the toxin could be neutralised with *C. sordellii* antitoxin, but later, the organism was confirmed as *C. difficile* (Rifkin et al., 1977, Bartlett et al., 1978, Tedesco, 1981).

Figure 4.2. Pseudomembranous colitis: pseudomembranes appear during colonoscopy of patients with *C. difficile* infection. Pseudomembranes are visible as raised yellow plaques scattered over the colorectal mucosa. (Figure obtained from Medscape. March 2014. *Clostridium difficile* colitis. [online]. Available from: http://emedicine.medscape.com/article/186458-overview#aw2aab6b2b4 [Accessed on 7 May 2014])

4.1.3 Epidemiology, risk factors and burden of *C. difficile* infection

Rates of *C. difficile* infection kept increasing from its discovery through to the 1990s, but it saw a dramatic increase in the incidence, severity and relapse in the 2000s due to the emergence of hypervirulent strains such as *C. difficile* ribotype 027 (Kuijper et al., 2008, Islam et al., 2012) which were resistant to fluoroquinolone antibiotics. The regions most affected were in North America (especially Quebec, Canada) and Europe (Kuijper et al., 2008). Within the United Kingdom, the rate of the infection was highest in 2007 with more than 50,000 cases reported and with 80% of affected patients aged over 65 years (Islam et al., 2012, Public Health England, 2014). Ribotype 027 of *C. difficile* was associated with epidemics and accounted for more than 40% of *C. difficile* isolates from English hospitals by 2007 (Islam et al., 2012). There has been a huge effort by the NHS which was also supported by the Department of Health to decrease the rates of the infection (Duerden, 2011, Islam et al., 2012). Interventions used include rapid diagnosis, root cause analysis, patient isolation, improved infection control practice, and antibiotic stewardship policies, which focuses on decreasing the use of "high risk antibiotics"(Gerding et al., 2008a, Owens and Ambrose, 2007). From 2007, the rate of *C. difficile* infection has fallen, with a decreasing prevalence of ribotype 027 strains being reported (Islam et al., 2012). However, between April 2013 and March 2014 alone, a total of 13,361 cases of *C. difficile* infection were reported across the NHS. This number represents a 9.1% reduction on the cases reported between 2012 and 2013 and

75.9% reduction on numbers reported between 2007 and 2008 (Public Health England, 2014). Whilst this indicates a significant reduction of cases reported across England, and a decreasing prevalence of ribotype 027 strains in the UK, other virulent strains have also been described (CDRN report for England and Northern Ireland, 2011-2013). *C. difficile* still remains a significant (up to 20-30%) cause of antibiotic-associated diarrhoea and is responsible for almost all cases of pseudomembranous colitis (Bartlett, 2002, Rea et al., 2013).

C. difficile infection predominantly occurs in older patients mainly because of their healthcare contact (McFarland et al., 1990, Bauer et al., 2011) and the reduced diversity of their microbiota, (predominantly, a reduction of the anaerobes and bifidobacteria) (Hebuterne, 2003) which reduces their colonization resistance. This occurs as a result of repeated courses of antibiotics and a background of an ageing immune system (Islam et al., 2012, Burke and Lamont, 2014). Other patients who are at risk include immunocompromised patients, patients on proton pump inhibitors (Dial et al., 2004) and patients who have undergone organ transplant (Munoz et al., 2007, Bouza, 2012).

C. difficile infection imposes a lot of burden on both patients and the healthcare systems. First of all, patients experience significant morbidity and in the worst case, mortality from the debilitating manifestations of the disease, which include profuse diarrhea, pseudomembranous colitis, abdominal pain, nausea, fever, intestinal perforations, peritonitis, megacolon and fulminant colitis (Hurley and Nguyen, 2002, Kuijper et al., 2006, Bouza, 2012, Burke and Lamont, 2014). *C. difficile* infection is more a frequent cause of nosocomial than community-acquired infections (Kuijper et al., 2006, Na and Kelly, 2011, Bouza, 2012). Patients in the hospital who develop the disease are often isolated and given specific antibiotics to treat the infection apart from the therapy they were already receiving for their underlying disease. Such measures are usually costly for the healthcare facility especially when the patients develop serious form of the disease or complications or have a recurring infection (Kuijper et al., 2006, Vonberg et al., 2008). Besides the cost associated with new antibiotics needed for the treatment of the infection, the rigorous hygiene in patient care, environmental decontamination, and in instances when an outbreak arises, the burden of closure of a ward (Kuijper et al., 2006) adds to the financial burden of healthcare facilities. The cost of longer hospital stays is also a burden both to the patient and the healthcare facility (Dubberke and Wertheimer, 2009). On average, patients with *C. difficile* infection could spend an extra one to three

weeks in hospital compared with patients who have not contracted the infection (Kuijper et al., 2006). This increase in hospital stay is the most significant factor on the cost of *C. difficile* infection (Dubberke and Wertheimer, 2009) to a healthcare facility. The total average cost of treating patients with *C. difficile* infection compared to a cohort with the same underlying condition was 78% more expensive (Vonberg et al., 2008). A study by Mc Farland et al., (1999) in the USA also reported a mean lifetime cost of $10,970 per patient for treatment of *C. difficile* infection and its complications and recurrences. In Europe, the management of *C. difficile* infection was estimated by Kuijper et al., (2006) to be around 3000 million euros per year and this they predicted to rise in line with the ageing population of Europe (Kuijper et al., 2006).

4.1.4 Management of *C. difficile* infection

Despite the discovery that *C. difficile* was the causative agent of pseudomembranous colitis in 1978, due to usage of broad-spectrum antibiotics (which included cephalosporins from the 1980s and fluoroquinolones, especially in the 2000s together with the lincosamides and penicillins which were implicated earlier; Hookman and Barkin, 2009, Bartlett, 2010, Britton and Young, 2012), antimicrobial treatments for the disease did not changed much in the intervening period. First line treatments for *Clostridium difficile* associated disease (CDAD) still remain limited to vancomycin and metronidazole; metronidazole more widely prescribed because of its lower cost and the need to prevent resistance to vancomycin (Gerding et al., 2008b, Best et al., 2012). More recently (2011) fidaxomicin was approved in the United States for the treatment of mild to moderate *C. difficile* and was approved for treatment of the infection in Europe in 2012 (Johnson and Wilcox, 2012) but its use has not been popular because of its cost and lack of trial data (Bartsch et al., 2013, Burke and Lamont, 2014). Faecal microbiota transplantation, by which donor faeces is infused into a patient's gastrointestinal lumen, has been one effective way of treating recurrent CDAD but its aesthetic acceptability and safety has been its major drawback (Burke and Lamont, 2013).

4.1.5. Probiotics for management of *C. difficile* infection

Although antibiotics are generally effective in achieving symptomatic recovery from *C. difficile* infection, the disease frequently relapses, partly because antibiotics do not only

kill *C. difficile,* but also disrupt the already compromised colonisation resistance of the gut microbiota (Blossom and McDonald, 2007, Britton and Young, 2012, Rea et al., 2013). For example, it was shown that, metronidazole reduces the depth of the mucus layer which consequently reduces its barrier function and causes a secondary infection (Wlodarska et al., 2011). It was also demonstrated by Brandl et al., (2008) that the combinations of neomycin, vancomycin and metronidazole in mice could induce infection by vancomycin-resistant enterococci (Brandl et al., 2008).

Usually about 15-35% of patients who have had a first episode of *C. difficile* infection develop another episode of the disease within 2 months (Pepin et al., 2005, Maroo and Lamont, 2006, Gerdings et al., 2008b, Bauer et al., 2011). After a cycle of antibiotic treatment of a recurrent infection, the risk of another relapse also increases again to about 33-65% (Gerding et al., 2008b). The high rate of recurrence of *C. difficile* infection in patients has been attributed to the decreased susceptibility of *C. difficile* to these antibiotics (Baines et al., 2008, Brazier et al., 2008, Huang et al., 2009) and the inability of antibiotics to restore the ecology of the gut to the normal state, but rather disturb it further (Blossom and McDonald, 2007, Giel et al., 2010, Rea et al., 2013, Britton and Young, 2014) and consequently, the deficiency of these antibiotics to efficiently manage the disease. There is therefore the need of finding other alternatives for the management of *C. difficile* infection. A good management for *C. difficile* infection would therefore aim at eliminating the organism and restoring the gut microbiota.

There have been clinical interests in using probiotics to manage *C. difficile* infection but this has received mixed reviews (Pattani et al., 2013, Rainkie and Kolber, 2013, Goldenberg et al., 2013, Allen et al., 2013, Hickson, 2011, Pillai and Nelson, 2008, Na and Kelly, 2011). To give an example, a review published by Pillai and Nelson in 2008 in the Cochrane library, which initially investigated the effect of probiotics either used alone or in conjunction with antibiotics for the treatment of *C. difficile* infection reported that only one study out of the four that met the inclusion criteria showed significant benefit of the probiotics. The authors concluded that there was insufficient evidence for the use of probiotics in the treatment of *C. difficile* infection as an adjunct in conventional therapy and that there was no evidence of its use for sole treatment. For most authors that have criticised the use of probiotics for management of *C. difficile* infection, the main issue that they raised, besides the limitations of the clinical studies

themselves (sample size, methodological problems, influence of funding), was the specificity of the strains. A large number of clinical studies done on probiotics are conducted without any reliable evidence from laboratory studies to show that they can address a specific condition (Allen et al., 2013, Smith, 2013). For instance, for the prevention or treatment of *C. difficile* infection, it will be useful to show from laboratory studies that a strain or product has anti-clostridial effect. Assuming that all products claimed to be probiotics can eliminate *C. difficile* or prevent the occurrence of the infection in patients is anecdotal and is preventing beneficial probiotics which could potentially be used in the management of the disease to be dismissed. Currently, the UK Health Protection Agency and the Society for Healthcare Epidemiology of America and the Infectious Diseases Society of America have not endorsed the use of probiotics for the management of *C. difficile* infection (Hickson, 2011, Rainkie and Kolber, 2013, Public Health England, 2013) even though these countries are among those that have been historically hardest hit with epidemics (McMaster-Baxter and Musher, 2007). Because of the very large number of commercially available products claiming to be probiotics and probiotic combinations that are now available, it would be useful if *in vitro* studies could be done to streamline those with anti-clostridial effect before further laboratory or clinical studies. This will save the clinical and economic cost of inapt studies.

Only few studies have reported on the anti-clostridial activity of potential or commercial probiotic strains *in vitro* (Schoster et al., 2013). Lee et al., (2003) demonstrated that out of 109 strains of lactic acid bacteria isolated from 32 healthy Korean infants, only 12 showed activity against a toxigenic *C. difficile* strain. Naaber et al., (2004) showed that only 5 out of 50 intestinal lactobacilli strains were inhibitory towards all 23 pathogenic strains of *C. difficile* tested on agar plates; 27 strains did not show antagonistic activity (Naaber et al., 2004). Trejo et al., (2006) also showed that all strains of bifidobacteria isolated from a healthy infant inhibited two strains of *C. difficile* but they also detected that the degree of inhibition was strain specific (Trejo et al., 2006). Tejero-Sarinena et al., (2013) using pH-controlled anaerobic batch cultures containing mixed faecal bacteria and analysing bacterial population by fluorescent *in situ* hybridation (FISH) also demonstrated that inhibition of *C. difficile* by probiotic strains and mixtures was strain specific. Schoster et al., (2013) have also studied and showed that some commercial strains of probiotics were capable of inhibiting *C. difficile* whilst others could not. Ten commercial probiotic strains out of seventeen strains they analysed using broth culture

and agar well diffusion assays inhibited a reference strain of *C. difficile*. They also commented on the lack of scientific studies scrutinizing potential and commercial probiotics for anticlostrial activity since they also believed this activity was strain specific (Schoster et al., 2013).

The work in this chapter aimed to investigate the *in vitro* anti-clostridial activity of the commercial probiotic strains and commercially available probiotic products either packed as a single strain or multi-strain using the isothermal microcalorimeter.

As introduced in Chapter 1, probiotics are usually members of the lactic acid bacteria, mainly members of the genera *Lactobacillus* and *Bifidobacterium* (but not exclusively) because they are known as members of the microbiota, which significantly contribute to the health effect and also because of their long history of safe use (Salminen et al., 1998, FAO/WHO 2001, Iannitti and Palmieri, 2010). Some researchers believed that it was unlikely a single strain could colonize the gut and achieve all therapeutic benefit and therefore suggested that probiotics should consist of multiple strains and also because probiotics could be used for targeting a number of diseases but each targeted disease will require a specific probiotic property, which cannot be found in a single strain (Dunne et al., 1999, Famularo et al., 1999, Sanders and Huis in't Veld, 1999, Timmerman et al., 2004). Probiotics packaged and used as multistrain or multispecies is now common practice and although some have not shown superior benefits (Tejero-Sarinena et al., 2013, Chapman et al., 2013), some evidence exist on their greater efficacy compared to single strain (Apella et al., 1992, Drago et al., 1997, Chapman et al., 2011, Chapman et al., 2012, Hell et al., 2013). To give examples, Chapman et al., (2013) and Tejero-Sarinena et al., (2013), determining the relative efficacy of multi-species and single-species probiotics against urinary pathogens (*E. coli* and *E. faecium*) and enteric pathogens (*C. difficile*, and *Salmonella typhimurium*) respectively reported insignificant difference between the two (Chapman et al., 2013) or better potency of inhibition by some single species than by probiotic mixtures (Tejero-Sarinena et al., 2013). Yet Chapman et al's previous study in 2012 demonstrated that 5 multi-species probiotic preparations had significantly ($P < 0.05$) greater inhibitions in 12 out of 24 cases towards *C. difficile*, *E. coli* and *S. typhimurium*, than 15 single-species probiotics (Chapman et al., 2012). Studies conducted by Apella et al., (1992) and Drago et al., (1997) also showed the superior potency of mixtures than single strains.

However, less is known about the stability of probiotic mixtures, i.e. whether there is possibility of inhibition of probiotic strains in a mixture (Chapman et al., 2011, Chapman et al., 2012). In this chapter, the potential of isothermal microcalorimetry to distinguish between a commercial multi-strain probiotic product and its component strains and their mixed culture was explored. This was to find out if all strains were present in the product at the point of consumption.

4.2 Objectives

The objectives of this chapter were:

- To use the isothermal microcalorimeter to test for anti-clostridial effect of commercial probiotic strains and products
- To investigate the microbial stability of a commercial probiotic product consisting of multiple strains

4.3 Materials and Methods

4.3.1 Microorganisms

Clostridium difficile NCTC 13565 was purchased from the National Collection of Type Cultures (NCTC), Public Health England. It is a toxigenic strain which had been previously isolated from the faeces of a patient with pseudomembranous colitis. *Lactobacillus acidophilus* LA-5® and *Bifidobacterium lactis* BB-12® were obtained as previously reported in section 3.3.1 of Chapter 3. Constituent species of a commercial probiotic product, Symprove™: *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus* and *Enterococcus faecium* referred to in this thesis as *L. plantarum* SYM A, *L. rhamnosus* SYM B, *L. acidophilus* SYM C and *E. faecium* SYM D were all obtained from Symprove Ltd., UK.

4.3.2 Commercial probiotics

The commercial products used included: Actimel® (Groupe Danone, France), Align® (Procter and Gamble, USA), Bio-kult® (Protexin Lopen Head, UK), Biobalance Support (Boots Company, UK), Symprove™ (Symprove Ltd, UK), VSL#3® (Ferring Pharmaceuticals Ltd), Yakult® (Yakult Ltd, UK) and Probio 7® (manufactured in France and distributed in the UK by Forever Young International, UK). Details of the products can be found in section 5.3.1 of Chapter 5.

4.3.3 Microbiological media and chemicals

Brain heart infusion (BHI) broth and agar, reinforced clostridial medium (RCM), *Clostridium difficile* agar base, were all from Oxoid Ltd, Basingstoke, UK and purchased from Fisher Scientific, UK. Defribinated horse blood (Oxoid, Basingstoke, UK) and *Clostridium difficile* selective supplement (Oxoid, Basingstoke, UK) were also purchased from Fisher Scientific. Sodium taurocholate was purchased from Sigma-Aldrich, UK.

4.3.4 Stock culture maintenance and purity

C. difficile NCTC 13565, received as a freeze dried culture was rehydrated with 0.5 mL RCM. A loopful of the rehydrated culture was streaked onto RCM agar and incubated anaerobically at $37^{o}C$ for 48 h. A few colonies were picked from the plate and used to make a lawn on a fresh plate using a sterile cotton swap. This was incubated anaerobically for 48 h after which the lawn of cells was inoculated into RCM supplemented with 15% v/v glycerol and frozen at $-80^{o}C$. Stocks of *L. acidophilus* LA-5® and *B. lactis* BB-12® were obtained as previously reported in section 3.3.3 of Chapter 3. The constituent strains of Symprove™: *L. plantarum* SYM A, *L. rhamnosus* SYM B, *L. acidophilus* SYM C and *E. faecium* SYM D, obtained as freeze dried powders were also each rehydrated with MRSc and sub cultured onto MRSc. Single colonies obtained were picked off and spread onto a fresh MRSc agar plate. Colonies obtained on the incubated plates were used to make a thick suspension of the cells in MRSc broth supplemented with 15% v/v glycerol, which were stored at $-80^{o}C$.

4.3.5 Cryopreservation and defrosting of bacteria

Cryopreservation of the organisms was done according to section 2.3.7. Parent stocks of *C. difficile* and the constituent strains of Symprove™ (*L. plantarum* SYM A, *L. rhamnosus* SYM B, *l. acidophilus* SYM C and *E. faecium* SYM D), were streaked onto RCM agar and MRSc agar respectively and incubated anaerobically for 48 h. Pure colonies obtained were inoculated into 7 mL RCM or MRSc and incubated anaerobically at 37°C to make starter cultures of the organisms. A 1 in 100 dilution of the starter cultures were made in fresh RCM or MRSc and was incubated anaerobically and harvested by centrifugation at 3500 *g* for 10 min at 4°C when the organisms reached their stationary phase of growth. The organisms were washed twice with PBS, resuspended with ¼ Ringer's solution supplemented with 15% v/v glycerol and dispensed into multiple aliquots in cryovials. Cryopreservation procedure, recovery determination, and defrosting procedures were carried out as previously described for the other species.

4.3.6 Determination of spore of *C. difficile* culture

10 µL of the thawed culture of *C. difficile* was used to make smears on glass slides, which had been cleaned with 70% v/v ethanol. The smeared glass slides were air dried for approximately 2 min and heat fixed. They were then stained with malachite green and safranin according to the Schaeffer–Fulton method (Schaeffer and Fulton, 1933). The cells on the fixed slides were firstly stained with malachite green by flooding the glass slides with the stain and heating over steam generated from a pan of boiling water for 5 min. The slides were washed under running tap water to remove excess stain. The cells on the slides were then counterstained with safranin for 30 s. The slides were rinsed with water and dried after which they were observed under oil immersion with a light microscope.

4.3.7 Determination of optimum condition for vegetative growth of *C. difficile* in the isothermal microcalorimeter

C. difficile is a spore-forming organism (Modaber, 1975, Wiegel, 2009). Unlike other species, it forms spores when there is a change in its environment that is unfavourable

for its growth. In order to cause diseases, *C. difficile* must be vegetative to produce the toxins. The influence of different medium and compounds on the germinantion of *C. difficile* was assessed. As per the supplier's instructions, *C. difficile* was inoculated into RCM. *C. difficile* was also inoculated into BHI and BHI supplemented with 0.1% w/v cysteine hydrochloride "BHIc", BHI supplemented with 0.1% w/v cysteine hydrochloride and 0.1% w/v sodium taurocholate "BHIct" and BHI supplemented with 0.1% w/v cysteine hydrochloride, 0.1% w/v sodium taurocholate and 5 mg/mL of yeast extract "BHIcty". Inoculations were done in 3 mL calorimetric glass ampoules containing the media. Samples were placed in the TAM and studied as previously described. Optical density measurements at 600 nm (OD600) of samples incubated under the same conditions were taken at 24 h and 48 h with a spectrophotometer (Heλiosα, Thermo Scientific).

4.3.8 Pure and mixed culture of *C. difficile* and probiotic strains and commercial probiotic products in the isothermal microcalorimeter

For pure culture experiments, the species were inoculated into BHIct to a population density of 10^6 CFU/mL in 3 mL calorimetric glass ampoules. 30 μL of commercial liquid probiotic products and hydrated lyophilised products (in 10 mL of PBS) was also inoculated into 2970 μL of BHIct in 3 mL calorimetric ampoules. For mixed culture experiments, 10^6 CFU/mL of the probiotic strains or 30 μL of hydrated or liquid commercial probiotic products were mixed with *C. difficile*. The sealed ampoules were vortexed for 10 s, and placed into the intermediate position in the TAM 2277 for 30 min after which calorimetric study was conducted as previously described in section 2.3.9.

4.3.9 Preparation of cell-free culture supernatants from probiotic cultures

The culture supernatants of the probiotic strains were prepared by cultivating the respective probiotic species in BHIct or MRSc over 48 h anaerobically. The cells were removed by centrifuging at 3500 *g* for 10 min at 4°C. The supernatant was collected and filter-sterilized using a 0.22 μm membrane syringe filter. The pHs of the supernatants were examined and equal aliquots modified by adjusting the pH to 5, 6, 7 and 8 with 5 M NaOH. Neutralised supernatants were also concentrated by freeze-drying (Modulyo D-230, Thermo Scientific, UK) and reconstituted with sterile distilled water to achieve

143

2.5-fold, 5-fold, 10-fold and 20-fold concentration. Modified supernatants were filter-sterilized before use.

4.3.10 Co-incubation of *C. difficile* with probiotic culture supernatants in the isothermal microcalorimeter

1.5 mL of unmodified or modified CFS of LA-5®, BB-12® and the strains of Symprove™ was added to 1.5 mL double strength BHIct "dsBHIct" in 3 mL calorimetric glass ampoule. The mixture was vortexed for 10 s. *C. difficile* was inoculated into this mixture and vortexed. The ampoule was placed in the TAM and power-time measurements were taken for as long as the sample was generating a signal in the TAM. A control experiment was done by replacing the CFS with sterile distilled water or MRSc.

4.3.11 Agar well diffusion assay

CFS of LA-5® and BB-12® were examined for their antibacterial activity against *C. difficile* by the agar well diffusion assay as described by Trejo et al., (2006) with some modifications. A *C. difficile* lawn was made by seeding a culture of the organism in molten BHIct agar (45°C). The agar was left to solidify. Wells of 9 mm diameter were made with a sterile borer and filled with 100 µL of unmodified and pH modified CFSs. The plates were kept in anaerobic jars and retained on the bench for 2 h after which they were incubated at 37°C for 48 h in the Don Whitley DG250 Anaerobic workstation. The zones of inhibition were measured after 48 h of incubation.

4.3.12 Microbial stability of multi-strain probiotics: pure and mixed cultures of the strains of Symprove™ in the isothermal microcalorimeter

The strains of Symprove™: *L. plantarum* SYM A, *L. rhamnosus* SYM B, *L. acidophilus* SYM C and *E. faecium* SYM D were each inoculated into BHIc or MRSc in 3 mL calorimetric ampoules to give individual population densities of 10^6 CFU/mL and loaded into the intermediate position of the TAM for 30 min. Measurements were taken as described in section 2.3.9. 30 µL each of 10 batches of the commercial product,

Symprove[TM] obtained from the manufacturer was also inoculated into BHIc or MRSc and individual microcalorimetric measurements taken.

4.3.13 Statistical Analysis

Statistical analysis was performed in Origin Pro 8.6 (Microcal Software Inc.). T-test or Analysis of Variance (ANOVA) with Turkey post-hoc analysis for means comparison was used where appropriate. P values less than 0.05 were regarded as significant difference between means.

4.4 Results and Discussion

4.4.1 Determination of optimum condition for vegetative growth of *C. difficile* in the isothermal microcalorimeter

As previously mentioned, *C. difficile* is a spore-forming bacterium (Modaber, 1975, Wiegel, 2009). It is an obligate anaerobe when in its vegetative state but its spores can persist in dry, aerobic environment and even on inanimate surfaces for several years (Cloud and Kelly, 2007, Gerding et al., 2008a, Wiegel, 2009, Paredes-Sabja et al., 2014). The spores play a very important role in the transmission of the disease (section 4.1.1) but to cause disease, the spores must return to the vegetative state and produce the two major toxins, toxins A and B (Gerding et al., 2008a, Kelly and LaMont, 2008, Burns et al., 2010, Burke and Lamont, 2014).

After the cryopreservation of *C. difficile,* there was no viable cell recovery on RCM agar plates. It was therefore suspected that *C. difficile* may have been killed or had formed spores during the procedure. A Schaeffer-Fulton spore stain indeed revealed spores of *C. difficile.* The spores may have formed as a means of the cells surviving the limiting nutrient in the medium and the metabolic waste products that had accumulated during the culture. Also, the cells were exposed to oxygen during the harvesting and cryopreservation procedure hence their ability to sustain vegetative growth may have been compromised due to these stressing factors. Efforts were therefore made to keep *C. difficile* in its vegetative state through the selection of a medium and conditions that

support vegetative *C. difficile* growth. As per the suppliers guide, RCM was used in maintaining *C. difficile* but this showed delayed growth of the organism in the microcalorimeter (Figure 4.3, peaking at ca. 40 h) and no recoveries on plates indicating that the medium condition was not adequate for supporting vegetative growth of *C. difficile*. BHI broth, which contains in g/L, brain infusion solids 12.5; beef heart infusion solids 5.0; proteose peptone 10.0; glucose 2.0 and disodium phosphate, 2.5, has been suggested to be a nutrient rich medium for germination of *C. difficile* (Paredes-Sabja et al., 2008). The use of this medium however did not boost the growth or germination of *C. difficile*; it even slowed the onset time of growth of the organism (lag time was increased, Figure 4.3) relative to RCM.
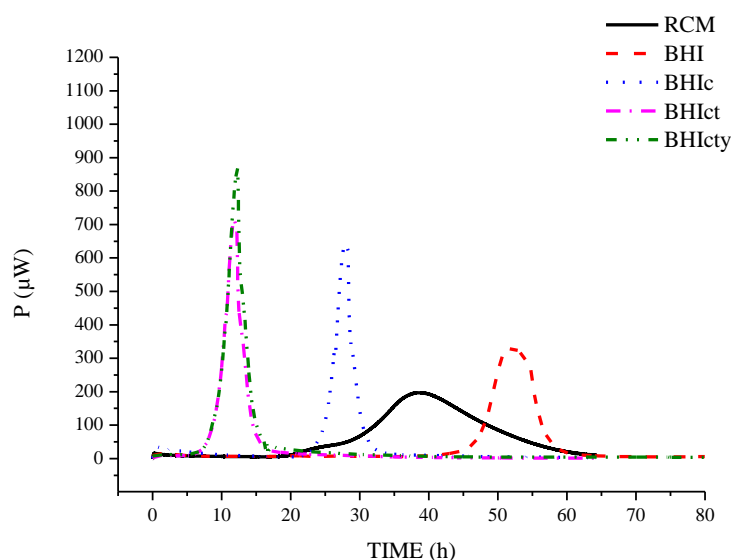
Figure 4.3. Power-time curves of *C. difficile* in different media and in the presence of germinants. RCM is Reinforced Clostridial Medium, BHI is Brain Heart Infusion Medium, BHIc is BHI supplemented with 0.1% w/v cysteine hydrochloride, BHIct is BHIc further supplemented with 0.1% w/v sodium taurocholate and BHIcty is BHIct supplemented with 5 mg/mL yeast extract. Growth was enhanced in the presence of the germinants

Addition of 0.1% w/v cysteine hydrochloride to BHI, which reduced the medium, increased growth. Further growth was observed when 0.1% w/v of sodium taurocholate, a primary bile salt was added to BHI together with cysteine hydrochloride. Further addition of yeast extract to BHI supplemented with 0.1% w/v cysteine hydrochloride and 0.1% w/v sodium taurocholate changed growth only slightly. The OD600 for 24 h and 48 h cultures of *C. difficile* in the respective media are shown in Table 4.1. The differences in the media in supporting vegetative growth of *C. difficile* can be observed

in the table; only BHIct and BHIcty showed OD values consistent with exponential growth during 24 h of incubation.

Table 4.1. Optical density of *C. difficile* culture in different medium after 24 and 48 h of incubation

Media	Optical Density at 600 nm	
	24 h incubation	48 h incubation
RCM	0.058	0.882
BHI	0.012	0.088
BHIc	0.039	0.716
BHIct	1.213	1.169
BHIcty	1.323	1.444

Germination of spores is understood to be initiated when the spores sense specific effectors known as germinants (Burns et al., 2010). For *Bacillus subtilis*, a well studied spore forming organism, germination is known to occur by the binding of nutrient germinants, L-alanine or a mixture of asparagine, glucose, fructose and potassium ions, to specific receptors: GerA, GerB and GerK which are located in the spore inner membranes (Paidhungat and Setlow, 2000). After binding, the spore is known to go through stages of events requiring the spore to encode specific proteins which subsequently results in the hydrolysis of the spore peptidoglycan cortex during which the spore is hydrated and is returned to the vegetative state for enzymatic activity, metabolism and growth (Paidhungat and Setlow, 2000). The mechanism of germination or the specific condition required for germination of *C. difficile* spore is however not well defined (Paredes-Sabja et al., 2008, Burns et al., 2010, Paredes-Sabja et al., 2011, Paredes-Sabja et al., 2014). So far, *in vitro* studies have pointed to the role of bile salts (cholate and its derivatives (taurocholate, glycocholate, cholate, and deoxycholate) (Wilson, 1983, Giel et al., 2010, Burns et al., 2010, Heeg et al., 2012) or glycine as co-germinant (Sorg and Sonenshein, 2008) or the use of nutrient media, mainly BHI with or without yeast extract for the germination of *C. difficile* spores (Paredes-Sabja et al., 2008, Heeg et al., 2012). The germination property of BHI was initially argued by Paredes-Sabja et al., (2008) to be due to the inorganic phosphate ions and potassium ions content of BHI because they were able to demonstrate that sodium phosphate and

potassium chloride could on their own germinate *C. difficile* (Paredes-Sabja et al., 2008). However their later study in 2009 (Paredes-Sabja et al., 2009) explained that spores of *C. difficile* used in their previous study (Paredes-Sabja et al., 2008) was contaminated with *Clostridium perfringens* spores and that *C. difficile* spores germinated poorly with potassium chloride or sodium phosphate (Paredes-Sabja et al., 2009).

In this work, the result suggests that germination occurred significantly in the presence of sodium taurocholate. The presence of the reducing agent, cysteine hydrochloride which made the medium anaerobic was required for growth. It should be noted that whilst the germination potential of sodium taurocholate has been demonstrated *in vitro* and *ex vivo* by other authors (Wheeldon et al., 2008, Giel et al., 2010, Heeg et al., 2012), germination of *C. difficile* has also been associated to be non-dependent on aerobic or anaerobic environment (Plowman and Peck, 2002, Wheeldon et al., 2008, Paredes-Sabja et al., 2008). Thus in the present study, the results suggest that the anaerobic condition was required for vegetative growth of *C. difficile*. The germination of *C. difficile* with sodium taurocholate *in vitro*, from results of present study and previous studies (Sorg and Sonenshein, 2008, Paredes-Sabja et al., 2009, Giel et al., 2010, Heeg et al., 2012) could be related to the germination of the spore of the organism in the human gut. As noted earlier, it is the spore form of *C. difficile*, which is usually ingested and normally survive transit through the stomach to the intestine (Mitty and LaMont, 1994, Gerding et al., 2008a). The vegetative cell can hardly survive in the inanimate environment nor can it survive gastric acid (Jump et al., 2007, Nerandzic et al., 2009). In the gut, proposed segments where germination and overgrowth could occur include the proximal small intestine which has significant amount of bile salt (Kalantzi et al., 2006) and the colon; a suitable environment for anaerobic growth (Roediger, 1980). However, the symptoms of *C. difficile* infection are largely derived from the colon and less so within the small intestine (Hurley and Nguyen, 2002). This may be probably because while the spores can start germinating in the small intestine due to the presence of the bile salt (Wilson et al., 1985) and the non-dependency of germination on aerobic or anaerobic environment (Plowman and Peck, 2002, Wheeldon et al., 2008, Paredes-Sabja et al., 2008) they might not outgrow there to cause disease but only outgrow in the colon, which presents a favorable condition for anaerobic growth (Roediger, 1980).

Previous studies have pointed to the germination of *C. difficile* within the small intestine or colon. Wilson et al., (1985) showed that 78% of spores inoculated intragastrically into

a hamster and studied with a ^{51}Cr tracer germinated within the small intestine (Wilson et al., 1985). Paredes-Sabja et al., (2008) also demonstrated germination of *C. difficile* by potassium salts and inorganic phosphate with an optimum pH of 6, while bile salt had little effect on the germination of their collection of *C. difficile* strains *in vitro* including clinical isolates. These authors previously concluded that *C. difficile* spore could either germinate in the early segments of the duodenum when the spores sensed inorganic phosphate or the colon, where the authors reasoned had high levels of potassium ions (Paredes-Sabja et al., 2008). However their work was unreproducible and later, they explained that their previous study was contaminated with spores of *C. perfringens.* Their final conclusion was that spores of *C. difficile* are able to germinate with sodium taurocholate and also with glycine (Paredes-Sabja et al., 2008, Paredes-Sabja et al., 2009). Findings from Nerandzic et al., (2009) also support that germination of *C. difficile* could occur in either the small intestine or colon. These authors showed that germination of *C. difficile* in mice occurred to the same degree in the small intestinal and caecal contents and demonstrated that germination could not occur in the stomach even when the pH of the stomach was modified to 5.4 with a Proton Pump Inhibitor (PPI) (Nerandzic et al., 2009).

Since germination of *C. difficile* spore is necessary for the pathogenesis of the disease, it is important that compounds that cause germination of the organism are researched and the mechanism surrounding the germination elucidated. The identification of these compounds and the mechanism of germination will help in the potential design of therapeutics which can inhibit or manipulate germination hence prevent CDAD. For instance, it is known that spores of *C. difficile* are resistant to all sorts of chemical and physical agents (Setlow, 2007, Gerding et al., 2008a) and this has been the mechanism of its persistence on inanimate surface which leads to infection when accidentally ingested (Gerding et al., 2008a). Identification of compounds that can cause germination could have positive implications in routine cleaning or decontamination and management of outbreak of the infection.

Subsequent experiments were focussed on the influence of probiotics on the germination and consequent growth of *C. difficile* in the microcalorimeter. Experiments as indicated in the methods were conducted in BHIct since this medium supported vegetative growth of *C. difficile*. Enumerations of CFU/mL were done by serial dilution in PBS

supplemented with 0.1% w/v sodium taurocholate and plating on BHIct. Viable counts were not affected by the presence or absence of yeast extract.

4.4.2 Pure and mixed culture of *C. difficile* and probiotic strains in the isothermal microcalorimeter

The power-time curves of pure cultures of *C. difficile*, LA-5® and BB-12® in BHIct are shown in Figures 4.4, 4.5 and 4.6 respectively. The power time curves were characteristic for the different species. Both LA-5® and BB-12® showed exponential growth in this medium without significant time lag unlike *C. difficile* which metabolised energetically prior ca. 7 h of dormancy in the microcalorimeter. The long lag phase that occurred for *C. difficile* could be associated with its slow rate of growth (Carroll, 2013). It could as well be associated with the period required for the spores to adapt, sense the suitable environment for germination and subsequently germinated and commenced vegetative growth (Stringer et al., 2011). The former events may have been associated with metabolic activity (Stringer et al., 2011) but possibly with related heat outputs, which was below the detection limit of the microcalorimeter.
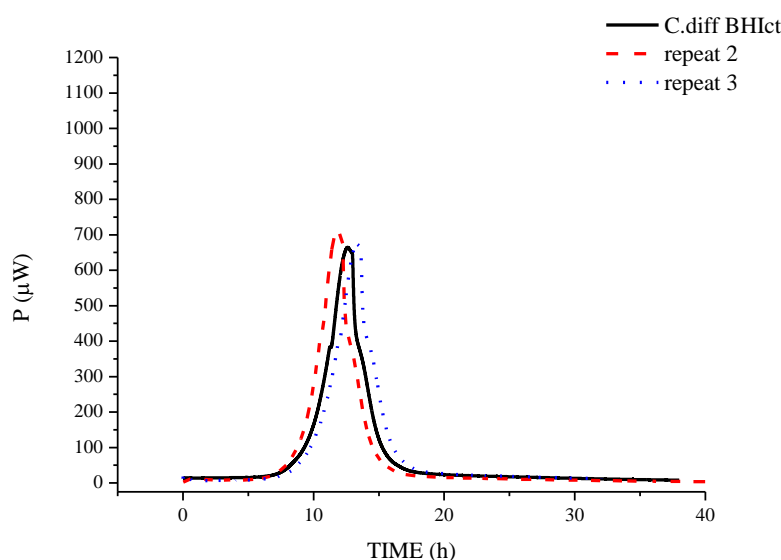
Figure 4.4. Power-time curves of 3 repeats of *C. difficile* in BHIct
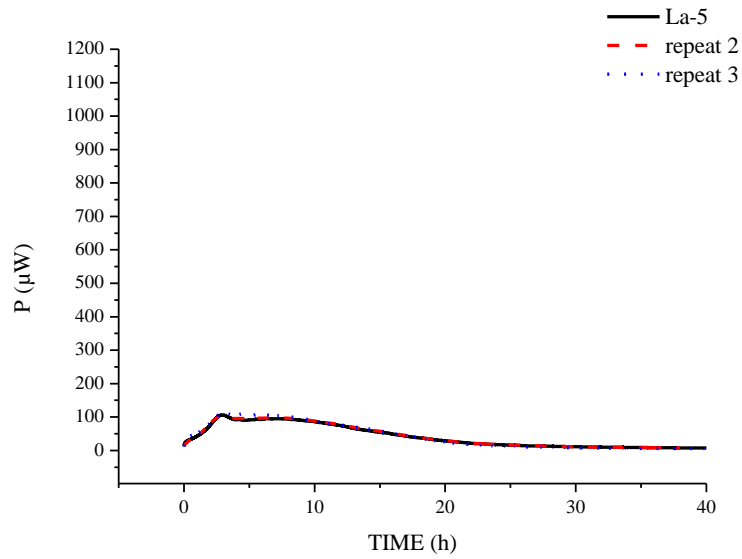
Figure 4.5. Power-time curves of 3 repeats of *L. acidophilus,* LA-5® in BHIct
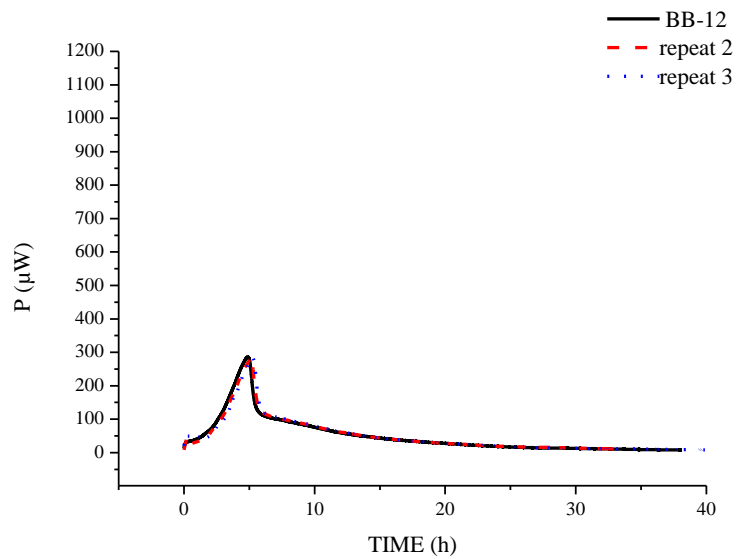
Figure 4.6. Power-time curves of 3 repeats of *B. lactis,* BB-12® in BHIct

The total heat outputs associated with the metabolism of *C. difficile* was 9.93 ± 0.38 J and was significantly higher ($P< 0.05$) than that of LA-5® and BB-12® in BHIct which were 6.05 ± 0.40 and 6.93 ± 0.36 J, respectively. The higher heat output associated with the metabolism of *C. difficile* relative to the probiotic species could be due to the cumulative effect of germination and metabolism leading to vegetative growth, which

together might have been a higher energy yielding process relative to sole vegetative growth. A previous study by Kalakoutskiai and Pozharitska, (1968) has shown that the emergence of germ tubes of spores of *Actinomyces streptomycini* in the microcalorimeter was associated with great heat output which was noted to be in the upper limit of heat generated by growing cultures of *E. coli*. The authors also demonstrated that the swelling prior to exponential growth was not associated with significant heat production (Kalakoutskiai and Pozharitska, 1968). Also, it is likely the medium might have favoured the growth *C. difficile* relative to the probiotic species, which could have been inhibited by the bile salt content of the medium (Begley et al., 2005). Exclusion of bile salt from the medium however did not change the power-time curves of the probiotic species.

A post TAM analysis of the culture showed a significantly less turbid culture of LA-5$^®$ relative to the other species and previous cultures of it in CMMg. It was also noted that the probiotic species did not produce as many acidic metabolites in BHIct as they had previously done in CMMg (Chapter 3). Nonetheless, they significantly reduced (P<0.05) the pH of the medium to 5.13 ± 0.04 and 4.92 ± 0.01 respectively for LA-5$^®$ and BB-12$^®$ whilst *C. difficile* also reduced the pH of the medium to 5.88 ± 0.05 (P<0.05) from an initial value of 6.77 ± 0.01.

The power-time curves of mixed cultures of *C. difficile* with LA-5$^®$ and *C. difficile* with BB-12$^®$ are shown in Figures 4.7 and 4.8. The power-time curves of the mixed cultures lacked the characteristic curve of *C. difficile* and showed only the growths of the probiotics.

In the previous section, it was demonstrated that *C. difficile* could germinate and grow in the environment created, BHIct, pH 6.8. This medium could simulate the environment of the small intestine (due to the bile salt) (Kalantzi et al., 2006) or the colon (Paredes-Sabja et al., 2008) due to the potassium and phosphate content of BHI (Paredes-Sabja et al., 2008). Also, the culture of *C. difficile* that was inoculated into BHIct consisted of mainly spores of *C. difficile,* which could simulate the oral transmission of the infection (Gerding et al., 2008a, Burns et al., 2010, Burke and Lamont, 2014). The results suggest that the consequent vegetative growth and production of toxins associated after germination may be prevented in the intestine by the probiotics since *C. difficile* germination may not have occurred in the presence of the probiotics.
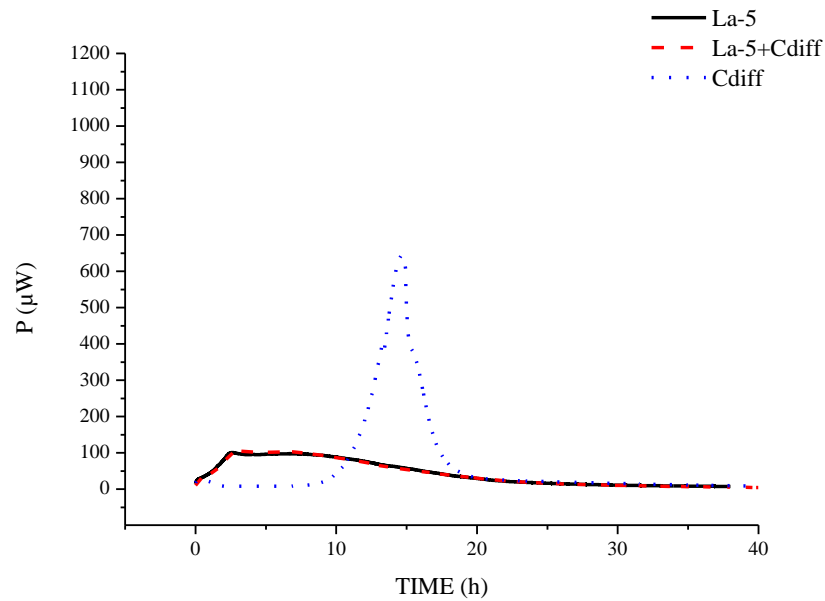
Figure 4.7. Comparison of the power-time curves of pure culture of *L. acidophilus,* LA-5® and *C. difficile,* Cdiff and their mixed culture (LA-5+Cdiff) in BHIct
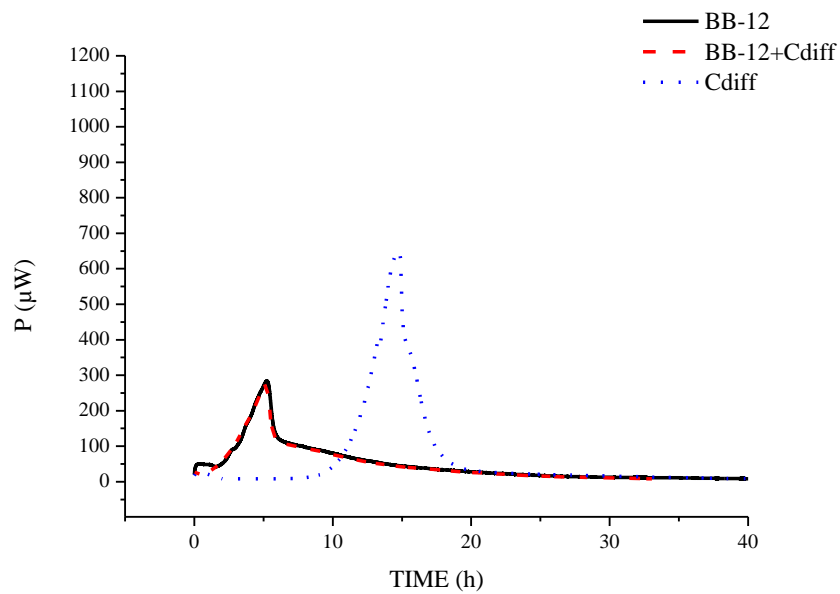
Figure 4.8. Comparison of the power-time curves of pure culture of *B. lactis,* BB-12® and *C. difficile,* Cdiff and their mixed culture (BB-12+Cdiff) in BHIct

As indicated earlier, spores of the organism are pivotal for transmission of the infection; but they need to return to the vegetative state through germination for consequent toxin

production and clinical manifestations of the infection. In the previous section, bile salt was demonstrated to play an important role in the germination of the organism but in the presence of the probiotic species, germination may not have progressed. These probiotic species could potentially play a major role in the prevention or treatment of the infection in susceptible individuals since no toxins would be consequently produced to cause CDAD.

The mechanism of inhibition of *C. difficile* by the probiotic species was likely due to the probiotic species competitively utilizing the nutrient sources in the media before *C. difficile* could be adequately capable of metabolic activity. Furthermore, the probiotics produced acids, or likely other non-acidic metabolites during their growth, which might have caused *C. difficile* to remain in spore form. The pH of the medium after growth of the mixed cultures of LA-5® and *C. difficile* and BB-12® and *C. difficile* were significantly reduced ($P<0.05$) to 5.24 ± 0.19 and 4.92 ± 0.03 respectively showing that they indeed still produced acidic metabolites in mixed culture which could have inhibited the growth of *C. difficile.*

In the previous experiments of mixed culture of the probiotic species with *P. aeruginosa*, *S. aureus* and *E. coli* in Chapter 3, it was observed that growth of the probiotics occurred after or concurrently with the growth of the other faster growing species. To determine whether *C. difficile* could germinate and grow in the presence of other species, *C. difficile* was also co-cultured with *P. aeruginosa.* The power-time curve showed the presence of both species (Figure 4.9), which implied that the inhibition of *C. difficile* by the probiotics might have been specific.

Enumerations of vegetative cells of *C. difficile* post TAM experiments on selective medium, *Clostridium difficile* selective agar supplemented with *Clostridium difficile* selective supplement (cycloserine 250 mg/L, cefoxitin 8 mg/L) and 0.7% v/v defibrinated horse blood "CDSAsb" gave no recoveries of *C. difficile* on plates for the mixed cultures with probiotics (Table 4.2).
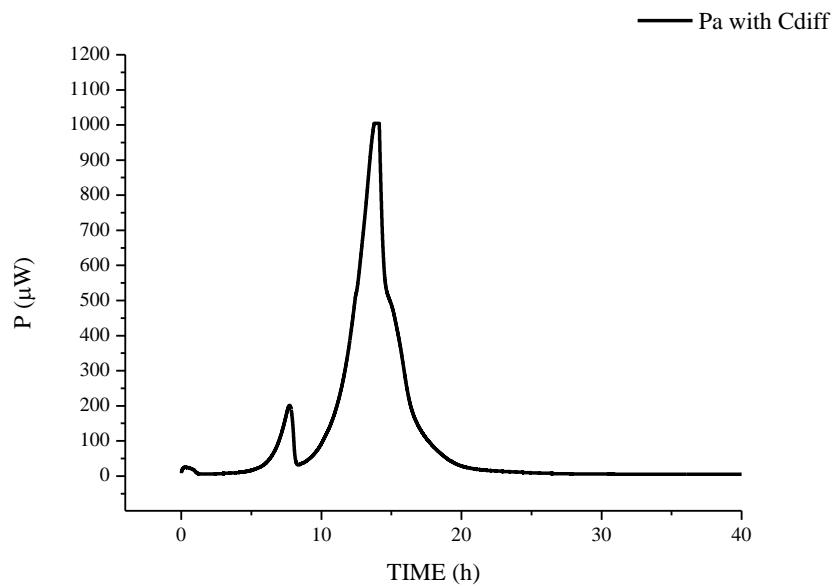
Figure 4.9. Power-time curve of mixed culture of *P. aeruginosa* (first peak) and *C. difficile* in BHIct

Table 4.2. A table comparing the mean heat output and post TAM profile of the pure culture and mixed culture of probiotics and *C. difficile*. n=3 for numerical values

Culture in BHIct	Heat output (J)	pH post TAM	Cell count post TAM (log CFU/mL)	
C. difficile	9.93 ± 0.38	5.88 ± 0.05	6.00 ± 0.60	
B. lactis BB-12®	6.93 ± 0.36	4.92 ± 0.01	6.47 ± 0.07	
L. acidophilus LA-5®	6.05 ± 0.40	5.13 ± 0.04	5.19 ± 0.19	
C. difficile and *B. lactis* BB-12®	6.63 ± 0.35	4.92 ± 0.03	Cdiff	BB-12®
			0	6.50 ± 0.03
C. difficile and *L. acidophilus* LA-5®	5.79 ± 0.52	5.24 ± 0.19	Cdiff	LA-5®
			0	5.04 ± 0.09

4.4.3 Co-incubation of probiotic supernatant with *C. difficile*

It had already been established in Chapter 3 that the probiotics produced acidic extracellular products, which were inhibitory towards *P. aeruginosa*, *S. aureus* and *E. coli*. To find out if inhibition of germination and growth of *C. difficile* was solely due to the acidic metabolites or was also due to the presence of other non-acidic or secondary metabolites produced by the probiotic species in BHIct, *C. difficile* was co-incubated with 1 in 2 dilutions (in double strength BHIct "dsBHIct") of unmodified and pH modified CFS of the probiotics. The results are shown in Figures 4.10 and 4.11 respectively for CFS of LA-5® and BB-12®.
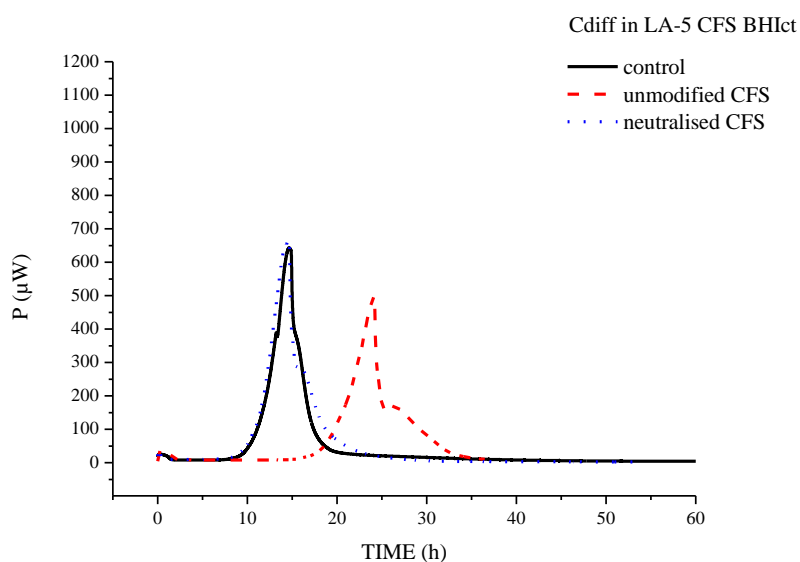
Figure 4.10. Power-time curves of *C. difficile* in LA-5® CFS produced in BHIct. Growth in 1 in 2 dilutions of the unmodified CFS (red) or neutralised CFS (blue) in dsBHIct is compared with a control of the organism (black)

156
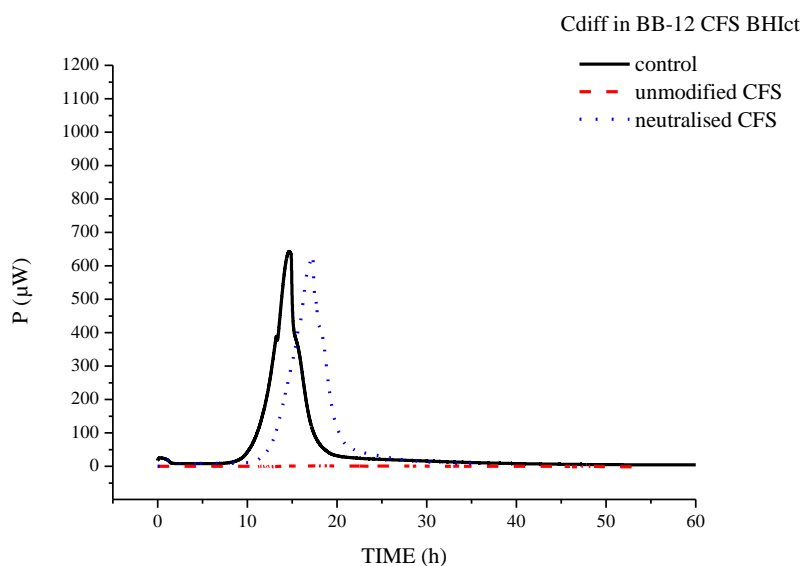
Figure 4.11. Power-time curves of *C. difficile* in BB-12® CFS produced in BHIct. Growth in 1 in 2 dilutions of the unmodified CFS (red) or neutralised CFS (blue) in dsBHIct is compared with a control of the organism (black)

The results indicate inhibition of *C. difficile* by the CFS of the probiotics. For the CFS produced by LA-5®, a 1 in 2 dilution of the CFS in dsBHIct did not completely inhibit *C. difficile* in the microcalorimeter but delayed growth. The growth of *C. difficile* in the CFS of LA-5® may be due in part to the role played by the live cells in inhibition which were not present in the CFS. Also, as noted earlier, the pH of BHIct after growth of LA-5® was 5.13 ± 0.04. After a 1 in 2 dilution with dsBHIct, the final pH of the medium pre-inoculation was 6.12 ± 0.02. BB-12® on the other hand had a pH of 4.92 ± 0.01 after incubation. After dilution with dsBHIct, the final pH of the CFS of BB-12® with dsBHIct was 5.97 ± 0.01 pre-inoculation. It was therefore most likely that inhibition was due to acidic metabolites produced, due to the pH-dependency of inhibition. Similarly, the pH of a 1 in 2 dilution of neutralised CFS of LA-5® and BB-12® in dsBHIct were 6.73 ± 0.03 and 6.72 ± 0.01 respectively. *C. difficile* growth was augmented (lag period was decreased) in the neutralised CFS of LA-5®. The CFS of BB-12® which had previously showed complete inhibition of *C. difficile* growth in the microcalorimeter for the duration tested also showed growth of *C. difficile* when neutralised.

It should be highlighted that, previous studies have also indicated the pH-dependency of inhibition of *C. difficile* by some probiotics. For instance, inhibition of the *C. difficile* strain used by Schoster et al., (2013) was reported to be only observed when the pH of

157

the supernatants obtained from the probiotic species was not neutralised. In a study by Trejo et al., (2006) the inhibition of the bifidobacteria strains against *C. difficile* were determined to be dependent on their production of lactic acid and acetic acid with the exception of one strain. A study by Naaber et al., (2006) also demonstrated the correlation of H_2O_2 and lactic acid production with inhibition of *C. difficile* by intestinal lactobacilli while Tejero-Sarinena et al., (2012) confirmed these substances to be lactic acid and acetic acid. As discussed in Chapter 1 and 3, lactic acid bacteria are capable of releasing specific antimicrobial metabolites (Anand et al., 1984, Anand et al., 1985, Kang et al., 1989) and low molecular weight compounds; organic acids (acetic, formic and lactic acids) and hydrogen peroxides (Piard and Desmazeaud, 1991, Piard and Desmazeaud, 1992) which directly target pathogenic bacteria including themselves or purportedly decreases the luminal pH of the intestine to prevent overgrowth of potential pathogens (Ng et al., 2009, Gerritsen et al., 2011). Some studies in humans have shown likely differential changes in the luminal pH of patients on probiotics relative to a control group based on breath test and changes in faecal SCFA (Butler et al., 2001). Some studies on animals have also directly assessed changes in luminal pH in the digestive tract. For instance, Fonseca et al., (2010) showed that the pH of the crop of chickens whose feed were supplemented with probiotics were significantly lower in comparison to a control group. Despite this, in ewes (Kumagai et al., 2004) no localised luminal pH reduction in the rumen was observed when the feed was supplemented with probiotics even though differences were observed in the microbial population between control groups and treatment groups of both studies (Fonseca et al., 2010, Kumagai et al., 2004). When considering the results from other related studies, and these animal studies, it would seem that localised reductions of the luminal pH of the intestine may or may not occur after ingestion of probiotics and there needs to be further studies to determine if indeed it could occur with these probiotics *in vivo*.

In the present study, it was anticipated that the probiotics could modify the primary bile salt, sodium taurocholate that was supplemented into the medium to secondary bile salts, such as deoxycholate through dehydroxylation, which would have inhibited *C. difficile*. Intestinal bacteria have been demonstrated by Giel et al., (2010), to be capable of such modification with inhibitory activity against *C. difficile*. In their experiment, Giel et al., incubated washed caecal contents of mice (either treated with antibiotics or not) with 0.1% w/v taucholate and observed that the bile salt in the caecal contents of untreated mice had been modified to the secondary form whilst the antibiotic treated mice had not

been converted to the secondary form (Giel et al., 2010). The authors were also able to demonstrate a decrease in colony formation of *C. difficile* spores using the secondary bile salt. It was therefore expected that neutralised CFS of the probiotics should still inhibit the germination and subsequent growth of *C. difficile* due to the presence of bile salt in the medium. The probiotic species were probably not capable of metabolizing the primary bile salt to secondary bile salts or otherwise, *C. difficile* was not inhibited by the secondary bile salts if it had been produced.

During the study, it was also noted that growth yields in BHIct for the probiotic species, especially LA-5® were poor relative to *C. difficile* and previous cultures of it in MRSc or CMMg. Both probiotic species were therefore also grown in MRSc and the CFS obtained. It is worth noting that MRS was developed by de Man, Rogosa and Sharpe (De Man et al., 1960) to provide a medium which would generally support good growth of lactobacilli, including related strains which showed poor growth in existing media. It is indeed known to produce more profuse growth of all strains of lactobacilli and related strains, especially difficult and slow growing ones which could be overgrown in general purpose media (De Man et al., 1960). It was believed that the probiotic species evaluated could potentially produce important metabolites in MRSc because they had previously shown better growth yields/more profuse growth in MRSc compared to BHIct.

CFSs were therefore obtained after 48 h of anaerobic incubation in MRSc. The final pHs of the dilutions of the CFS with dsBHIct are given in Table 4.3.

Table 4.3. Mean pH (n=3) of media and CFS - media mixtures used.

Uninoculated media	pH
BHIct	6.77 ± 0.01
dsBHIct	6.63 ± 0.02
dsBHIct + LA5® CFS (BHIct) unmodified 1in 2 dilution	6.12 ± 0.02
dsBHIct + LA5® CFS (BHIct) pH 7	6.73 ± 0.03
dsBHIct + BB-12® CFS (BHIct) unmodified 1 in 2 dilution	5.97 ± 0.01
dsBHIct + BB-12® CFS (BHIct) pH 7	6.72 ± 0.01
dsBHIct + MRSc	6.30 ± 0.04
dsBHIct + LA-5 CFS (MRSc) unmodified	4.73 ± 0.04
dsBHIct+ LA-5 CFS (MRSc) 1 in 4 dilution	5.60 ± 0.04
dsBHIct+ LA-5 CFS (MRSc) 1 in 6 dilution	6.00 ± 0.01
dsBHIct + LA-5 CFS (MRSc) pH 5	5.86 ± 0.06
dsBHIct + LA-5 CFS (MRSc) pH 6	6.45 ± 0.07
dsBHIct + LA-5 CFS (MRSc) pH 7	6.71 ± 0.01
dsBHIct + LA-5 CFS (MRSc) pH 8	6.90 ± 0.04
dsBHIct + BB-12 CFS (MRSc) unmodified	5.20 ± 0.03
dsBHIct + BB-12 CFS (MRSc) 1 in 4 dilution	5.93 ± 0.05
dsBHIct + BB-12 CFS (MRSc) 1 in 6 dilution	6.19 ± 0.03
dsBHIct + BB-12 CFS (MRSc) pH 5	5.84 ± 0.09
dsBHIct + BB-12 CFS (MRSc) pH 6	6.48 ± 0.02
dsBHIct + BB-12 CFS (MRSc) pH 7	6.76 ± 0.01
dsBHIct + BB-12 CFS (MRSc) pH 8	6.89 ± 0.03

The power-time curves of *C. difficile* inoculated into the MRSc produced CFS-dsBHIct mixtures are shown in Figures 4.12, 4.13, 4.14 and 4.15. The probiotic species indeed produced more acidic metabolites in MRSc. The pH of the CFSs produced were 4.12 ± 0.01 (P<0.05) and 4.62 ± 0.01 (P<0.05) respectively for LA-5® and BB-12®. For LA-5®

CFS, complete inhibition of *C. difficile* in the microcalorimeter was observed at 1 in 2 dilutions of the CFS. But partial inhibition was observed at 1 in 4 and 1 in 6 dilutions. Upon pH modification of the CFS of LA-5®, inhibitory activities were lost at pH 6, 7 and 8 (Figure 4.13) of the modified CFS.
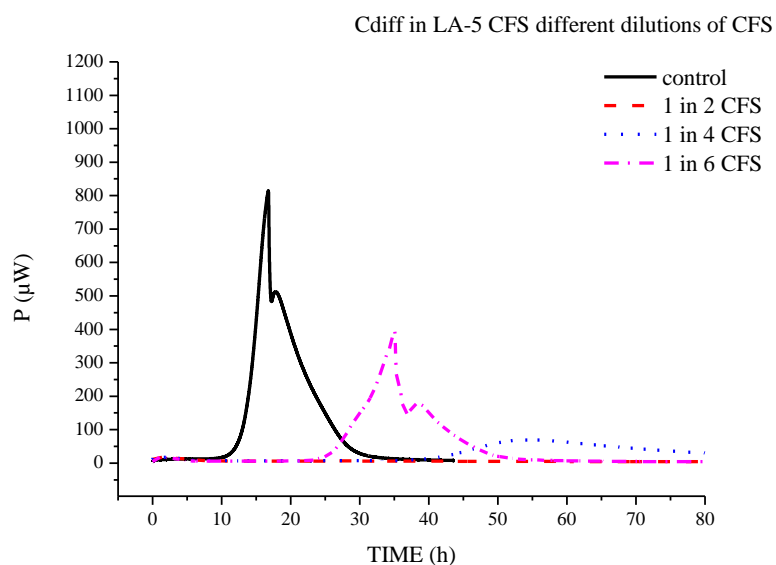
Figure 4.12. Power-time curves of *C. difficile* in LA-5® CFS: 1 in 2 dilution (red), 1 in 4 dilution (blue) and 1 in 6 dilution (magenta) compared to a control of the organism in dsBHIct-MRSc (black)
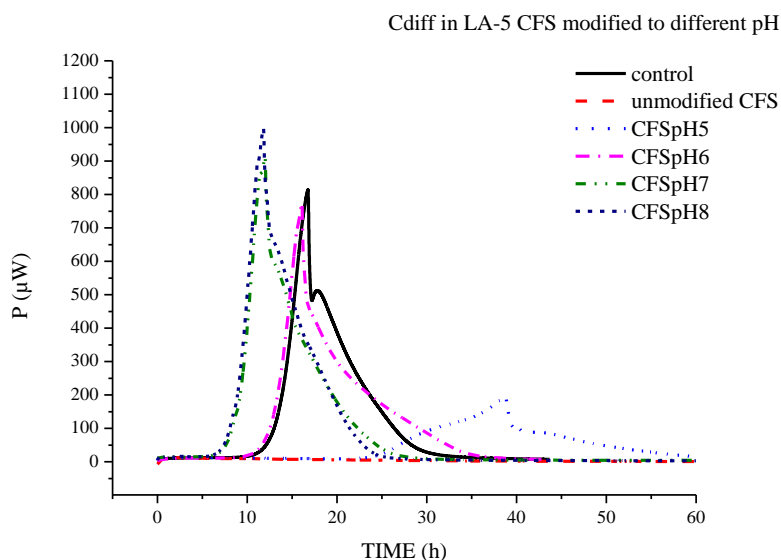
Figure 4.13. Power-time curves of *C. difficile* in LA-5® CFS unmodified (red), pH modified to 5 (light blue) 6 (magenta), 7 (green), 8 (dark blue) compared to a control of the organism in dsBHIct-MRSc (black)
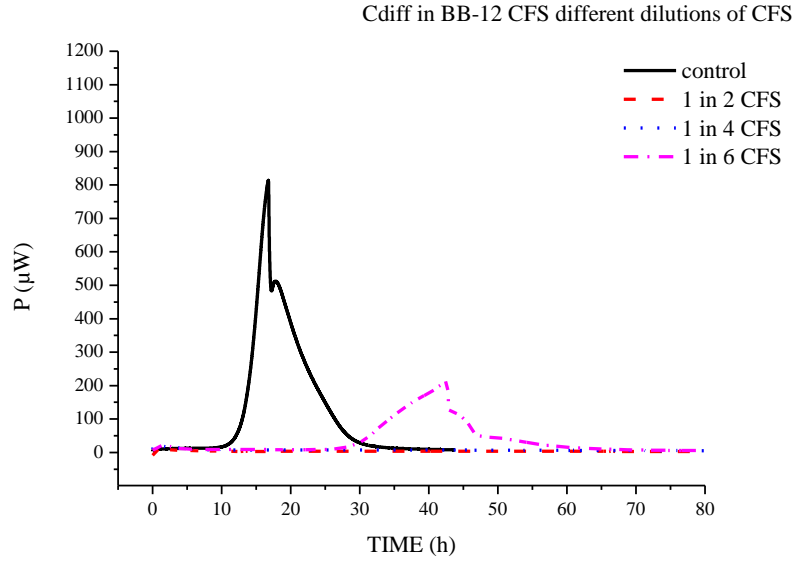
Figure 4.14. Power-time curves of *C. difficile* in BB-12® CFS:1 in 2 dilution (red), 1 in 4 dilution (blue) and 1 in 6 dilution (magenta) compared to a control of the organism in dsBHIct-MRSc (black)
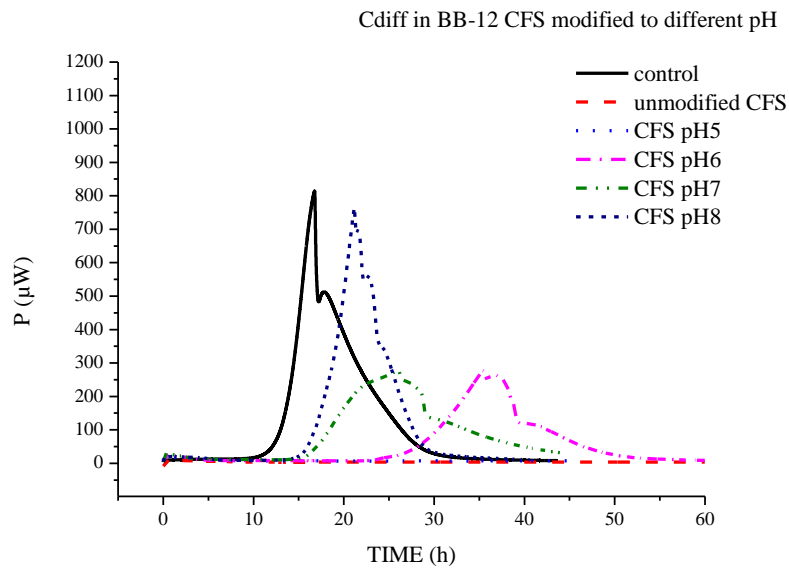
Figure 4.15. Power-time curves of *C. difficile* in BB-12® CFS unmodified (red), pH modified to 5 (blue) 6 (magenta), 7 (green), 8 (dark blue) compared to a control of the organism in dsBHIct and MRSc (black)

LA-5® CFS adjusted to pH 7 and 8 surprisingly enhanced the growth of *C. difficile* as the onset time of growth was decreased. This indicated that not only was inhibition dependent on pH but also germination and growth of *C. difficile* was pH-dependent and

162

also further that possibly there was an optimum pH required for germination and growth of *C. difficile*. Table 4.3 tabulates the final pH of the medium and mixtures of CFS with dsBHIct.

LA-5® CFS modified to pH 7 and 8 when mixed with equal volumes of dsBHIct had final pH of 6.71 ± 0.01 and 6.90 ± 0.04 respectively. The controls for the MRSc produced CFS were conducted in dsBHIct diluted with MRSc and had pH of 6.30 ± 0.04. It is likely the optimum pH for germination and growth of *C. difficile* is ca. pH 6.7 as pH above this value did not significantly increase growth. This concurs with previous findings by Wheeldon et al., (2008) who reported an optimum pH between 6.5 and 7.5 for germination of *C. difficile* with decreased rate and extent of germination occurring at pH 5.5 and 8.5 (Wheeldon et al., 2008). It further supports that germination of *C. difficile* could occur anywhere between the proximal small intestine and the descending colon but likely in the small intestine. The pH of the proximal small intestine is between 6.15-7.35. That of the distal small intestine is 6.80-7.88. The ascending colon has variable pH range between 5.26-6.72 and the descending colon 5.20-7.07 (Press et al., 1998, Cook et al., 2012).

An interesting observation was also made with the CFS of BB-12®. A 1 in 4 dilution of BB-12® CFS showed complete inhibition of *C. difficile* (Figure 4.15) in the microcalorimeter unlike the CFS of LA-5® even though it had a significantly higher pH of 5.93 ± 0.05 compared to 5.60 ± 0.04 (P<0.05) of the 1 in 4 dilution of LA-5® CFS. Also, CFS of BB-12® modified to pH 5 showed no growth of *C. difficile* for the time period studied unlike that for LA-5®. At neutral pH of the CFSs, the anticlostridial effect of BB-12® CFS was also not completely lost. This confirmed with results obtained in Chapter 3 and corroborates with other findings (Messens and De, 2002, Ruiz et al., 2009, Sourabh et al., 2011, Cruz-Guerrero et al., 2014) that there could be non-acidic antimicrobial metabolites (bacteriocins or bacteriocin-like compounds) (Anand et al., 1984, Anand et al., 1985, Kang et al., 1989, Cheikhyoussef et al., 2008) produced by the probiotic species.

One major concern of the experiments was the dilution of the CFS that occurred when modifying the pH and when obtaining the CFS-media mixture for inoculation of *C. difficile* (which also occurred in Chapter 3). Since nutrient source was necessary for the detection of the growth of the species in the microcalorimeter, the CFS had to be always

diluted with growth media because of the exhausted nutrient sources in the CFS. To compensate for the effect of dilution, the neutralised CFSs were concentrated by freeze-drying and evaluated as described by Lee et al., (2003). The freeze-drying was done with a Modulyo D-230 (Thermo Scientific, UK). At the end of the freeze-drying, the dried powder was diluted with 2.5, 5, 10 or 20-fold less water and filter sterilized. Figures 4.16 and 4.17 compare the power-time curves of *C. difficile* inoculated into dsBHIct with neutralised CFS unconcentrated, 2.5-fold, 5-fold, 10-fold and 20-fold concentrated of LA-5® and BB-12® CFS, respectively. The power-time curves demonstrate the presence of other non-acidic antimicrobial metabolites in LA-5® and BB-12® CFS whose activity had been obscured because of dilution caused during pH modification and during the growth experiments. The non-acidic antimicrobial metabolites were likely produced in larger quantities in BB-12® than LA-5® demonstrated by the higher concentration of the neutralised CFS of LA-5® needed for complete inhibition in the microcalorimeter. Also, dilutions and pH modifications of the CFS of BB-12® consistently inhibited *C. difficile*.
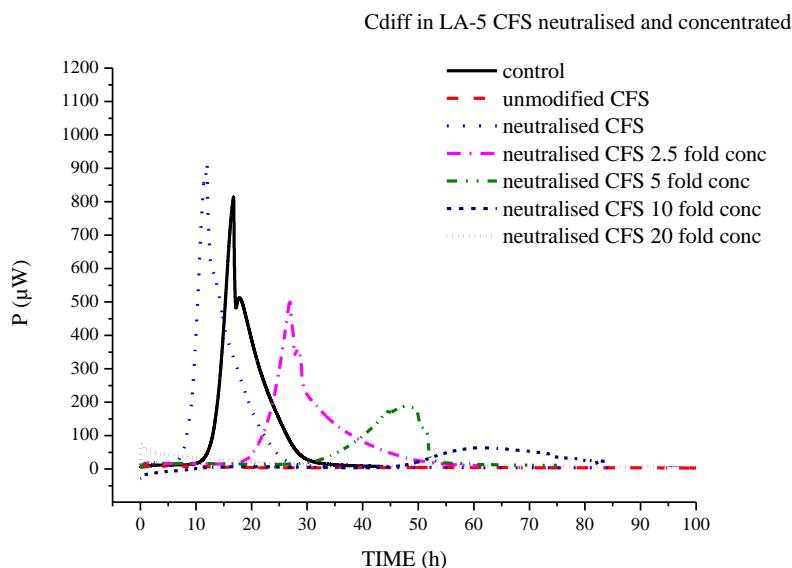
Figure 4.16. Power-time curves of *C. difficile* in LA-5® CFS unmodified (red), neutralised (blue), neutralised but concentrated by 2.5 folds (magenta), neutralised and concentrated by 5 folds (green), neutralised and concentrated by 10 fold (violet) and neutralized and concentrated by 20 fold (light blue) compared to a control of the organism in dsBHIct and MRSc (black)
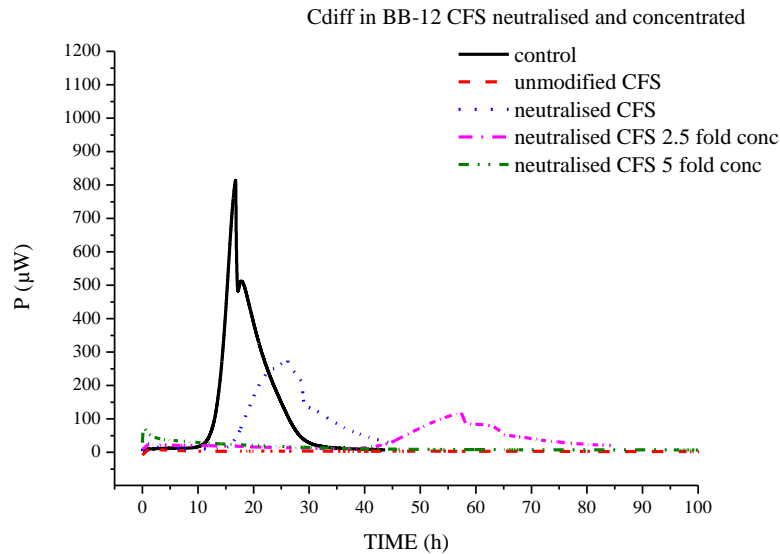
Figure 4.17. Power-time curves of *C. difficile* in BB-12® CFS unmodified (red), neutralised (blue), neutralised but concentrated by 2.5 folds (magenta), neutralised and concentrated by 5 folds (green) compared to a control of the organism in dsBHIct and MRSc (black)

As discussed previously, some probiotics species have been shown to produce specific proteinaceous antimicrobial compounds (bacteriocins and bacteriocin-like compounds), which can inhibit undesirable pathogens (Anand et al., 1984, Anand et al., 1985, Kang et al., 1989, Cheikhyoussef et al., 2008). It has been demonstrated in these experiments that the commercial strains that were tested had such potential and that microcalorimetry could detect it. This supports previous findings by Lee et al., (2003) and Kos et al., (2008) who also showed the likely presence of these compounds in some screened lactic acid bacteria and selected probiotics using optical density measurements and agar diffusion assay respectively. In Lee et al's study, they also noticed about 41% increased inhibition of growth of *C. difficile* by a 20-fold concentrated CFS of one of the selected strains they evaluated (Lee et al., 2003).

It must be mentioned that these compounds have a history dating back to the early 1920s (Nes, 2011). Anand et al., (1984, 1985), Kang et al., (1989) and Barefoot and Klaenhammer (1983) isolated them from *B. bifidum* (bifidin) and *B. longum* (biflong) and *L. acidophilus* (lactacin B). Indeed Bifilact BB-12 and lactacin B have been respectively reported to be produced by *B. lactis* BB-12® and *L. acidophilus* LA-5® by Abd El-Salam et al., (2004) and Tabasco et al., (2009). Lactacin B produced from *L. acidophilus* LA-5® was found to display inhibitory activity against taxonomically related

bacteria with sensitive strains limited to members of the genus *Lactobacillus* (Tabasco et al., 2009). Bifilact BB-12 was however shown to be inhibitory towards *S. aureus, S. typhimurium, Bacillus cereus* and *E. coli* (Abd El-Salam et al., 2004). The present study suggests that these compounds inhibit *C. difficile* and could have a prospective role in the management of *C. difficile* infection. However the specificity of their action needs further studies. Generally the mechanism of action of these compounds has been proposed to include direct killing of pathogens, allowance of probiotics to compete with the resident microbiota and acting as a signalling factor to recruit other bacteria in the gut or immune system to fight and eliminate pathogenic bacteria (Rea et al., 2013). Thuricin CD isolated from a strain of *Bacillus thuringiensis* was shown by Rea et al., (2010) to be capable of specifically targeting *C. difficile* and not significantly upsetting the wider microbiota of the gut unlike vancomycin and metronidazole which were shown to lead to abundance of Enterobacteriaceae at the expense of other beneficial microbiota (Rea et al., 2010, Rea et al., 2011). However some have been shown to be non-specific. For instance, nisin, produced by *Lactococcus lactis* although showed strongest bactericidal activity against *C. difficile* compared to metronidazole or vancomycin, was also shown to have broad-spectrum inhibitory acitivity against some Gram-positive bacteria (Bartoloni et al., 2004, Field et al., 2010).

4.4.4 Agar well diffusion assay

Microcalorimetric experiments were correlated with conventional methods. The diameters of growth inhibition zones of *C. difficile* (with the CFSs of the probiotic strains produced in MRSc) are shown in Table 4.4. By this test, it was established that the CFSs were inhibitory towards *C. difficile* but inhibitory activities of the CFSs were lost upon neutralisation. CFS obtained from BB-12® demonstrated superior inhibition (P<0.05) and this could be as the result of the greater quantities of the other non-acidic inhibitory metabolites produced by BB-12® relative to LA-5® which was demonstrated in the previous section.

Table 4.4. Zones of inhibition of unmodified and modified cell free supernatant (CFS) of LA-5[®] and BB-12[®] against *C. difficile*. The values are mean ± SD. n=4 for zone of inhibition values

Cell Free Supernatants (CFS)	pH	Zone of inhibition of *C. difficile* (mm)
L. acidophilus CFS unmodified	4.12 ± 0.01	7 ± 0.0
L. acidophilus CFS neutralised	7.0	0.0 ± 0.0
B. lactis CFS unmodified	4.62 ± 0.01	7.5 ± 0.72
B. lactis CFS neutralised	7.0	0.0 ± 0.0

It should be pointed out that although the results from the agar well diffusion assay correlates well with the microcalorimetric data and previous findings (Naaber et al., 2004, Trejo et al., 2006, Schoster et al., 2013), if this was the only assay employed in the study, the other findings may have been missed. To illuminate this point, the agar well diffusion assay suggests that inhibitory activities were totally lost for both species when the CFS was neutralised whilst the microcalorimetric data indicates that this is not the case. A study by Schoster et al., (2013), which also determined inhibitory activity by both agar well diffusion and broth culture assays showed that while the CFS of some strains showed no inhibitory activity by agar well diffusion assay, the CFS of those same strains demonstrated inhibitory activity against *C. difficile* or *Clostridium perfringens* in broth. As discussed previously, this may be due to the capacities of agents within CFS to diffuse through agar.

4.4.5 Screening of commercially available probiotic products for anticlostridial activity

Commercial probiotic products: Actimel[®], Align[®], Biobalance-support, Bio-kult[®], Probio 7[®], Symprove[TM], VSL#3[®] and Yakult[®], were screened to determine if they could also inhibit germination and growth of *C. difficile* in the microcalorimeter. The results are presented in Figures 4.18a to 4.18h. Each commercial product showed individual characteristics that could be used for identification; each showed a different time of

onset of growth, which could be related to their differences in viable cell concentration and strain(s) characteristics. With the exception of Probio 7® (Figure 4.18e), all commercial probiotics tested showed power-time curves that suggested inhibition of germination and growth of *C. difficile*. Correspondingly, with the exception of Probio 7®, all products did not give colonies on "CDSAsb". The phase contrast image of one of the mixed cultures of the commercial products (Symprove™) with *C. difficile* after microcalorimeteric experiment is shown in Figure 4.19. The image shows some spores of *C. difficile* supporting that spores of *C. difficile* could not complete germination in the presence of the probiotic product.
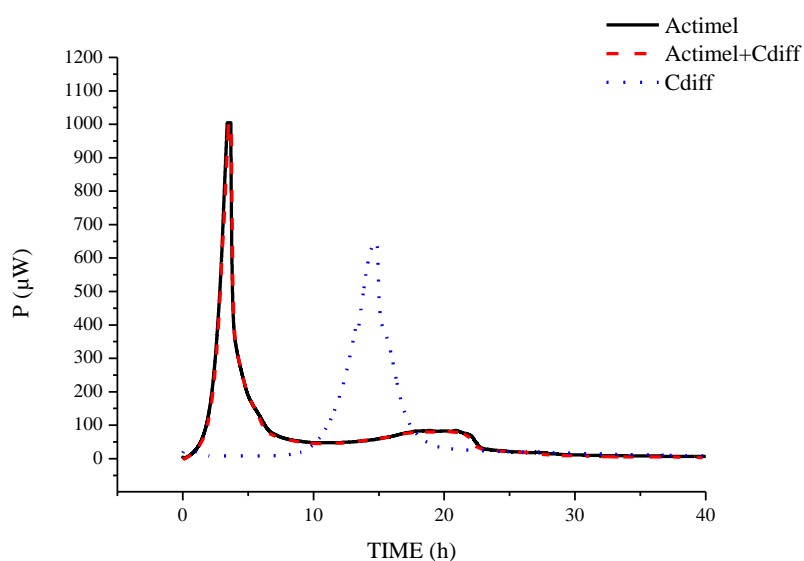
Figure 4.18a. Power-time curves of pure Actimel® (black) and *C. difficile* (Cdiff) and their mixed culture (Actimel+Cdiff, red) in BHIct
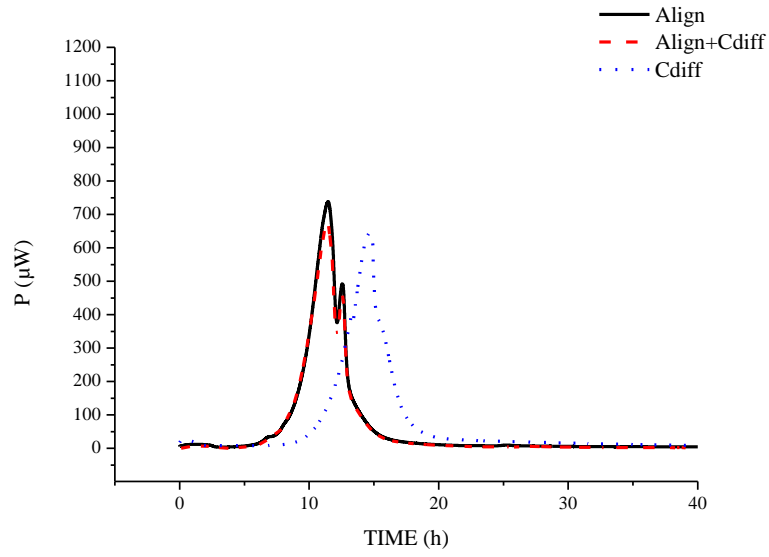
Figure 4.18b. Power-time curves of pure Align® (black) and *C. difficile* (blue) and their mixed culture (Align+Cdiff, red) in BHIct
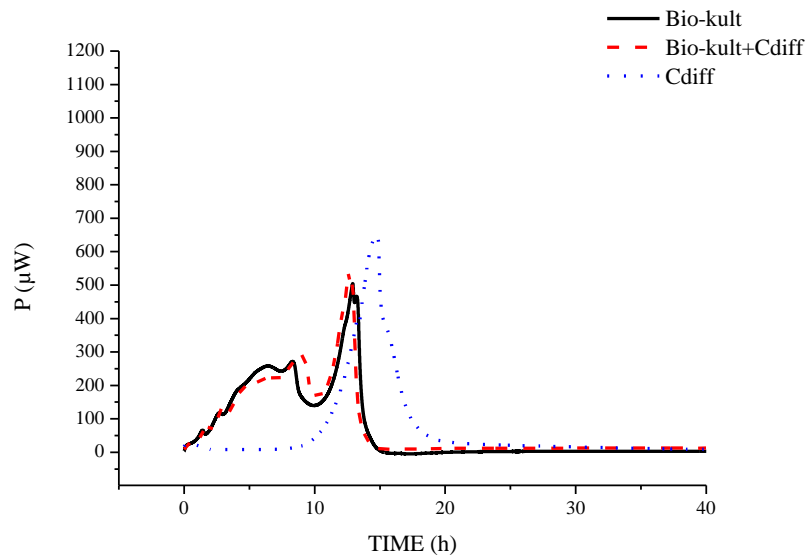
Figure 4.18c. Power-time curves of pure Bio-kult® (black) and *C. difficile* (blue) and their mixed culture (Bio-kult+Cdiff, red) in BHIct
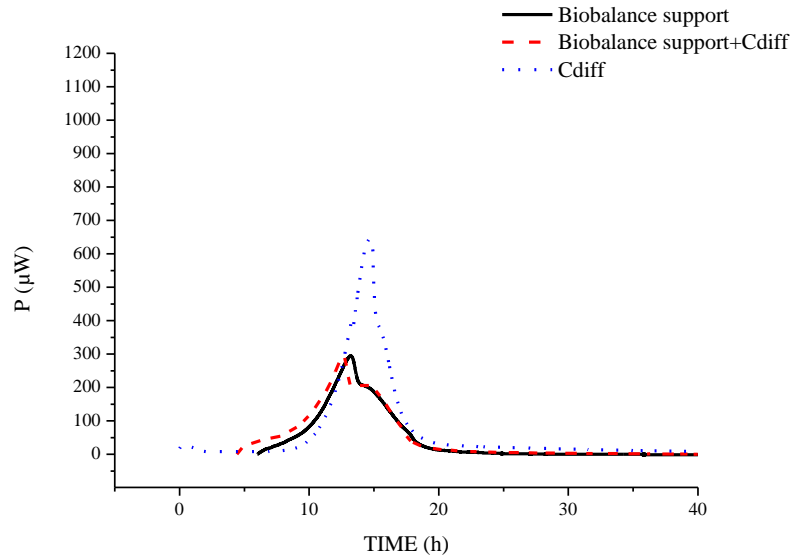
Figure 4.18d. Power-time curves of pure Biobalance support (black) and *C. difficile* (blue) and their mixed culture (Biobalance support+Cdiff, red) in BHIct
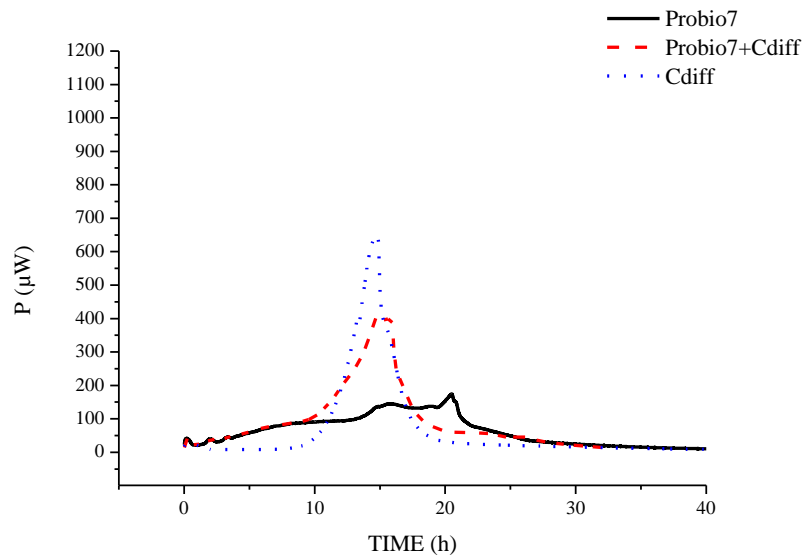
Figure 4.18e. Power-time curves of pure Probio 7[®] (black) and *C. difficile* (blue) and their mixed culture (Probio7+Cdiff, red) in BHIct
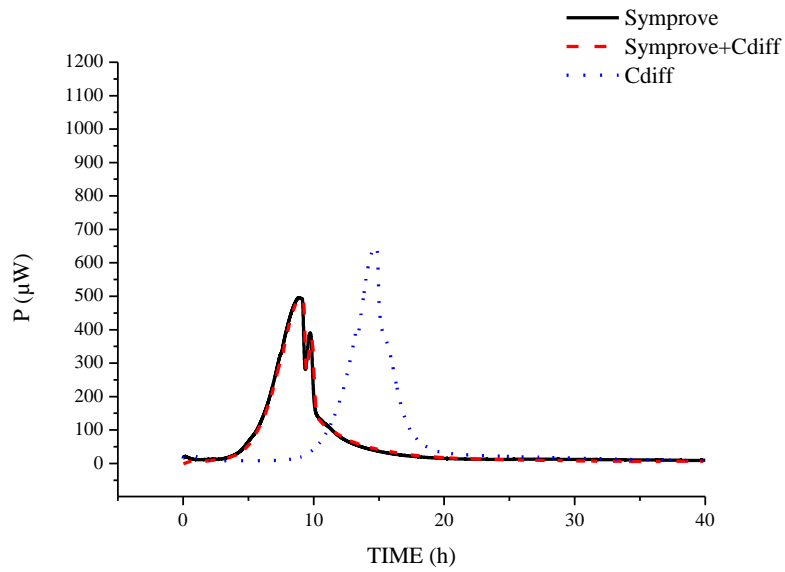
Figure 4.18f. Power-time curves of pure Symprove^{TM} (black) and *C. difficile* (blue) and their mixed culture (Symprove+Cdiff, red) in BHIct
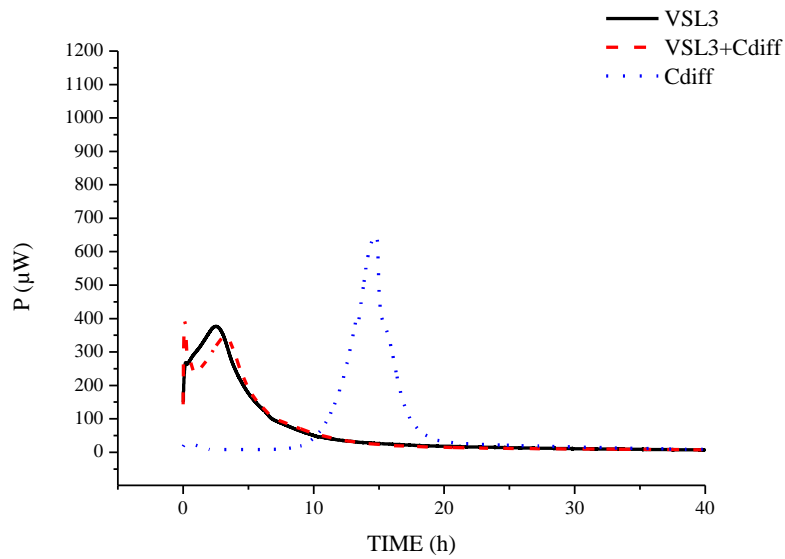
Figure 4.18g. Power-time curves of pure VSL#3® (black) and *C. difficile* (blue) and their mixed culture (VSL3+Cdiff, red) in BHIct
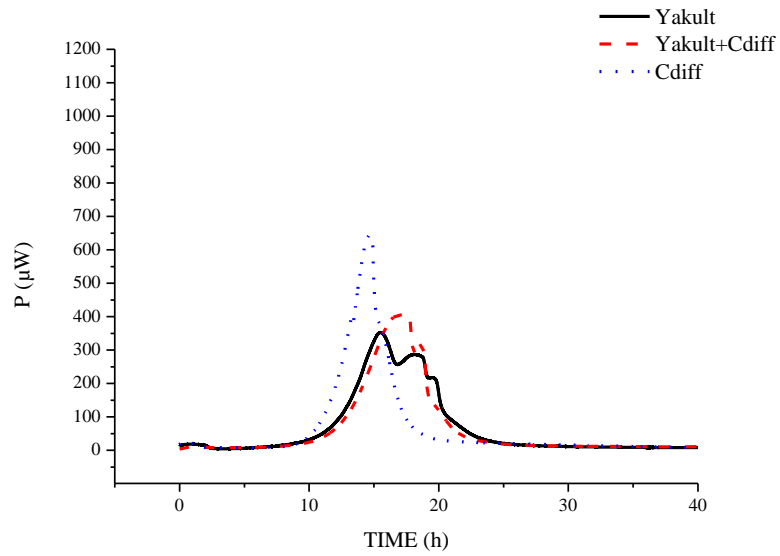
Figure 4.18h. Power-time curves of pure Yakult® (black) and *C. difficile* (blue) and their mixed culture (Yakult+Cdiff, red) in BHIct
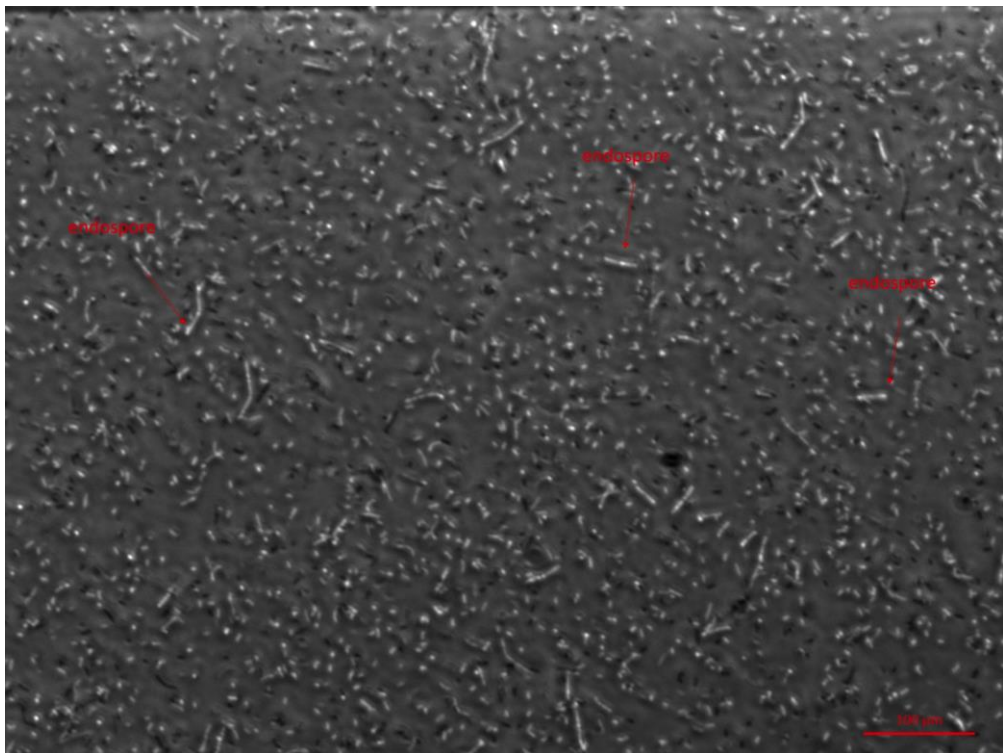
Figure 4.19. Phase contrast image of a mixed culture of *C. difficile* with Symprove[TM] post TAM experiment showing some endospores of *C. difficile*

The results from this work therefore suggest that these commercial products with the exception of Probio 7® could have a potential role in the management of *C. difficile* infection in susceptible individuals. As discussed in the introduction of this chapter, although antibiotics are the main treatment option of *C. difficile* infection (Gerding et al., 2008b), the infection is known to be often recurrent in patients due to decreased susceptibility of the pathogen to the antibiotics (Baines et al., 2008, Brazier et al., 2008, Huang et al., 2009) and reinfection; associated with the inability of the antibiotics to restore the compromised colonization resistance of the gut, a property directly (eg. through antimicrobial production) or immunologically related to its microbial composition and diversity (Britton and Young, 2012, Lawley and Walker, 2013, Walker and Lawley, 2013, Briton and Young, 2014). From the results of the present study it seems that these probiotics could potentially prevent germination and growth of *C. difficile in vivo.* If indeed they are able to, and also able to potentially restore the gut microbiota then, consequently the colonization resistance of the microbiota could be restored and this could prevent occurrence or recurrence of the infection when they are used. As already indicated in the introduction of this chapter, the UK Department of Health has not endorsed the use of probiotics for management of *C. difficile* infection even though faecal transplant (due to its treatment success) has been strongly recommended for recurring *C. difficile* infection (Public Health England, 2013, Debast et al., 2014). It would therefore be useful if further laboratory studies (also with different strains of *C. difficile*) or clinical test could be done to determine if indeed these probiotics could play evidential role in prophylaxis or treatment of the infection.

4.4.6 Microbial stability of multi-strain probiotics: pure and mixed cultures of the strains of Symprove™

The power-time curves of 10 batches of the commercial multi-strain probiotic product, Symprove™ in BHIc are shown in Figure 4.20. The power-time curves are reproducible but show different lag period among some batches suggesting reproducibility of content but differences in the concentration of viable bacteria in the batches. The power-time curves of 10^6 CFU/mL of the component strains of the product: *L. plantarum* SYM A, *L. rhamnosus* SYM B, *L. acidophilus* SYM C and *E. faecium* SYM D in the same medium are shown in Figures 4.21, 4.22, 4.23 and 4.24, respectively. The power-time curves were characteristic for each strain. *E. faecium* SYM D metabolised the medium most

energetically and *L. acidophilus* SYM C the least. It is likely that the medium was more favourable for the growth of *E. faecium* than the lactobacilli strains. The reason for this is not yet known.
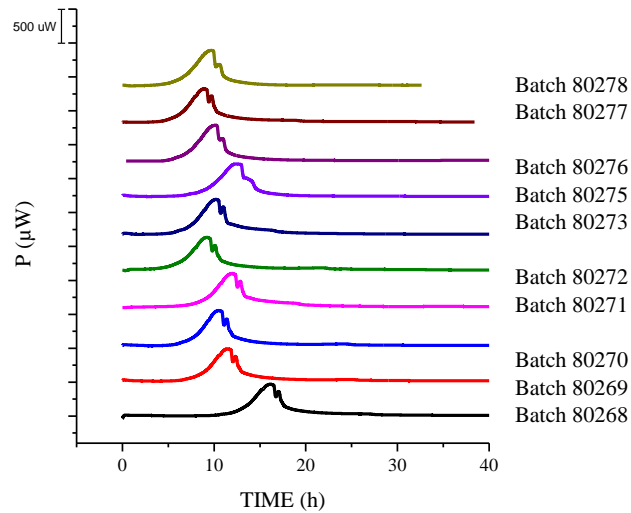
Figure 4.20. Power-time curves of 10 batches of Symprove® inoculated into BHIc at 1 in 100 dilution
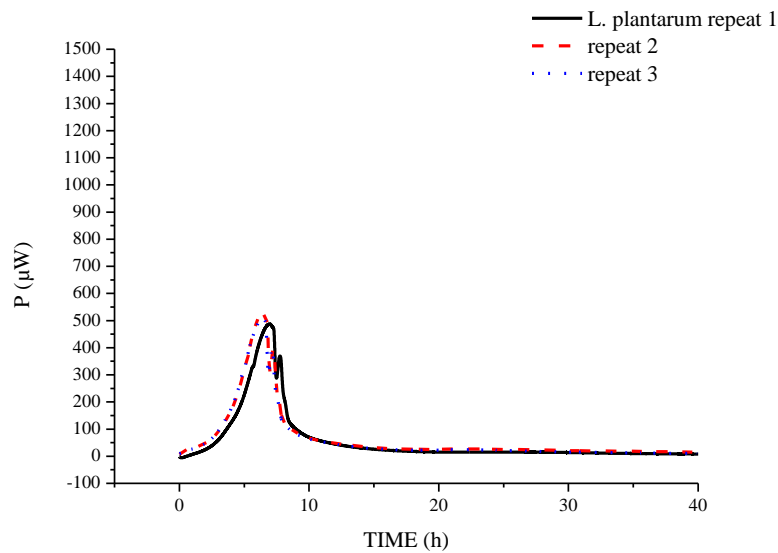
Figure 4.21. Power-time curves of 3 repeats of *L. plantarum* SYM A inoculated into BHIc at a cell density of 10^6 CFU/mL
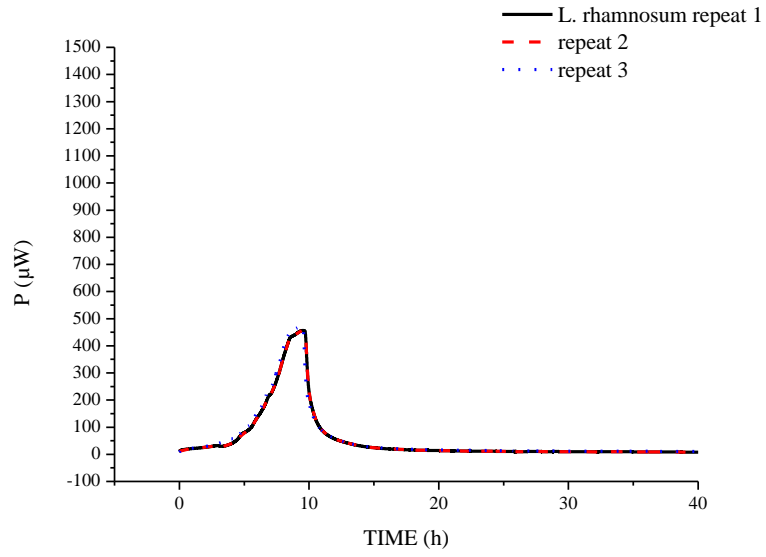
Figure 4.22. Power-time curves of 3 repeats of *L. rhamnosus* SYM B inoculated into BHIc at a cell density of 10^6 CFU/mL
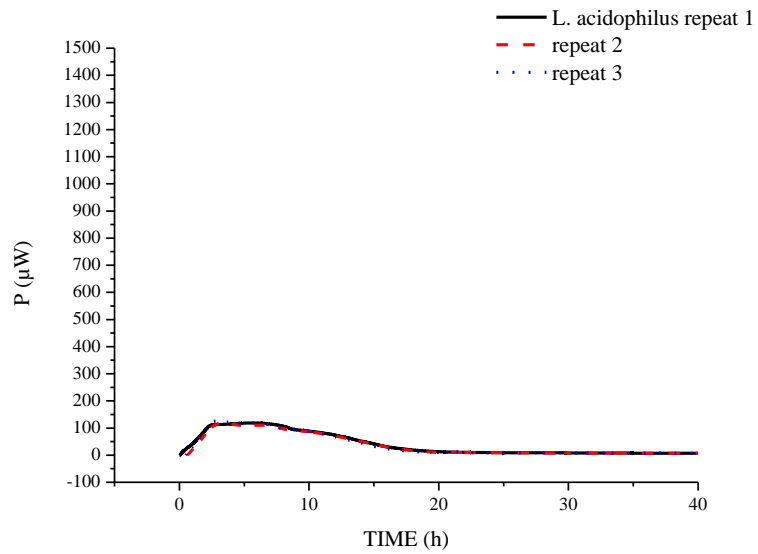
Figure 4.23. Power-time curves of 3 repeats of *L. acidophilus* SYM C inoculated into BHIc at a cell density of 10^6 CFU/mL
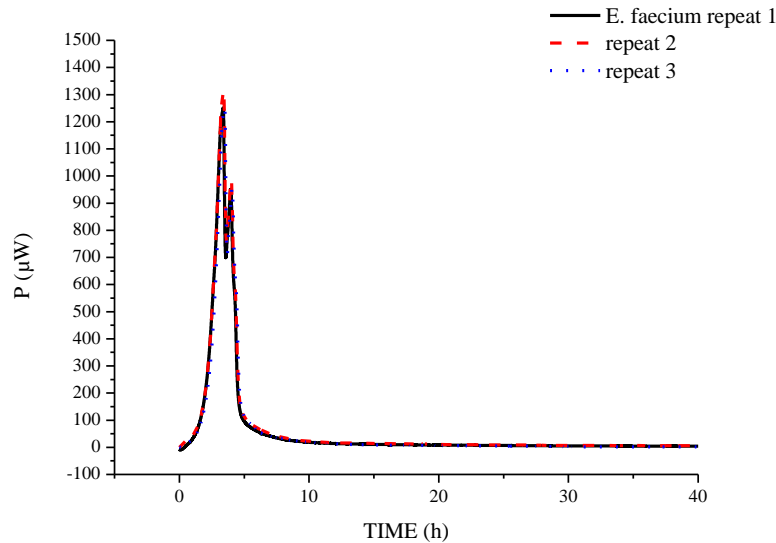
Figure 4.24. Power-time curves of 3 repeats of *E. faecium* SYM D inoculated into BHIc at a cell density of 10^6 CFU/mL

Figure 4.25 compares the power-time curves of 10^6 CFU/mL of the component strains of Symprove™, a 1 in 100 inoculation of a batch of the commercial product and a mixed culture of the four strains at equal cell density in BHIc. The mixed culture of all strains suggested that *E. faecium* SYM D might have dominated the mixed culture in BHIc. However, the power-time curves of the batches of Symprove™ showed similarities to the power-time curve of sole culture of *L. plantarum* SYM A. It is likely *L. plantarum* SYM A could have inhibited the other strains and dominated the commercial product or was present in higher concentration in the product. When the medium was changed to MRSc, the same trend of observation was noted; the power-time curves of batches of the product had similarities to the sole curve of *L. plantarum* SYM A (Figure 4.26). In contrast to BHIc, a defined mixed culture of all the strains in MRSc under the same conditions was not likely dominated by *E. faecium* SYM D however.

Relative to the other strains, *E. faecium* SYM D and *L. acidophilus* SYM C did not have a significant period of lag before exponential growth proceeded in BHIc, which was very energetic in the former species. It was therefore likely that the dominance of *E. faecium* SYM D in the mixed culture in BHIc could have been more of an "adaptive effect" or "competitive nutrition" in BHIc than of superior antimicrobial effect against the other species. Also as discussed in section 4.4.3 of this chapter, it is likely that the general-purpose medium, BHI was more favourable towards the growth of *E. faecium* SYM D

176

than the other lactobacilli strains (De Man et al., 1960) as repitition of the experiment in MRSc did not show superior growth of *E. faecium* SYM D.
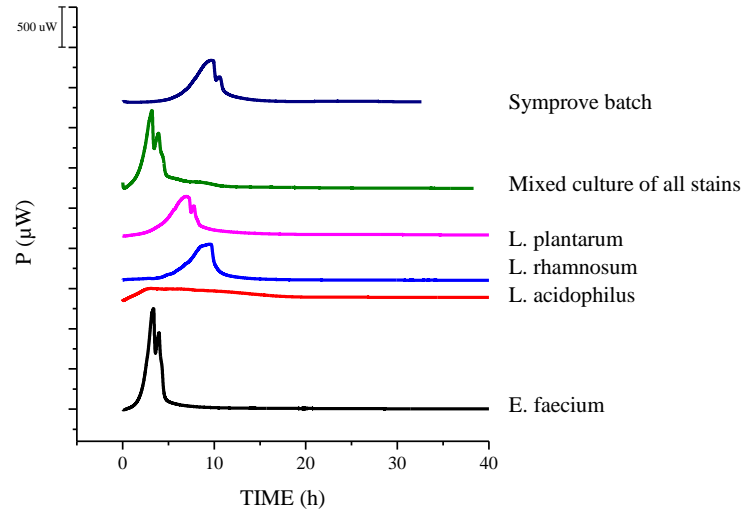
Figure 4.25. Comparison of the power-time curves of the individual strains of Symprove[TM] inoculated to respective cell densities of 10^6 CFU/mL, their mixed culture at equal cell density and a 1 in 100 dilution of a batch of the commercial product in BHIc
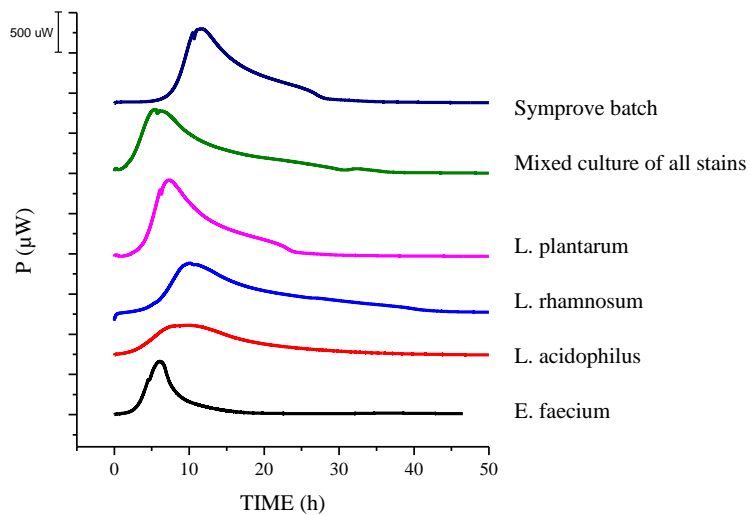
Figure 4.26. Comparison of the power-time curves of the individual strains of Symprove[TM], inoculated to respective cell densities of 10^6 CFU/mL, their mixed culture at equal cell density and a 1 in 100 dilution of a batch of the commercial product in MRSc
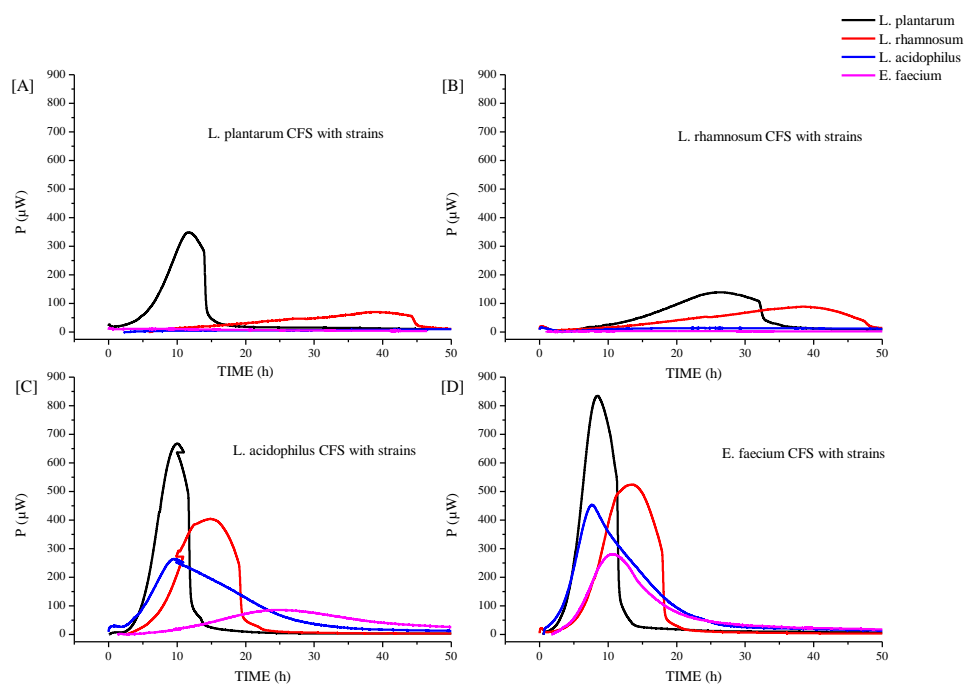
Figure 4.27. Power-time curves of Symprove[TM] strains in the CFS of each other; the strains in the CFS of [A], *L. plantarum*, [B], *L. rhamnosus*, [C], *L. acidophilus*, [D], *E. faecium*

Table 4.5. Cell count of the respective species of Symprove[TM] after incubation in the CFSs of each other

CFS of strains	Cell count (CFU/mL) of species post CFS incubation			
	L. plantarum SYM A	*L. rhamnosus* SYM B	*L. acidophilus* SYM C	*E. faecium* SYM D
L. plantarum SYM A CFS	1.9×10^7	1.2×10^7	2.0×10^5	3.5×10^4
L. rhamnosus SYM B CFS	1.6×10^7	1.8×10^7	3.4×10^5	2.3×10^5
L. acidophilus SYM C CFS	1.9×10^7	2.4×10^7	3.1×10^6	1.2×10^7
E. faecium SYM D CFS	9.9×10^7	3.8×10^7	3.5×10^7	2.2×10^7

178

CFS obtained from the other lactobacilli strains of Symprove™ inoculated into cultures of the species in double strength BHIc, dsBHIc actually inhibited the growth of *E. faecium* SYM D (Figure 4.27, Table 4.5). Its CFS did not on the other hand inhibit the lactobacilli strains.

The pure strains of Symprove™ and the product were also assessed on MRSc agar supplemented with 0.002% w/v of bromophenol blue "MRSc-BPB" (Lee and Lee, 2008). Bromophenol blue is an acid-base indicator with a useful indicator range of pH 3.0 and 4.6. At pH 3.0, it appears yellow and appears blue at pH 4.6 (Product specification, Sigma-Aldrich). The dye was used to detect the different strains in Symprove™ product due to the different pH values they develop during culture. It was noted that cultures of *L. plantarum* SYM A, *L. rhamnosus* SYM B, *L. acidophilus* SYM C and *E. faecium* SYM D produced acidic metabolites and decreased the pH of MRSc to mean values of 3.76, 3.83, 3.99 and 4.37 respectively (measured with a pH probe).

The respective colonies of the pure strains of Symprove™: *L. plantarum* SYM A, *L. rhamnosus* SYM B, *L. acidophilus* SYM C and *E. faecium* SYM D on MRSc-BPB are shown in Figure 4.28. Though each strain had individual colony morphology characteristics on MRSc-BPB agar plate, only two types of colonies were isolated in a commercial product, the numerically dominant organism isolated in the product was consistent with the colony morphology of *L. plantarum* SYM A (also identified as *L. plantarum* with an API 50 CHL biochemical identification test). The other organism, which was present in lower numbers, had been identified by the API 50 CHL test as *L. rhamnosus*. It therefore seems plausible that the other strains in the commercial product were inhibited by *L. plantarum* SYM A and *L. rhamnosus* SYM B since they were not isolated from the commercial product. Amongst the strains of Symprove™, *L. plantarum* SYM A produced the greatest quantity of antimicrobial substance, evident with the lowest pH of the medium after growth comparative to the other strains. Also the antimicrobial substances produced by *L. plantarum* SYM A and *L. rhamnosus* SYM B had the greatest inhibitory activity towards the other strains (Figure 4.27, Table 5.5). It is therefore likely that at the point of consumption, the commercial product, Symprove™ contained the dominant species *L. plantarum* SYM A, with *L. rhamnosus* SYM B present in lower quantities.

[A]

[B]

[C]

[D]

[E]
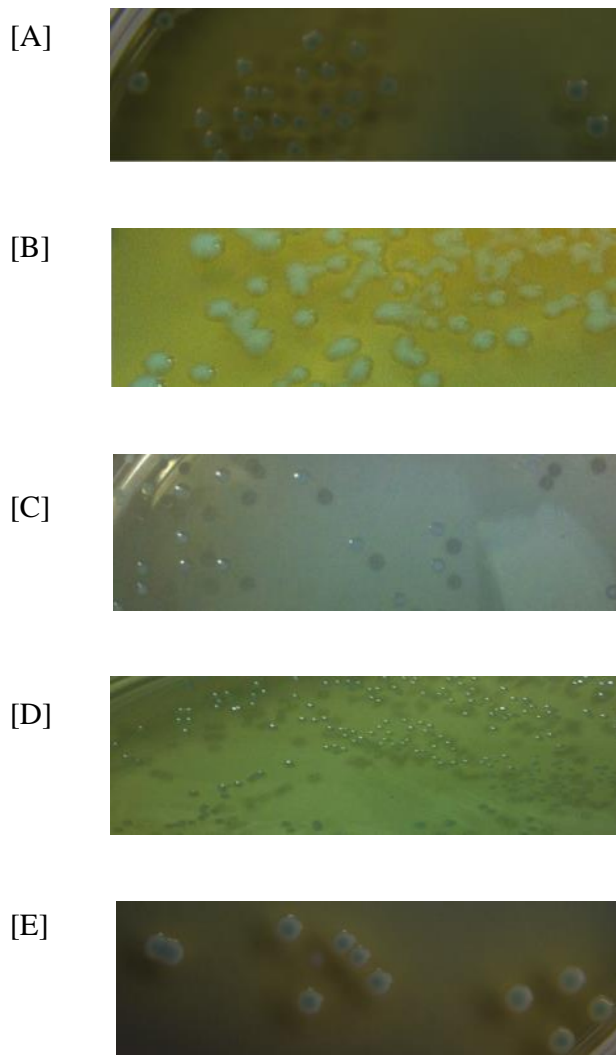
Figure 4. 28. Colonies of strains of Symprove™ on MRSc agar supplemented with Bromophenol blue (MRSc-BPB). Following anaerobic incubation of the plates for 48 h, each strain produced individual colony characteristics on MRSc-BPB agar. Photographic images were taken: [A], *L. plantarum* SYM A; [B], *L. rhamnosus* SYM B; [C], *L. acidophilus* SYM C; [D], *E. faecium* SYM D. Colonies of *L. plantarum* SYM A were yellowish and circular with bluish green centered spots. *L. rhamnosus* SYM B appeared creamy white and had the tendency to merge even on low density when multiple colonies were present. Colonies of *L. acidophilus* SYM C and *E. faecium* SYM D were smaller, circular and light blue. The commercial product, Symprove™ had one dominant type of colony [E] isolated. The colony characteristics were similar to *L. plantarum* SYM A. The other colony isolated from the commercial product was consistent with the colony morphology of *L. rhamnosus* SYM B. Its numerical number was lower relative to the other colony.

Species specificity of probiotics for inhibition of pathogens is a valid argument. While it has encouraged the use of different probiotics for targeting different pathogens based on evidence (Chapman et al., 2011, Chapman et al., 2012, Hell et al., 2013), it has also encouraged the use of cocktail of strains in commercial products by manufacturers who may have misinterpreted it as "the more the strains, the better the product" without a proper understanding of interspecies interaction. As demonstrated with Symprove™, and throughout this thesis, interactions of species do occur with consequent inhibition (or possible variant selection; Biswas et al., 2009). A study by Chapman et al., (2012) which also tested 14 single species of probiotics by cross-streak and agar spot assays also demonstrated inhibition of all probiotic combinations (Chapman et al., 2012). Their findings and findings from the present study are consistent with that from Be'er et al., (2009) who also observed inhibition between sibling strains, thus closely-related strains

of *Paenibacillus dendritiformis* (Be'er et al., 2009). The results from the present study and that of Chapman et al., (2012) show the importance and the need that manufacturers research into the interspecies interaction of potential strains before putting them together as a product.

The multistrain product, Symprove™ demonstrated inhibitory activity against *C. difficile*. Its component strains were also screened for activity against *C. difficile* in the microcalorimeter and they all showed anticlostridial activity (results not shown). The results from the microcalorimeter did not however demonstrate whether the mixture or the individual single species had greater activity against *C. difficile*. But whilst it indicates that each strain in the commercial Symprove™ product had probiotic characteristics and specifically, anticlostridial activity, the same cannot be said for all multistrain commercial products. For instance, it was shown by Lema et al., (2001) who wanted to demonstrate the efficacy of different probiotics in reducing *E. coli* O157:H7 shedding by sheep, that whilst one of the monostrain probiotic; *S. faecium* was observed to lower the count of *E. coli*, another, *L. acidophilus* did not relative to a control. But as a combined preparation, both probiotic species, *L. acidophilus* and *S. faecium* did not reduce *E. coli* shedding in sheep as much as sole *S. faecium* supplementation (over the entire experimental period of 7 weeks) although a 5-probiotic mixture of *L. acidophilus*, *S. faecium*, *L. casei*, *L. fermentum* and *L. plantarum* reduced shedding the most (Lema et al., 2001). The point being made is that, if competitive inhibition of strains occurs in some multistrain products, a consumer would derive no benefit if the strain(s) inhibited are those with specific activity. From other related studies, what is also possible is that the strains in a multistrain or multispecies product could affect each others potency, inhibit each other or adversely react hence defeating the purpose, issues also echoed by Myllyluoma et al., (2008) when studying the effects of multispecies probiotic combinations on *Helicobacter pylori* infection *in vitro* and Grandy et al., (2010) when studying two different probiotic preparations for treatment of acute rotavirus diarrhoea.

4.5 Chapter summary

In this chapter, the anticlostridial activity of some commercial probiotics was assessed. The results demonstrate that two commercial probiotic strains and seven out of the eight commercial products that were assessed inhibited germination and growth of *C. difficile* as assessed by microcalorimetry. These strains and products inhibited *C. difficile*

germination and growth in a pH-dependent manner, which suggested the likely production of organic acids. This correlates with previous studies: Naaber et al., (2004), Trejo et al., (2006) and Schoster et al., (2013). The results also suggested that the strains, which were further tested, may have produced other antimicrobial substances apart from the acids, which could have inhibited the germination and growth of *C. difficile*. The other antimicrobial substance, likely bacteriocins, may have been produced in larger quantities by bifidobacteria than the lactobacilli strain since inhibitory effect of the CFS of the former organism was always greater and needed less concentration for total inhibition of *C. difficile* in the microcalorimeter when it was neutralised. The work also demonstrated that the germination and growth of *C. difficile* was greatly influenced by pH. The work suggests that the optimum pH for germination and growth of *C. difficile* could be ca. pH 6.7; correlating with and lending weight to results obtained by Wheeldon et al., (2008).

Multi-strain probiotics are very common due to their advocacy (Sanders and Huis in't Veld, 1999, Dunne et al., 1999, Timmerman et al., 2004, Chapman et al., 2011). There has been however few studies on the inter/intra-species compatibility and stability in multistrain probiotic products (Timmerman et al., 2004, Chapman et al., 2012). In this chapter, it was also demonstrated that some strains could be inhibitory to others in a mixture using the component strains of a commercially available product. The results obtained in the study was consistent with previous findings of Be'er et al., (2009) and Chapman et al., (2012) who reported the inhibition of closely related strains and species/genera respectively.

The studies in this chapter had several limitations. Firstly, the *C. difficile* work, although showed the potential of the evaluated probiotic strains or products in inhibition of *C. difficile,* cannot be directly extrapolated to the *in vivo* situation. For treatment of *C. difficile* infection, there should be an established infection to which the probiotics could be targeted to treat but this could not be simulated in the closed system of the calorimetric ampoule which was also a very simple *in vitro* medium compared to the complexity *in vivo*. When a biorelevant intestinal juice obtained from an animal was used as the medium to simulate the *in vivo* environment, loss of the structure of the power-time curve occurred hence different species gave the same thermal curve in the medium.

Also, for the stability study, the concentration of each strain dosed in the commercial multi-strain product could not be exactly replicated in the experiments.

Notwithstanding these limitations, the study suggests that, component strains of commercial multi-strain probiotics could compete for nutrient sources and could also mutually inhibit each other. Despite the fact that they could inhibit each other, in this instance, one strain may have dominated the product under study and was still effective in inhibiting *C. difficile*. Also, the study suggests that the probiotics that were tested, except one, could have relevance for the management of *C. difficile* infection. However, further laboratory studies need to be conducted to determine whether they can achieve a therapeutic benefit.

Chapter 5 Microbiological and microcalorimetric evaluation of commercial probiotic products: *in vitro* gastric tolerance

5.1 Introduction

As discussed in Chapter 1, probiotics have become a huge commercial success due to their claimed health benefits, which stems from their relationship with the gut microbiota. Although the work in this thesis has addressed the potential use of some commercial probiotics for gastrointestinal disease; particularly antibiotic associated diarrhoea as the microcalorimetric studies have shown, their real therapeutic benefit will also principally depend on their ability to withstand gastrointestinal barriers and be able to colonise the intestinal tract (FAO/WHO 2001, Gismondo et al., 1999). The main barriers of the gastrointestinal tract, which they need to withstand include the gastric secretion, intestinal motility, pancreatic enzymes, lysozyme, and intestinal secretion and microbiota (Sarker and Gyr, 1992).

The stomach and its secretion: gastric juice is a crucial barrier to most bacteria. The juice consists of HCl, salts, water, pepsinogens, intrinsic factors (Pocock et al., 2006) and can kill bacteria within 15 min when the pH is less than 3.0 (Giannell et al., 1972). The stomach displays large variability in characteristics under different conditions, for example during the fed and fasted states (Fordtran and Walsh, 1973, Kalantzi et al., 2006). Under fasted conditions the pH in a healthy human stomach is acidic, generally ranging between 1 and 3 (Dressman et al., 1990, Kalantzi et al., 2006). After food, the stomach environment varies considerably over the course of gastric residence of the meal (Kalantzi et al., 2006) and the pH climbs briefly to a median peak value of 6.7 and then declines back to the fasted state value within two hours or less (Dressman et al., 1990). The stomach also displays large variability in emptying times both between fed and fasted states and within these states there are also large inter-individual variations (Coupe et al., 1991, Kalantzi et al., 2006, Mudie et al., 2010). In the fasted state, gastric emptying is largely controlled by the migrating myoelectric complex (MMC), which cycles every 90 to 120 min (McConnell et al., 2008b), while in the fed state, gastric emptying is controlled by low-amplitude contractions, duodenal feedback mechanism and pyloric resistance (Mudie et al., 2010). Generally, emptying time in both the fasted and fed state is also influenced by the size, amount and nature of liquid or solid ingested and the phase of contraction during which it was ingested (Mudie et al., 2010). Emptying

times in the fasted state are known to be considerably quicker than those in the fed state. Mean time for half gastric emptying of 12 min and 15.8 min have been reported respectively after the ingestion of saline solution and 300 mL of water in the fasted state (Granger et al., 1985, Steingoetter et al., 2006) while mean half gastric emptying time of 32±7, 46±9, 67±9 and 76±6 min have been reported for liquids (Marciani et al., 2001) and 105±21 min for solids respectively in the fed state (Coleman et al., 2003, Mudie et al., 2010).

There is also variability in the complexity of the chemical constituents of the gastric juice and in the volume of the gastric contents (Pocock et al., 2006); the capacity of the stomach has been noted to be 1.5 L (Aulton, 2013) but can contain up to 63 mL of free fluid in the fasted state (McConnell et al., 2008b).

Gastric tolerance assays for potential new probiotic formulations (Chandramouli et al., 2004, Iyer and Kailasapathy, 2005, Ding and Shah, 2009, Mokarram et al., 2009, Cook et al., 2011) and commercial probiotic products (Sahadeva et al., 2011) or organisms (Jensen et al., 2012) routinely use either buffer or saline solutions adjusted to pH 1.2 to 4, or growth media, adjusted to these same pH's to simulate the gastric juice. These simulated fluids often lack the complexity of the real fluids and often, gastric volumes are not accounted for or ignored. In addition, gastric tolerance assays as discussed in Chapter 1 frequently use the plate count technique to assess the survival of bacteria after exposure and this has inherent problems discussed previously.

The study in this chapter was designed to investigate the *in vitro* gastric tolerance of commercially available probiotics to a bio-relevant fluid and simulated fluids with the specific aims of developing a more realistic test and understanding the factors that must be controlled in delivering viable species to the gut. The study investigated selected commercial probiotics representative of those available to consumers and of the different classes of product available. As previously mentioned, due to their extrapolated clinical significance (without substantial evidence in all cases), probiotics have had a huge commercial success in the last decades. New probiotic products are hence introduced onto the market at a fast pace, inevitably competing for the consumers' income. In this study, it was also set out to firstly identify and enumerate the viable organisms present in the products to determine if the label claim was representative of the actual contents of the products before gastric tolerance was assessed. Previous studies have indicated that

the product quality of commercial probiotics is poor; with most products not accurately meeting their label claim (Temmerman et al., 2001, Weese, 2002, Elliot and Teversham, 2004, Drago et al., 2004, Drago et al., 2010, Aureli et al., 2010, Weese and Martin, 2011). For example, Weese and Martin, (2011), assessing 25 commercial probiotics used in animal health reported that only 4 out of 15 products that stated the concentration of viable content on the label, met their label claim. Similarly, a previous study by Weese in 2002 on both human and verterinary probiotics reported that only 15% of products accurately described and contained their claim content (Weese, 2002). Also, Drago et al., (2010) evaluating commercial probiotic products available on the USA market in 2009 reported that only 4 out of the 13 products fulfilled their label claim. These authors recommended the need for adequate control of probiotic production and the periodical screening of probiotic products for effect of storage on product quality (Drago et al., 2010).

It was believed that due to the competitive market, the quality of probiotics might have possibly improved. The study therefore aimed to investigate this.

The study, as the main focus of this thesis explored the use of microcalorimetry to measure gastric tolerance of the probiotics in or after exposure to simulated gastric fluid. The stability of lyophilised *Lactobacillus acidophilus*, LA-5$^{®}$ was studied at two different storage conditions to investigate the effect of storage on viability.

5.2 Objectives

The objectives of this chapter were:

- To identify and enumerate viable organisms present in commercial probiotic products
- To determine gastric survival of commercial probiotics using a biorelevant and simulated gastric fluid
- To explore the potential of isothermal microcalorimetry to measure gastric survival of probiotics
- To study the effect of storage on the viability of a lyophilised probiotic

5.3 Materials and Methods

5.3.1 Commercial probiotic products

Eleven commercial products, in total were used in the study (Table 5.1). They were either purchased from local supermarkets or pharmacies or obtained from the manufacturers or marketers. After purchase, the products were stored appropriately in cool, dry places, away from light or in the fridge at 4°C as per the information on their labels. They were all used before the expiry dates printed on the labels.

5.3.2 Probiotic control strains

Probiotic strains, *Lactobacillus acidophilus* LA-5[®], *Bifidobacterium lactis* BB-12[®] and *Bifidobacterium bifidum* ATCC 11863 were used as control strains or as live free cells for the gastric tolerance assay. 10^8 CFU/mL cell suspensions of these strains were obtained as described in section 3.3.4.

Table 5.1. Commercial probiotic products evaluated in this study

Product	Manufacturer /Marketer	Form	Probiotic strains	Volume or weight measured per content or dose	Claimed Culture concentration	Other Ingredients	Dosage/adminstration instructions
Actimel®	Groupe Danone, France	Liquid (milk)	*Lactobacillus casei* DN 114 001 (main strain), *Lactobacillus bulgaricus* and *Streptococcus thermophilus*	100 mL	10 billion per 100 mL	Yogurt (skimmed milk, skimmed milk concentrate/powder, cream, yogurt cultures), skimmed milk, sugar/liquid sugar (sucrose 7.3%), dextrose, milk mineral concentrate, vitamins (B6, D)	One bottle per day as part of breakfast
Align®	Procter and Gamble, USA.	Solid (Capsule)	*B. infantis* 35624	0.174 g	1 billion per capsule	Microcrystalline cellulose, hypromellose, sucrose, magnesium stearate, sodium caseinate, titanium dioxide, trisodium citrate dihydrate, propyl gallate	One capsule should be taken per day
Biobalance support	Boots Ltd.	Solid (Capsule)	*B. bifidum*, *L. acidophilus*, and *B. lactis*	0.300 g	12.5 billion per capsule	Fructooligosaccharides, microcrystalline cellulose, hydroxypropyl methylcelluose, silicon dioxide, magnesium stearate	Capsules are to be taken once daily with liquid

Product	Manufacturer/Marketer	Form	Probiotic strains	Volume or weight measured per content or dose	Claimed Culture concentration	Other Ingredients	Dosage/adminstration instructions
Biobalance Travel	Boots Ltd.	Solid (Capsule)	*B. bifidum*, *L. acidophilus*, and *B. lactis*		10 billion per capsule	Ginger powder, capsule shell (hydroxypropyl methylcellulose), silicon dioxide, magnesium stearate	One capsule a day with liquid
Bio-kult®	Protexin Lopen Head, UK.	Solid (Capsule)	*Bacillus subtilis* PXN 21, *Bifidobacterium* spp. (*B. bifidum* PXN 23, *B. breve* PXN 25, *B. infantis* PXN 27, *B. longum* PXN 30), *Lactobacillus* spp. (*L. acidophilus* PXN 35, *L. delbrueckii* spp. *bulgaricus* PXN 39, *L. casei* PXN 37, *L. plantarum* PXN 47, *L. rhamnosus* PXN 54, *L. helveticus* PXN 45, *L. salivarius* PXN 57), *Lactococcus lactis* ssp. *lactis* PXN 63, *Streptococcus thermophilus* PXN 66	0.171 g	2 billion per capsule	Cellulose (bulking agent), vegetable capsule (hydroxypropyl methyl cellulose), traces of soya, milk products at level that would not affect lactose intolerance sufferers	One or two capsules, once or twice daily. Capsule can be opened and the contents can be sprinkled on food, taken in a drink or swallowed whole
Digestive Health	Boots Ltd	Solid (Capsule)	*L. acidophilus, B. bifidum, B. lactis*	0.298 g	12.5 billion per capsule	Fructo-oligosaccharides, cellulose, hydroxypropyl methylcellulose, anti-caking agents (silicon dioxide, magnesium stearate). Contains milk	Take one or two capsules with food or drink

Product	Manufacturer /Marketer	Form	Probiotic strains	Volume or weight measured per content or dose	Claimed Culture concentration	Other Ingredients	Dosage/adminstration instructions
OptiBac	Wren Laboratories Ltd.	Solid (powdered sachet)	*L. acidophilius* Rosell-52, *L. casaei* Rosell-215, *Lactococcus lactis* Rosell-1058, and *B. bifidum* Rosell-71.	-	5 billion per sachet	Fructooligosaccharides (FOS), stabilizer (maltodextrin)	One sachet daily with breakfast. Pour sachet into an inch of water in a tall glass, leave to dissolve and swirl vigorously
Probio 7®	Forever Young International, UK.	Soid (Capsule)	*L. casei, L. rhamnosus, L. plantarum, L. acidophilus, Streptococcus thermophilus, B. bifidum, B. breve and B. longum.*	0.439 g	10 billion probiotic organisms plus 1 billion *Saccharomyces boulardii* per capsule	Chicory inulin fibre (Fructo-oligosaccharide), vegetable cellulose (capsule shell), maltodextrin, soy fibre, magnesium stearate, asocobic acid	One capsule per day. Capsule should be swallowed whole
Symprove™	Symprove Ltd.	Liquid (non-milk)	*L. rhamnosus, L. planatarum, L. acidophilus, and Enterococcus faecium*	60 mL	10 billion per 50 mL	Barley, Vitamin C, potassium sorbate, trisodium citrate	1 mL per kilogram of bodyweight as a liquid drink to be taken prior to food in the morning

Product	Manufacturer /Marketer	Form	Probiotic strains	Volume or weight measured per content or dose	Claimed Culture concentration	Other Ingredients	Dosage/adminstration instructions
VSL#3®	Ferring Pharmaceuticals Ltd.	Solid (powdered sachet)	*Streptococcus thermophilus* DSM 24731, *B. breve* DSM 24732, *B. longum* DSM 24736, *B. infantis* DSM 24737, *L. acidophilus* DSM 24735, *L. plantarum* DSM 24730, *L. paracasei* DSM 24733 and *L. delbrueckii* subsp. *bulgaricus* DSM 24734.	4.447 g	450 billion per sachet	Maltose, silicone dioxide (anti-caking agent)	One sachet once or twice daily. Sachets can either be spinkled on food or reconstituted with cold water or any non-fizzy drink and consumed
Yakult®	Yakult, UK	Liquid (milk)	*L. casei* Shirota	65 mL	6.5 billion per 65 mL	Skimmed milk solids	One or two bottles per day

5.3.3 Chemicals

HCl (37%), Skim milk powder and D-(+)-Trehalose dihydrate were purchased from Sigma-Aldrich, UK. NaCl (AnalaR BDH 58.44g/mol) was obtained from VWR International, UK.

5.3.4 Isolation and identification of probiotic species in commercial products

Seven of the commercial products: Actimel®, Biobalance Support, Biobalance Travel, Digestive Health, OptiBac, Symprove™ and Yakult® were used for this test. They were obtained from a visit to a local pharmacy and supermarket at Brunswick Centre (London, UK) or from the manufacturer. The solid (lyophilised) commercial products (Biobalance Support, Biobalance Travel, Digestive Health and OptiBac) were hydrated in 3 mL MRSc broth. A loopful of the hydrated lyophilised products or the liquid products was streaked onto MRSc agar plates and incubated at 37°C anaerobically. After 48 h of incubation, the colonies obtained, which could be differentiated by size and appearance were sub-cultured to obtain pure cultures of the selected colonies. Gram staining was performed on the pure cultures isolated. Gram staining was also performed on pure colonies of *Lactobacillus acidophilus* LA-5® and *Bifidobacterium bifidum* ATCC 11863 which were used for reference or controls for identification by microscopic imaging. Biochemical tests for identification of the bacteria were carried out using the commercial kits API 50 CHL for *Lactobacillus* and related genera and API Rapid ID 32A for anaerobes. The tests were conducted according to the manufacturers instructions.

5.3.5 Enumeration of viable organisms in commercial probiotic products

Only one product per manufacturer was employed for this experiment and the subsequent experiments. Eight products were used and included: Actimel®, Align®, Biobalance Support, Bio-kult®, Probio 7®, Symprove™, VSL#3® and Yakult®. The content of a capsule or sachet of the lyophilised products (Align®, Biobalance Support, Bio-kult®, Probio 7® and VSL#3®) was dispersed in 10 mL sterile PBS. The mixture was left to hydrate for 10 min, was vortexed to homogeneity, serially diluted in PBS and

spread-plated on MRSc agar. Liquid products (Actimel®, Symprove™ and Yakult®) were also serially diluted and spread-plated on MRSc agar. All plates were then incubated at 37°C anaerobically. Colonies were counted after incubation for 48 h. The actual contents of a dose of these products were calculated using the formula:

Actual content (CFU/dose) = (CFU/mL)*dose   Equation 5.1

for the different form of the product, lyophilised or liquid, content of viable cells per dose was calculated using Equations 5.1.1 and 5.1.2 respectively

content per capsule or sachet = (CFU/mL)*10 mL   Equation 5.1.1

content per dose =(CFU/mL)* recommended volume   Equation 5.1.2

Experiments were performed in triplicate and the number of colony forming units was expressed as log values.

5.3.6 Obtainment of porcine gastric fluid and preparation of simulated gastric fluids

Three types of gastric fluid were used for the study; porcine gastric fluid (PGF), simulated gastric fluid (SGF*) and fasted state simulated gastric fluid (FaSSGF). PGF was obtained from the stomach of an adult animal, *fed ad libitum*; obtained from an abattoir. Juices were dispensed into multiple aliquots and frozen at -80°C. Prior to use, they were thawed at room temperature, centrifuged at 10000 rpm (Mini spin, Eppendorf, Germany) for 10 min at 25°C. The supernatant obtained was sterilized by filtration using a membrane filter of 0.22 μm pore size.

SGF* was prepared by adding HCl to a 2 g/L solution of NaCl and adjusting pH (pHenomenal®, UK) to 1.2 ± 0.1 and 3.4 ± 0.1.

FaSSGF was prepared according to the composition presented by Vertzoni and co-workers (Vertzoni et al., 2005) with SIF® powder (Biorelevant Ltd.). Briefly, NaCl (2 g) was dissolved in purified water (1 L) and the pH adjusted to either 1.6 or 3.4 as appropriate with HCl. 0.06 g of SIF® powder was added to the prepared HCl/NaCl

solution to a volume of 1 L to give a final composition of sodium taurocholate 80 µM; lecithin 20 µM; pepsin 0.1 mg/mL; sodium chloride 34.2 mM.

The artificial fluids were used within 48 h of preparation.

5.3.7 Measurement of surface tension, osmolality, buffer capacity and pH of the porcine and simulated gastric fluids

The tensiometer, Delta-8, Kibron Inc, Finland was employed for measurement of the surface tension of the fluids using the Delta-8 manager software (version 3.8). The temperature of the instrument was between 27.7-27.9^{o}C. Measurements were taken on 50 µL/well of 8 samples of each fluid in 96-well plates. Calibration was done with deionized water prior to the experiments.

The osmolality of the fluids were measured using the freezing point depression technique with a digital micro-osmometer, Hermann Reobling Messtechnik, Germany on 100 µL samples. Calibration was done with deionized water and measurements made in triplicates.

The buffer capacities of the fluids were measured by adding 0.1 M NaOH standard solution (Sigma-Aldrich, Germany) to 3 mL of the fluids and noting the volume of NaOH needed to change the pH by 0.5 unit. The buffer capacity was determined using the equation,

$$\beta \ (mmol / L / \Delta pH) = \Delta AB / \Delta pH \hspace{3cm} \text{Equation 5.2}$$

where ΔAB is the small increment in mol/L of the amount of acid or base added to produce a pH change of ΔpH in the buffer. This equation can be rewritten as:

$$\beta \ (mmol / L / \Delta pH) = ((M_a * V_a) / \Delta pH) * (1000 / V_b) \hspace{2cm} \text{Equation 5.3}$$

where M_a is the molarity of the acid or base, V_a is the volume of acid or base in mL, V_b is the volume of buffer in mL, ΔpH is the change in pH unit. The equation is multiplied by 1000 to express the volume in litre.

5.3.8 *In vitro* gastric tolerance of probiotics mimicking the fasted state

For these tests, it was assumed that the stomach has a fluid volume of 60 mL in the fasted state (McConnell et al., 2008b). Doses of the commercial products were mixed with 60 mL of the artificial fluids (SGF* and FaSSGF) to simulate *in vivo* condition upon ingestion of a dose of the commercial product in the fasted state. Thus 60 mL of Symprove[TM], entire contents of bottles of Actimel[®] and Yakult[®], the entire content of capsule/sachet of Align[®], Bio-kult[®], Biobalance Support, Probio 7[®] and VSL#3[®] were added to 60 mL of the artificial fluids. The entire contents of the lyophilised products were also hydrated with 10 mL of sterile distilled water for 10 min and mixed with the simulated gastric fluids at pH 3.4. This was to mimic situations where the manufacturer instructs the consumer to reconstitute with liquid before consumption.

For the porcine gastric fluid, representative dose of the commercial products was mixed with the fluid in a ratio representative of the artificial fluids to the commercial products (4.4 mg of Align[®], 7.5 mg of Biobalance support, 4.4 mg of Bio-kult[®], 11 mg of Probio7[®], 111.7 mg of VSL#3[®], 2.5 mL of Actimel[®], 1.5 mL of Symprove[TM] and 1.63 mL of Yakult[®] was mixed with 1.5 mL of porcine gastric fluid).

The samples were vortexed at maximum settings for 10 s and incubated at 37°C. Aliquots of 50 µL were withdrawn at 5, 10, 20, 30 and 120 min, serially diluted in PBS and plated on MRSc agar plates for the determination of viable count.

To determine gastric tolerance of live free cells of probiotic strains in gastric fluid, 17 µL of a 10^8 CFU/mL cell suspensions of LA-5[®] and BB-12[®] were added to 1 mL porcine gastric fluid and tolerance assay performed as above.

5.3.9 *In vitro* gastric tolerance of probiotics mimicking the fed state in PGF

Gastric tolerance in the fed state was done on the assumption that the fed stomach had a fluid capacity of 1.5 L (Aulton, 2013). A representative dose of the commercial products was mixed with porcine gastric fluid in a ratio depictive of *in vivo* situation upon ingestion. The total volume of porcine fluid used was 1.5 mL. Thus, 100 µL of Actimel[®], 60 µL of Symprove[TM], 65 µL of Yakult[®], 0.174 mg of Align[®], 0.300 mg of Biobalance Support, 0.171 mg of Bio-kult[®], 0.439 mg of Probio 7[®] and 4.45 mg of

VSL#3® were added to 1.5 mL PGF. The entire contents of the lyophilised products were also hydrated with 10 mL of sterile water for 10 min, vortexed to homogeneity and 10 µL mixed with 1.5 mL of PGF. The mixture was vortexed at maximum settings for 10 s and incubated at 37°C. Aliquots of 50 µL were withdrawn at 5, 10, 20, 30, 90, 120 and 180 min, serially diluted in PBS and plated on MRSc agar plates for the determination of viable count.

5.3.10 Microcalorimetric gastric tolerance assay mimicking the fasted state

The fasted state gastric tolerance of the products in PGF was simulated in the microcalorimeter, by mixing the respective ratio of PGF to product in 3 mL sterile calorimetric glass ampoule and recording the heat output. The volumes of PGF and quantities of products used were as follows:

Product	Volume of PGF (mL)	Volume /Weight of Product
Actimel®	1.125	1.875 mL
Symprove™	1.500	1.500 mL
Yakult®	1.440	1.560 mL
VSL#3®	3.000	0.220 g

Alternatively, the products were exposed to the porcine and simulated gastric fluids (PGF, SGF* and FaSSGF, at pH 3.4, 1.2 or 1.6) mimicking the fasted state for 30 min as described in section 5.3.6 after which 30 µL of the mixtures were inoculated into 2970 µL of pre-warmed MRSc broth in a sterile 3 mL calorimetric glass ampoule. The ampoules were sealed with crimped caps and vortexed for 10 s. They were placed in the thermal equilibration position of the TAM and allowed to equilibrate to temperature. Data were captured after 30 min with Digitam 4.1 every 10 s over an amplifier range of 1000 µW or 3000 µW until the power-time data returned to baseline.

5.3.11 Pharmaceutical consideration: Freeze-drying and storage of lyophilised probiotic, LA-5®

LA-5® was grown anaerobically in MRSc broth at 37°C. At the end of incubation, the culture was divided into equal aliquots and centrifuged at 3500 g for 10 min at 4°C. The pellet obtained in each aliquot was washed twice with PBS. The pellets were resuspended and mixed in different protectants:

- 15% v/v glycerol,
- 10% w/v trehalose,
- 10% w/v reconstituted skim milk

1 mL of each suspension was dispensed into vials and frozen over liquid nitrogen vapour as described in section 2.3.7 to a temperature of -50°C. At the end of the freezing process, vials from each protectant were thawed at 40°C for 3 min and viable cells enumerated as described previously. Viable count was compared with the count before freezing. The rest of the vials were transferred to a freeze dryer, Modulyo D-230 (Thermo Scientific, UK) whose sample shelf was equilibrated at -50°C. The vials were kept at this temperature and under 0.5 mbar vacuum for 22 h for the primary drying stage after which a secondary drying stage began for a further 6 h at 10°C. At the end of the freeze drying process, the samples were separated into two; one set was stored at 4°C in the fridge and the other stored at room temperature (RT) to replicate the storage condition instructed by manufacturers of the lyophilised products evaluated. The viability of the freeze dried probiotic strain in the different protectants was determined immediately after freeze-drying and after 3 days, 7 days, 10 days, 14 days, 28 days, 2 months, 3 months and 6 months of storage either in the fridge at 4°C or at RT.

5.3.12 Statistical Analysis

Statistical analysis was performed in Origin Pro 8.6 (Microcal Software Inc.). T-test or Analysis of Variance (ANOVA) with Turkey post-hoc analysis for means comparison was used where appropriate. P values less than 0.05 were regarded as significant difference between means.

5.4 Results and Discussion

5.4.1 Isolation and identification of probiotic species in commercial products

Colonies were formed on all the incubated MRSc plates streaked with liquid or hydrated lyophilised product. The colonies isolated were similar in appearance i.e. creamy white, smooth and circular. They were further sub-cultured on the basis of the size/characteristics of the colonies to obtain pure strains. The Gram stain appearance of the control strains and the classification of the colonies isolated from each product according to the API identification software, based on their biochemical profile are summarized in Tables 5.2 and 5.3 respectively.

Almost all the products contained at least one probiotic bacteria indicated on their label. According to the label, Biobalance Support, Biobalance Travel and Digestive Health contained three probiotic bacteria mixture: *Lactobacillus acidophilus, Bifidobacterium bifidum* and *Bifidobacterium lactis.* In this study only two types of colonies were isolated from these products. A Gram stain of the colonies revealed Gram-positive, straight, rounded end rods in chains or presented singularly which were similar to colonies of LA-5®. The API 50 CHL test confirmed that both colonies were *L. acidophilus.* The API rapid ID 32A test however suggested that they could be bifidobacteria except one of the isolates of Digestive Health.

OptiBac, which was labelled to contain *L. acidophilus*, *Lactobacillus casaei*, *Lactococcus lactis* and *B. bifidum,* also gave two isolates. The API rapid ID 32A test suggested that the isolates could either be *L. acidophilus* or *Bifidobacterium* spp. The API 50 CHL however suggested that both isolates were *Lactobacillus* species.

Symprove™, as indicated in the previous chapter, was labeled to contain four strains: *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus acidophilus* and *Enterococcus faecium* but only two of the strains indicated on the label were isolated. The API 50 CHL identified the strains as *L. plantarum* and *L. rhamnosus*.

Actimel® also indicated it contained *Lactobacillus casei* DN 114 001 as the main strain. Only one type of colony was isolated from it. The colony was identified as *L. paracasei*

with the API 50 CHL test kit. The other yoghurt cultures, *Lactobacillus bulgaricus* and *Streptococcus thermophilus* were not isolated.

A single isolate was obtained from Yakult® which was also labeled to contain *L. casei* Shirota. The strain was identified as *L. paracasei* with the API 50 CHL test kit.

It is worth mentioning that *L. casei* and *L. paracasei* form a closely related taxonomic group within the heterofermentative lactobacilli (Ward and Timmins, 1999). These two species even though well distinguishable from other lactobacilli species (except *L. rhamnosus*), are known to be difficult to differentiate using traditional fermentation profiles (Ward and Timmins, 1999) which most often, identify *L. casei* was *L. paracasei* (Yeung et al., 2002).

Table 5.2. Gram stain of control strains, *L. acidophilus* LA-5® and *B. bifidum* ATCC 11863
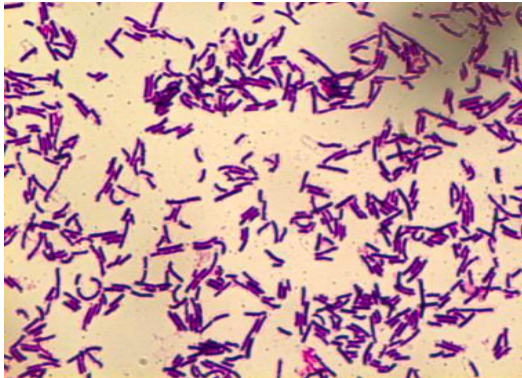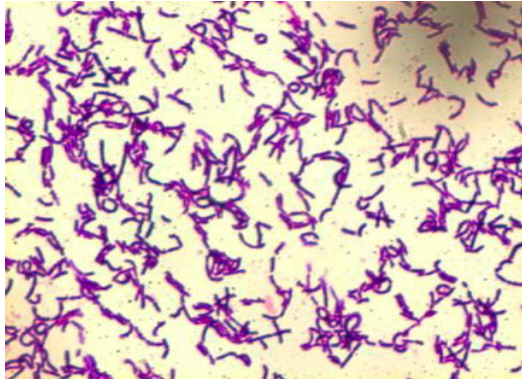
Control Strain	Gram stain morphology	Gram stain image
Lactobacillus acidophilus, LA-5®	Gram-positive, straight, solid, single or in chains, rounded end rods	
Bifidobacterium bifidum ATCC 11863	Gram-positive, thin, short, curved, single or in chains, rods	

Table 5.3. Gram stain appearance and biochemical identification of colonies isolated from commercial probiotic products

Product	Isolates	Gram Stain Appearance	API 50 CHL Identification	API Rapid ID 32A Identification
Actimel®	1	Gram-positive, straight, solid rounded end rods in chains or singles	*Lactobacillus paracasei*	-
Biobalance Support	2	Gram-positive, straight, solid rounded end rods single or in chains	*Lactobacillus acidophilus*	*Bifidobacterium* spp.
		Gram-positive, straight, solid rounded end rods single or in chains	*Lactobacillus acidophilus*	*Bifidobacterium* spp.
Biobalance Travel	2	Gram-positive, straight, solid rounded end rods single or in chains	*Lactobacillus acidophilus*	*Bifidobacterium* spp.
		Gram-positive, straight, solid rounded end rods single or in chains	*Lactobacillus acidophilus*	*Bifidobacterium* spp.
Digestive Health	2	Gram-positive, straight, solid rounded end rods single or in chains	*Lactobacillus acidophilus*	*Lactobacillus acidophilus*
		Gram-positive, straight, solid rounded end rods single or in chains	*Lactobacillus acidophilus*	*Bifidobacterium* spp.

Table 5.3. Gram stain appearance and biochemical identification of colonies isolated from commercial probiotic products (continued)

Product	Isolates	Gram Stain Appearance	API 50 CHL Identification	API Rapid ID 32A Identification
OptiBac	2	Gram-positive, straight, solid rounded end rods single or in chains	*Lactobacillus paracasei*	*Lactobacillus acidophilus*
		Gram-positive, straight, solid rounded end rods single or in chains	*Lactobacillus acidophilus*	*Bifidobacterium* spp.
Symprove™	2	Gram-positive, straight, solid rounded end rods single or in chains	*Lactobacillus plantarum*	-
		Gram-positive, straight, solid rounded end rods single or in chains	*Lactobacillus rhamnosus*	-
Yakult®	1	Gram-positive, straight, solid rods single or in chains	*Lactobacillus paracasei*	-

The results of this study suggest that bifidobacteria cannot be identified using the API 50 CHL tests, which has also been recognized by Carmen Collado and Hernandez, (2007). Strains that the API 50 CHL identified as lactobacilli were also identified as bifidobacteria by the API rapid ID 32A. This may indicate the selectively of the API 50 CHL for identification of lactobacilli and the API rapid ID 32A for identification of bifidobacteria than lactobacilli and the likely limitation of both tests; mainly misidentification. However, the Gram stain showed that the morphology of all the isolates matched the lactobacillus control strain than the bifidobacterium strain. Also, the API 50 CHL test depended on the metabolism of carbohydrates by the strains and relied on colour changes caused by pH modifications during fermentation of the substrates in the kits by the growing strains (Moll et al., 1996, Herbel et al., 2013) whilst the API rapid ID 32A test depended on colour changes caused by preformed enzymes (Moll et al., 1996). These colour changes were noted visually after either 48 h or 4 h of incubation for the respective tests. For the API 50 CHL, the pH indicator in all the tests were originally purple and were meant to change to yellow to indicate a positive result after 48 h of incubation except for one tube where a colour change from purple to black indicated a positive result. These changes were easier to note than the API rapid ID 32A, where colour changes were subtle; for example, colour change from red to yellow-orange after shorter incubation time (4 h). Hence it was more likely that the API rapid ID 32A could result in erroneous result; that from the API 50 CHL could therefore be more accurate. Generally, the specificity of these identification tests has been challenged by a number of authors. For instance, Boyd et al., (2005) found out that 33 out of 97 strains of lactobacilli were misidentified by the API 50 CHL test as *L. acidophilus* or *L. fermentum*. Other authors such as Nagy et al., (1991), Alvarez-Olmos et al., (2004) and Collado et al., (2005) have also reported such likely misidentification. It is possible these misidentifications occurred because the same strain could have had different carbohydrate fermentation pattern for the different protocol or assay condition used for the different test or different strains could have had the same enzymatic profile therefore may have showed the same fermentation pattern. Or the test could have been influenced by the wholesomeness of the cultures used. Moll et al., (1996) analysing fresh, frozen and lyophilised isolates have reported that the API rapid ID 32A test worked better with fresh cultures than lyophilised cultures and remarked that the use of this system to identify organisms from freeze-dried products, which needed multiple sub culturing to recover could lead to inaccuracy (Moll et al, 1996). They further criticized that the incubation period for the kit was not sufficient for identification of some species.

The results of the present study also suggest that bifidobacteria could be more difficult to isolate and appropriately identify using MRSc medium and the API tests respectively or may not have been contained in products claiming their content. It must be noted that the detection and enumeration of probiotic microorganisms in a product can be performed in a number of ways. In some instances, the respective species or genera in a product can be selectively isolated and enumerated using selective media or one plating medium can be used which supports the growth of the different species or genera (Charteris et al., 1997). Differentiation between the species or genera is then carried out on the basis of the morphological characteristics of the colonies (Charteris et al., 1997). A number of media have been suggested for selectively isolating and growing species of lactobacilli and bifidobacteria and yoghurt starter cultures which are too numerous to list (Charteris et al., 1997, Nebra and Blanch, 1999, Shah, 2000, Tharmaraj and Shah, 2003, Tabasco et al., 2007, Ashraf and Shah, 2011, Saccaro et al., 2011). But generally, MRS agar is used for the specific isolation and growth of lactobacilli with added antibiotics, bile, maltose or trehalose (Charteris et al., 1997) and incubated under aerobic condition for sole proliferation of lactobacilli (Charteris et al., 1997). Media used for isolation of bifidobacteria apart from the main fermentable substrates which also generally includes MRS (routinely), Reinforced clostridial medium (Champagne et al., 2011), Trypticase phytone yeast extract (Barrett et al., 2012), Columbia blood agar (Nebra and Blanch, 1999, Weese and Martin, 2011), may require fortification with substances such as cysteine, ascorbic acid and sodium sulphite to lower redox potential and keep the medium anaerobic with antibiotics such as kanamycin and mupirocin and supplements such as horse or sheep blood, raffinose, added for the selective growth. Also, Transgalactosylated oligosaccharides (TOS) agar and TOS agar supplemented with neomycin sulphate, paromycin sulphate, nalidixic acid and lithium chloride (NPNL) and BLOG agar, which is based on blood-glucose-liver medium with additional selective ingredients (oxgall and gentamicin) have been used for selective enumeration of bifidobacteria (Sonoike et al., 1986, Wijsman et al., 1989, Lim et al., 1995). Bifidobacterium medium (BFM) (Nebra and Blanch, 1999) has also been used. They are generally incubated anaerobically (Champagne et al., 2011, Charteris et al, 1997). But MRS (supplemented with 0.05% L-cysteine hydrochloride) and Homofermentative-heterofermentative differential (HHD) medium (based on bromocresol green pH indicator) has also been used for isolation of mixed cultures of lactobacilli, bifidobacteria and yoghurt starter cultures (Charteris et al., 1997, Camaschella et al.,

1998, Lin et al., 2006, Aureli et al., 2010, Champagne et al., 2011, Sahadeva et al., 2011).

Previous studies reported in the other chapters of this thesis had shown growth of *L. acidophilus*, *L. plantarum*, *L. rhamnosus*, *B. lactis*, *B. bifidum* and *E. faecium* on MRSc, which were the main species contained in the products evaluated besides *L. casei* and *Lactococcus lactis*, which were contained in a few other products. In this study, it is likely *L. lactis* was not isolated. It is also likely the bifidobacteria strains in the products could not grow well or were outgrown by the lactobacilli species on MRSc agar or were not isolated in the products claiming their content since as indicated previously, the Gram stain results corroborated well with the API 50 CHL results which was also a more detailed and rigourous tests, consisting of fermentation tests with longer growth incubation duration compared with the API rapid ID 32A, which depended on preformed enzymes by mostly previously lyophilised species, hence whose results may have only confirmed the species isolated were anaerobic. Moreover, Moll et al., (1996) have also reported in their study that some lactobacilli species were identified as bifidobacteria by the API rapid ID 32A.

Also, it has been reported by other authors (Aureli et al., 2010, Drago et al., 2010) that bifidobacteria were not usually found in products claiming their content. For instance, Drago et al's evaluation of 13 commercial probiotics on the USA market highlighted that products containing mixed species with bifidobacteria did not contain them, which they attributed, could be due to the higher sensitivity of bifidobacteria to inadequate processing, packaging and storage than other bacteria (Drago et al., 2010). Aureli et al., (2010) also reported that *B. bifidum* was usually sporadically detected (4 out of 25) in products claiming its contents and when detected, were mostly dead (Aureli et al., 2010). In Aureli et al's assay, they used both phenotypic and genotypic (polymerase chain reaction, PCR) methods for species identification, which was more confirmatory than the Gram staining, morphological characteristics and biochemical profiling used in the present study and in Drago et al's study. PCR and PCR-denaturing gradient gel electrophoresis (PCR-DGGE) (Charteris et al, 1997, Tannock 1999), quantitative real time PCR (qPCR), pulsed field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD) are among the genotypic methods that have been used or recommended (FAO/WHO, 2001) for identification of species in probiotic products. The sole use of the biochemical test kits for identification was one of the main limitations of

this study. These biochemical tests could be unreliable and should therefore be always accompanied by other phenotypic or genotypic tests or physico-chemical identification method for the accurate determination of specific species within a product.

In summary, the evaluation showed that all the probiotic products contained viable probiotic organisms. Both Actimel[®] and Yakult[®] contained one species identified by the API test as *L. paracasei*. Symprove[TM] contained two species identified as *L. plantarum* and *L. rhamnosus*. Biobalance Support, Biobalance Travel and Digestive Health contained *L. acidophilus* and a *Bifidobacterium* spp. OptiBac contained *L. paracasei*, *L. acidophilus* or a *Bifidobacterium* spp. The API test kits could not accurately discern the phenotypic variability within members of the different genus. But it is also likely that bifidobacteria were not isolated from the products: those that were supposedly identified with API rapid ID 32 A could not be differentiated at the species level. But overall, the identification test gave some information about the quality of the products, which agrees with previous studies about the microbial composition of commercial probiotics (Temmerman et al., 2001, Weese, 2002, Elliot and Teversham, 2004, Drago et al., 2004, Drago et al., 2010, Aureli et al., 2010, Weese and Martin, 2011).

5.4.2 Enumeration of viable organisms in commercial products

The purpose of this test was to compare the viable organisms present per dose in each probiotic product to the viability stated on their respective packaging. The products used for this test and the subsequent tests included: Actimel[®], Align[®], Biobalance Support, Bio-kult[®], Probio 7[®], Symprove[TM], VSL#3[®] and Yakult[®]. The products could be classified into two categories: lyophilised products (i.e. lyophilised powders packed into capsules or sachet which included Align[®], Biobalance Support, Bio-kult[®], Probio 7[®] and VSL#3[®]) and liquid products (i.e. milk based: Actimel[®] and Yakult[®] and water based, Symprove[TM]).

Full cell recovery was observed for the liquid products: Actimel[®], Symprove[TM] and Yakult[®] (Figure 5.1). Recovery in excess of 1-log unit was obtained for Actimel[®] and 0.5-log for Yakult[®] respectively. The lyophilised products had lower viable content compared to their stated claim. Align[®], Bio-kult[®] and Biobalance support had 0.4 to 1.5-log less in viability and Probio 7[®] showed almost a major 4-log reduction in viability.
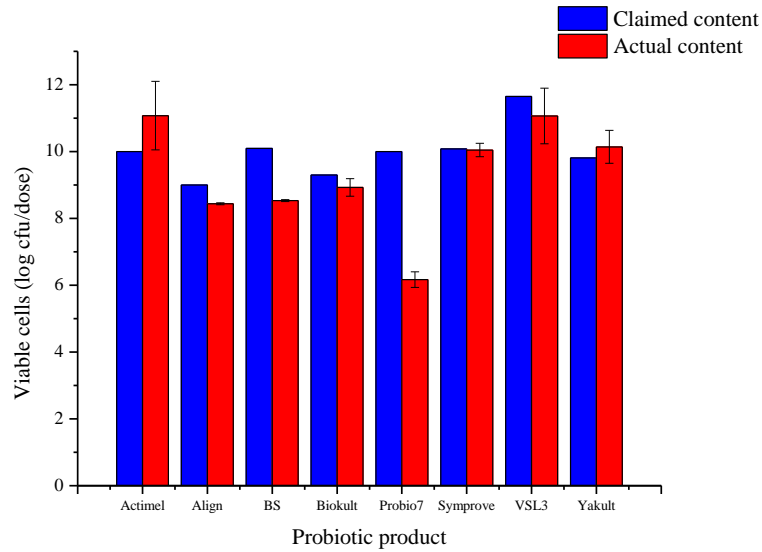
Figure 5.1. Claimed content and actual content of 8 commercial probiotic product evaluated. BS is Biobalance support. Values are expressed as log 10. Bars are SD of means of three determinations. Actual content was the same as or more than the claimed content for Actimel[®], Yakult[®], and Symprove[TM]

Even though there is no global agreement on the number of bacteria a probiotic product should contain, it is generally agreed that a probiotic product should contain $\geq 10^6$ CFU/g or mL of product and that for any therapeutic benefit, a total of 10^9 probiotic microorganisms should be consumed per serving or daily (Minelli and Benini, 2008, Champagne et al., 2011, Health Canada, 2009, Italian Ministry of Health, 2013). While most of the lyophilised products contained $<10^9$ CFU/dose, the entire products tested contained $>10^6$ CFU/dose, which implies they could have a potential clinical efficacy. However, it is only reasonable to assume that a product with a high number of viable organisms is much more likely to have a potential clinical efficacy than a product with a low number. Thus, the higher the initial cell concentration, the higher the chances of intestinal colonization and health benefit due to the barriers they need to withstand before establishing in the lower intestinal tract.

Previous studies (Temmerman et al., 2003, Lin et al., 2006) have also reported higher recovery of cells in liquid products compared to lyophilised or solid products. For instance, Lin et al., (2006) evaluating 8 brands of commercial lactic acid products, in the form of milk and yoghurt products, granule powders, and tablet formulations reported higher recovery for the liquid products than the solid products. Also, 2 of the solid

products they evaluated did not contain any viable count of lactic acid bacteria although these products were labelled to contain them. Temmerman et al., (2003) also tested 10 commercially available probiotic products, 5 of which were lyophilised products, 4 dairy products, and one fruit drink. They utilised both culture-dependent and independent analysis to determine cell counts. Their results also indicated that cell counts were much lower in the lyophilised products, yielding values between 10^5 and 10^7 CFU/g of product, while the count in dairy products were between 10^7 and 10^9 CFU/mL.

While it is acknowledged that discrepancies could occur between different media used for the determination of viable content or the techniques employed, for instance culture-dependent versus culture-independent methods (Temmerman et al., 2003), there seems to be a significant difference in recoverable viable content between liquid and lyophilised products irrespective of the methods employed which is strengthened in the present study. It is likely the lower viable content may be contributed by injured cells, suboptimal reconstitution of the products or possible instability of the lyophilised products amongst other factors (Ray et al., 1971, De Valdez et al., 1985a, Champagne et al., 1991, Zhao and Zhang, 2005, Champagne et al., 2011).

A lot of research has been carried out on the reconstitution of lyophilised bacteria due to their susceptibility to injury during lyophilisation (Ray et al., 1971, De Valdez et al., 1985a, Champagne et al., 1991, Zhao and Zhang, 2005, Santivarangkna et al., 2007, Muller et al., 2010, Champagne et al., 2011). While some authors have demonstrated or argued that the reconstitution medium was important to the recovery of bacteria (De Valdez et al., 1985a, Charteris et al., 1997, Zhao and Zhang, 2005) and recommended careful recovery of lyophilised products to levels comparable to those of wet cultures not previously dried (Charteris et al., 1997), others have shown otherwise. For instance, Teixeira et al., (1995) found out that the recovery of *Lactobacillus bulgaricus* spray dried in milk matrix was not significantly affected when rehydrated in skim milk, phosphate buffer, MRS broth or water. Ray et al., (1971) in their work on effect of rehydration on recovery, repair and growth of injured freeze-dried *Salmonella anatum* also demonstrated that added milk during lyophilisation provided the needed nutrients for the cells during reconstitution and masked the effect of any additional nutrients added to reconstitution media (Ray et al., 1971). Other authors have otherwise demonstrated or reviewed that not only is the reconstitution medium important but also all other substances carried over from the drying process, for example the milk used as

the matrix or other cryoprotective agents used during the formulation, the medium used for harvesting the bacteria and time of harvesting of the bacteria all played significant role in the recovery of dried products and even further advised that reconstitution medium was strain specific. They also argued that lyophilised bacteria could contain dead cells, unharmed cells and sub-lethally injured cells, which could repair and regain normal function if rehydrated under adequate conditions which includes besides an optimal rehydration medium, the temperature of hydration, the volume of rehydration medium and the rate of hydration (De Valdez et al., 1985a, De Valdez et al., 1985b, Zhao and Zhang, 2005, Morgan et al., 2006, Muller et al., 2010, Zhang et al., 2012).

In the present work, a buffered saline solution was used for reconstitution as most of the dried products already contained milk or other protectants or stabilizers (Table 5.1). Rehydration was carried out for 10 min at room temperature. Other comparative studies on commercial products have also used buffered saline solution (Coeuret et al., 2004, Lin et al., 2006) or peptone physiological solution (PPS) (0.1% w/v peptone, 0.85% w/v NaCl) (Temmerman et al., 2003) or Ringers (Maukonen et al., 2006). Other studies that have mostly assessed dried probiotic products have also routinely used normal saline or media (but some did not comment on the duration or temperature of hydration) and have generally reported lower viable content in relation to claimed content (Elliot and Teversham, 2004, Drago et al., 2004, Drago et al., 2010). The observed lower viable content with the dried products could therefore be reasoned to be as a result of the stress the organisms are subjected to during freeze-drying or the method employed for their recovery (reconstitution medium, duration of reconstitution, temperature of reconstitution etc.) or perhaps their instability during storage. Aureli et al., (2010) assessed probiotic products produced and distributed on the Italian market between 2005 and 2006 which they collected from the processing plants or from retailers after 3 months, 8 months and 13 months and reported instability of the products. Only 7 out of the 24 collected after 3 months were of the same composition as those obtained from the manufacturer. By the 13 months, only 1 product had the same composition (Aureli et al., 2010).

More research is therefore needed for full recovery of viable content of these products or ensuring stability of their viable content. It is likely manufacturers also perform their own viability checks of microbial composition and populations in their products hence they should also advise on the optimum means of reconstitution prior ingestion for their

specific product or means of ensuring the stability of their products. For the lyophilised products evaluated, manufacturers who advised on reconstituiton instructed the consumer to reconstitute in water or a drink (which may be detrimental to the cells if a consumer chooses a fruit juice or a drink which may be acidic). Storage was only advised to be in cool and dry places, with some stating positively that refrigeration was not needed.

5.4.3 Measurement of surface tension, osmolality, buffer capacity and pH of the porcine and simulated gastric fluids

The measurements of surface tension, osmolality, pH and buffer capacity of the porcine and simulated gastric fluids are tabulated in Table 5.4. Among the fluids, only PGF closely mimicked all the characteristics of the human gastric fluid. The other simulated fluids, SGF* and FaSSGF showed some differences to the human gastric fluid.

A lot is known about the fluid in the human stomach; it is known to have an ionic strength of 0.1 ± 0.025 mM and concentrations of potassium, sodium, chloride, calcium, at 13.4 ± 3.0 mM, 68 ± 29 mM, 102 ± 28 mM, and 0.6 ± 0.2 mM respectively (Lindahl et al., 1997). Its bile salt concentration has been reported to be approximately 0.06 mM in the fed (Rhodes et al., 1969) and 0.2 ± 0.2 mM in the fasted state (Lindahl et al., 1997) but Kalantzi et al., (2006) have reported insignificant levels in both the fasted and fed states (below their quantification limit of 500 μM). Pepsin level has also been reported to be in the range from 0.11 to 0.22 mg/mL by Kalantzi et al., (2006) while others have reported it to be in the range of 0.1 and 1.3 mg/mL in the fasted state (Schmidt et al., 1970, Lambert et al., 1968). In the fed state, pepsin level has been reported to be in the range from 0.26 to 1.72 mg/mL (Lambert et al., 1968, Kalantzi et al., 2006). Lipase activity has also been shown to be in the range from 11.4 to 43.9 U/mL in the fed stomach (Armand et al., 1996) while in the fasted state, Vertzoni and coworkers state that its activity may not be important because it is known to be active in the pH range of 3–6 and thought to be present at concentrations of 0.1 mg/mL (Vertzoni et al., 2005). Other characteristics of the human gastric fluid are given in Table 5.4.

Table 5.4. Buffer capacity, osmolality, pH and surface tension of the porcine and simulated gastric fluids

Simulated fluid	pH	Buffer capacity $(mmol.L^{-1}.\Delta pH^{-1})$	Osmolality $(mOsm.Kg^{-1})$	Surface tension $(mN.m^{-1})$
PGF**	3.38 ± 0.03	12.85 ± 0.68	255.33 ± 0.58	46.08 ± 2.55
SGF* 1.2	1.21 ± 0.02	43.33 ± 0.71	255.67 ± 0.58	74.18 ± 3.56
FaSSGF 1.6[+]	1.60 ± 0.01	41.70 ± 0.40	138.00 ± 1.00	52.04 ± 2.20
SGF* 3.4	3.36 ± 0.02	1.70 ± 0.42	82.25 ± 1.89	73.18 ± 0.25
FaSSGF 3.4	3.37 ± 0.04	1.23 ± 0.24	83.50 ± 1.92	65.38 ± 1.86
Human gastric fluid	1- 2.5 [a]	7-18 (fasted)[c]	559-217[c]	30-31[c]
	up to 5 (fed)[b]	14-28 (fed)[c]		35-45[d]

[a] Evans et al., 1988; [b] Fortran et al., 1973; [c] Kalantzi, et al., 2006; [d] Efentakis & Dressman, 1998. **PGF was taken from a freely fed animal; volume was the only variable for the fed versus fasted test system. [+]FaSSGF is made to pH 1.6 to represent the fasted state

To date, various media have been developed to simulate the fasted gastric content (British Pharmacopoeia, 2013, FDA, 2000, United States Pharmacopoeia, 2008). Often, these media try to mimic the salt concentration of the gastric fluid through the addition of 0.5% w/v of sodium chloride to HCl (Charteris et al. 1998). Surface tension and bile content is also simulated through the addition of bile salt, pepsin and/or surfactants to HCl (Vertzoni et al., 2005, Aburub et al., 2008). These characteristics and compositions

have been shown to be very important, and have a major influence on substrates (eg. drugs) or processes in the stomach (Nicolaides et al., 1999, Jantratid et al., 2008, Mudie et al., 2010). For example, the pH and buffer capacity affects the dissolution of ionizable drugs. Pepsin is also known to interfere with the stability of proteins and peptides (Mudie et al., 2010). Lipase activity affects drug release from lipid-based dosage forms (Dressman et al., 1998) whilst bile salt concentration decrease the surface tension and enhance wetting of drugs as well as increase the solubility of some drugs (Dahan and Amidon, 2008). These compositions and characterisitics could also have a major influence on the survival capacity of probiotics. For instance Zhu et al., (2006) have shown that the rate of killing of *E. coli* and *Helicobacter pylori* at pH 2.5, 3 and 3.5 in solutions was increased significantly upon the addition of pepsin (effect of pepsin-mediated proteolysis).

SGF* (Simulated Gastric Fluid, USP), which has traditionally been used to simulate the gastric conditions showed measured differences to the human gastric fluid in this study. The FaSSGF fluid, representing Fasted-State Simulated Gastric Fluid, was proposed by Vertzoni et al., in 2005 to simulate the human basal gastric juice with a glass of water, which is often taken with a dosage form. This fluid has constituted a good simulation of fasting gastric contents and has gained decent acceptance for use for *in vitro* dissolution/solubility studies (Vertzoni et al., 2005, Vertzoni et al., 2007). Our evaluation of this fluid however showed that it differed at different pH from reported characteristics of the human gastric fluid characterized in this study in terms of buffer capacity, osmolality and surface tension.

The results obtained concurs with a recent study by Pedersen et al., (2013) who also evaluated and highlighted differences between FaSSGF, HCl (pH 1.2) and their measured characteristics of aspirates of human gastric fluid obtained from volunteers between 20-79 years during gastroscopic examinations (Pedersen et al., 2013). Apart from the differences in the pH, buffer capacity, osmolality and surface tension between the simulated fluids and the human gastric fluid they reported, which is in agreement with the results obtained in the present study, these authors also demonstrated lower viscosity of FaSSGF and HCl compared to the human gastric aspirates which they attributed could be due to the presence of mucus components present in the aspirate but absent in the simulated fluids.

As mentioned previously, these non-acidic characteristics or constituents of the human gastric fluid may majorly influence the survival of probiotics and may have been the deficiency of previous studies that have assessed survival of probiotics (Chandramouli et al., 2004, Ding and Shah, 2009, Mokarram et al., 2009, Sahadeva et al., 2011, Jensen et al., 2012).

5.4.4 *In vitro* gastric tolerance of commercial probiotic products and strain in porcine and simulated gastric fluid, fed and fasted states; plate technique

The gastric tolerance of the commercial products in porcine gastric fluid simulating the fed state is presented in Figure 5.2. Four of the lyophilised products (Align®, Biobalance support, Bio-kult® and Probio 7®) and one liquid product (Yakult®) exhibited complete loss of viability within 90 min of simulated gastric transit. Symprove™, VSL#3® and hydrated Bio-kult® showed retained viability for the duration tested. Actimel® showed retained viability for 90 min but significant reduction ($P<0.05$) in viability after 90 min. Symprove™ exhibited the best tolerance relative to the other products in the fed state with no significant reductions throughout the test duration.

The fasted state gastric tolerances of the commercial products in the porcine and simulated gastric fluids at similar pH are also compared in Figure 5.3a to 5.3c. In general, the viabilities of the products within the first 30 min were improved relative to the fed state. For all test systems at this pH (3.4), the results also highlighted important differences between the more bio-relevant fluid, PGF (Figure 5.3a), and the other simulated fluids: SGF* (Figure 5.3b) and FaSSGF (Figure 5.3c), particularly evident for some products.
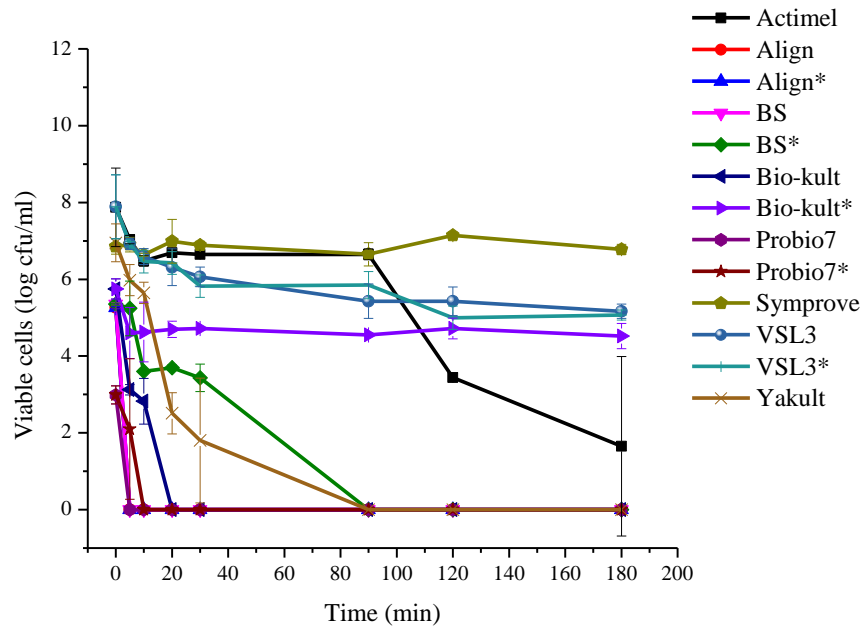
Figure 5.2. Gastric tolerance of commercial probiotic products in porcine gastric fluid mimicking the fed state. Viable count (log CFU/mL) of each product at 5, 10, 20, 30, 90, 120 and 180 min were compared with log CFU/mL in the product at 0 min. The results are expressed as mean ± SD (n=3). *means hydrated
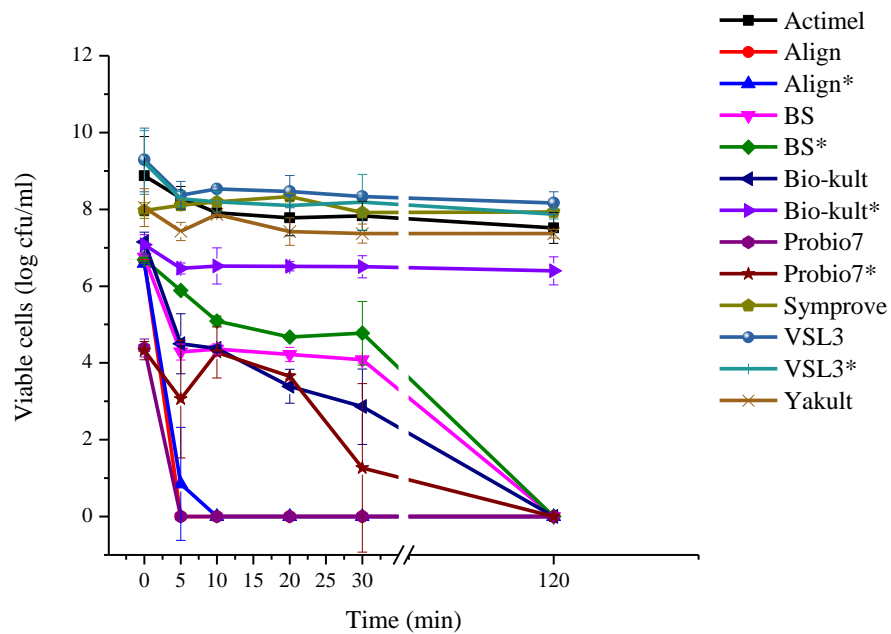
Figure 5.3a. Gastric tolerance of commercial probiotic products in PGF pH 3.4 mimicking the fasted state. Viable count (log CFU/mL) of each product at 5, 10, 20, 30, and 120 min were compared with log CFU/mL in the product at 0 min. The results are expressed as mean ± SD (n=3). *means hydrated.

213
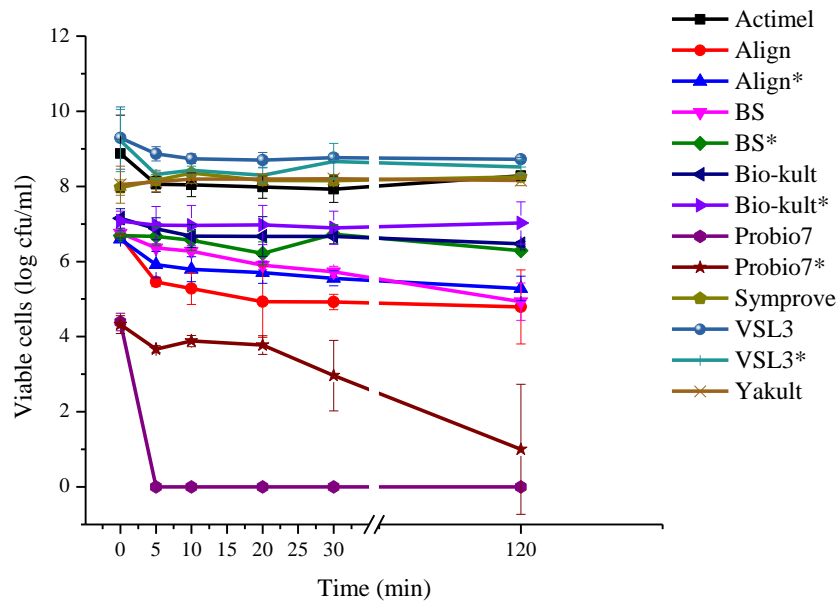
Figure 5.3b. Gastric tolerance of commercial probiotic products in SGF* pH 3.4 mimicking the fasted state. Viable count (log CFU/mL) of each product at 5, 10, 20, 30, and 120 min were compared with log CFU/mL in the product at 0 min. The results are expressed as mean ± SD (n=3) *means hydrated.
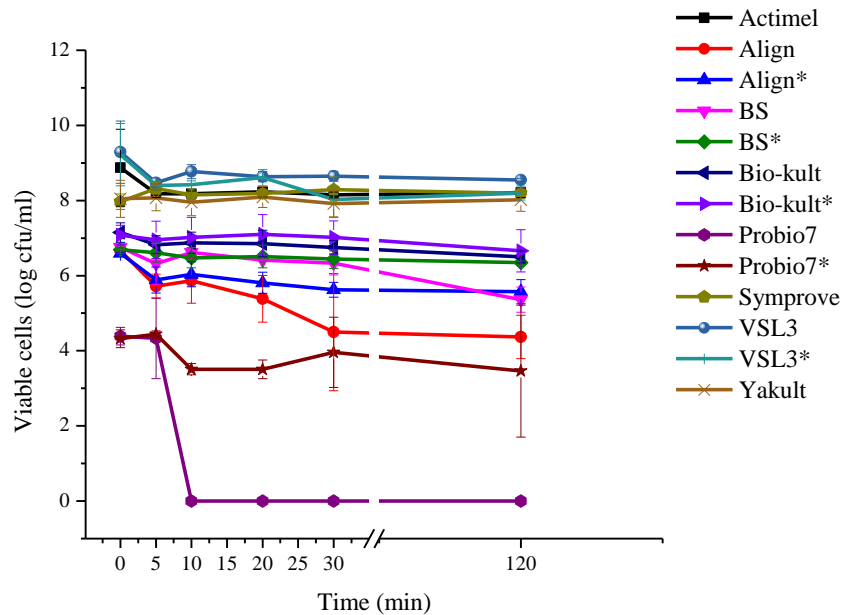
Figure 5.3c. Gastric tolerance of commercial probiotic products in FaSSGF pH 3.4 mimicking the fasted state. Viable count (log CFU/mL) of each product at 5, 10, 20, 30, and 120 min were compared with log CFU/mL in the product at 0 min. The results are expressed as mean ± SD (n=3). *means hydrated.

In PGF, Align®, and unhydrated Probio 7® showed complete loss of viability within 10 min whilst unhydrated Biokult®, Biobalance support and hydrated Probio 7® showed loss of viability within 2 h. The other products retained viability for the duration of the assay in PGF (Figure 5.3a). In SGF* and FaSSGF adjusted to pH 3.4, the product performances were significantly improved with only Probio 7® showing complete loss of viability within 2 h of product tolerance (Figure 5.3b and 5.3c).

The results from gastric tolerance testing at the lower pH for SGF* (1.2) and FaSSGF (1.6) are also shown in Figure 5.4a and 5.4b. The artificial fluids at this pH, especially FaSSGF (1.6) were more in congruent with PGF than at pH 3.4.

The fasted state gastric tolerance of the two commercial strains, *L. acidophilus,* LA-5® and *B. lactis*, BB-12® are shown in Figure 5.5. Both strains had poor resistance in PGF but they showed different degrees of tolerance. The lactobacilli strain showed complete loss of viability within 10 min of exposure but the bifidobacteria strain showed gradual exponential loss of viability in PGF.
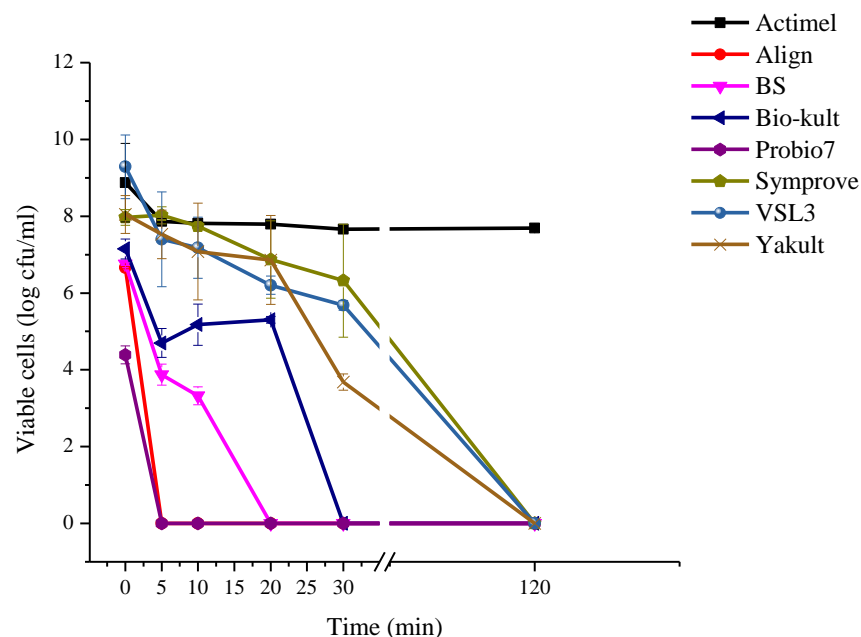
Figure 5.4a. Gastric tolerance of commercial probiotic products in SGF* 1.2. Viable count (log CFU/mL) of each product at 5, 10, 20, 30, and 120 min were compared with log CFU/mL in the product at 0 min. The results are expressed as mean ± SD (n=3).
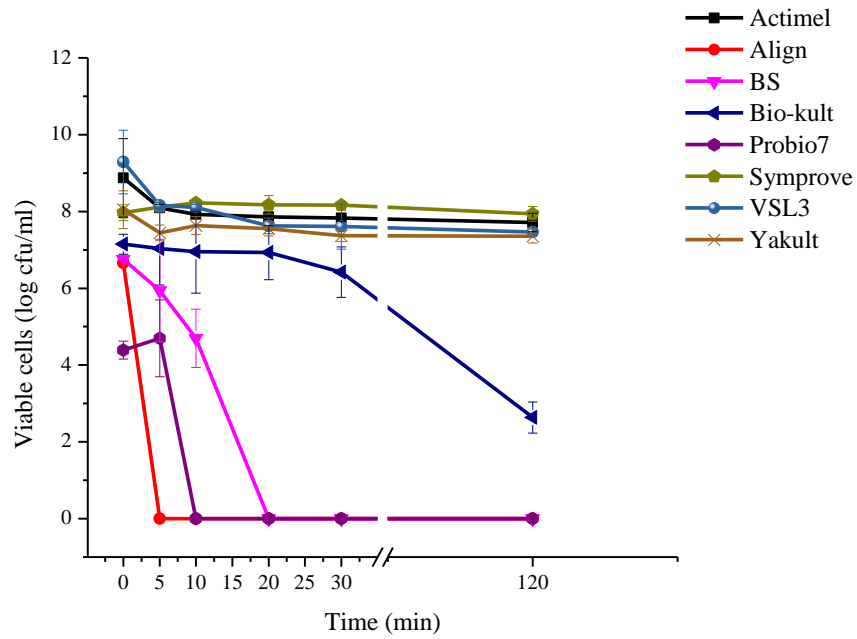
Figure 5.4b. Gastric tolerance of commercial probiotic products in FaSSGF pH 1.6. Viable count (log CFU/mL) of each product at 5, 10, 20, 30, and 120 min were compared with log CFU/mL in the product at 0 min. The results are expressed as mean ± SD (n=3).
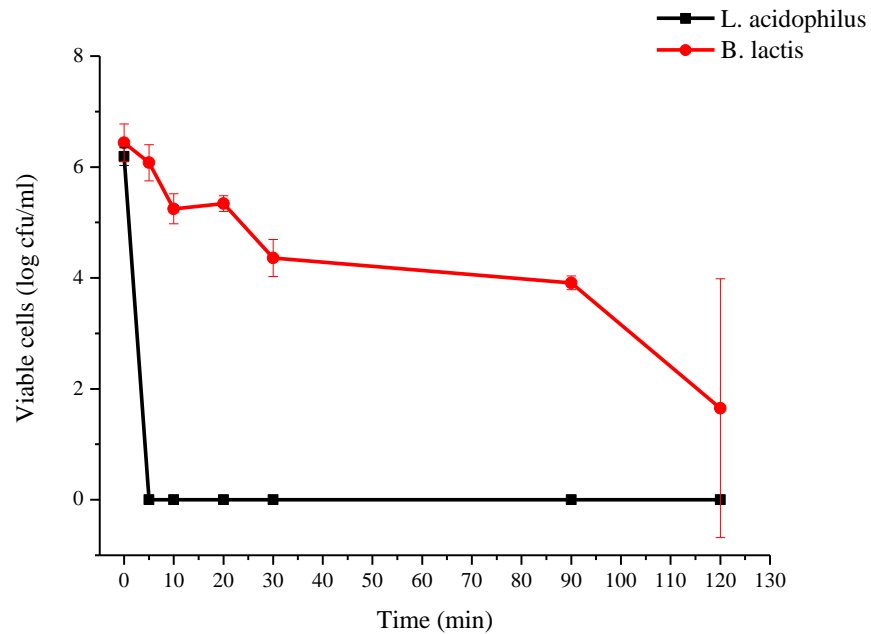
Figure 5.5. Gastric tolerance of free cells of La-5[®] and BB-12[®] in simulated gastric fluid, PGF mimicking the fasted state. Viable count (log CFU/mL) of each strain at 5, 10, 20, 30, 90 and 120 min was compared with log CFU/mL at 0 min. The results are expressed as mean ± SD (n=2)

The findings from this work suggest a number of things. Firstly that the probiotics evaluated survived better in the fasted state than the fed. As pointed out earlier, in the fed state, large volumes of gastric juice is secreted to digest the food ingested. Volumes as large as 800 mL have been reported to be secreted in response to 400 mL of ingested solid meal (Malagelada et al., 1976, Goetze et al., 2009). In the fasted state, however, volumes of gastric juice are much lower. At the same pH for the fed and fasted, the results show that the products reacted differently. For the liquid products, the volumes per dose were equivalent to the volume of gastric fluid in the fasted state or more. This meant some of these products may have diluted the gastric fluid and produced a final pH that was more tolerable to the probiotics contained in the product. For example, a dose of Actimel® was 100 mL; more than 60% the volume of simulated gastric fluid. This volume was quite adequate to dilute the simulated fluid in the fasted state during the study compared to the other products or even the other liquid products given that the pH of a freshly opened Actimel® (4.26 ± 0.01) was significantly higher (P<0.05) than Symprove™ (3.83 ± 0.01) or Yakult® (3.67 ± 0.01). For instance, upon addition of Actimel® to SGF* pH 1.2, the pH of the final mixture was 3.30 compared to 1.68 for a final SGF*-Yakult® mixture. This buffering effect however might not occur in *in vivo* situation, as the stomach may react to this and may reduce the pH further so negating any benefit. At higher volume of gastric fluid however, the effect of product volume was not as pronounced even though the liquid products still fared better. This increased volume also affected VSL#3® whose strains may also have depended on other properties instead of their intrinsic resistance to the gastric fluid. This product, relative to the others except Actimel®, contained higher initial load of organisms per dose of product with a dose weight more than ten times of any of the other lyophilised products. In a tolerance assay simulating the fasted state in PGF, the viability was equivalent or better than the liquid products, which could have been due to the buffering effects of its excipients in the small volume of gastric fluid (unlike other lyophilised products, VSL#3® significantly altered the pH of gastric fluid in the fasted state) however in the fed state, when exposed to greater volume of gastric fluid, the overall loss was greater.

The residence time is also a major important factor for viability in both the fasted and fed state. The residence time of ingested products is much longer in the fed state than in the fasted state. In the fed state, normal gastric transit times are 90-180 min even though they could also range between 70-130 min (Dressman, 1986) and are usually influenced by contractions, pyloric resistance and feedback mechanisms in the duodenum resulting

in slower and linear emptying pattern (Mudie et al., 2010). In the fasted state however, gastric emptying is known to be mostly controlled by the migrating myoelectric complex (MMC), and therefore is known to be much shorter (mean half- time for emptying water is 15.8 min) and exponential but is also affected by the nature, size, or amount of liquid or solid ingested (Mudie et al., 2010). For example, Dressman, (1986) was able to demonstrate that a 25% glucose solution influenced the MMC and consequently emptied in 75 min when ingested. This implies that in *in vivo* situation, the stomach may treat some of these products as food and may retain them for longer even in the fasted state. In that vein, consumers might still benefit from the liquid products as these showed sustained tolerance for periods of 120 min in the fasted state unlike the lyophilised products in the bio-relevant fluid. Even among the liquid products, the water-based probiotic (Symprove[TM]) might fare better *in vivo* as it will be expected to transit faster due to the less amount of food ingredient it contains.

The findings from this work secondly suggest that gastric survival of probiotics majorly depends on the way the products are formulated. The investigation looked at representative samples of the different formulations available to consumers, namely solid lyophilised preparations both capsules and powder and liquid formulations, both dairy and non-dairy based. Free-living unformulated commercial probiotic strains were also investigated. Even though it is a prerequisite that commercial probiotic organisms should be able to survive in simulated gastric juices (FAO/WHO, 2001), some commercial strains, as demonstrated by the results and other studies (Collado et al., 2005, Jensen et al., 2012) rarely possess this property. For instance, Jensen et al., (2012) investigating the gastric tolerance of eighteen commercial and potential probiotic lactic acid bacteria to simulated gastric fluid at pH 3, also demonstrated significant reductions within 90 min of gastric tolerance for half of the strains with four strains showing less than 1.2 log-CFU/mL of viable cells.

From results obtained in this study and previous studies, it seems that survival of probiotics in gastric fluids and survival during transit may consequently depend on the way they are formulated. In other words, gastrointestinal challenges can also be overcome if a non-intrinsically gastric resistant probiotic is adequately provided with protection systems for the low pH. Symprove[TM] for instance, may depend on the barley (Table 5.1), a prebiotic, not digested by pancreatic enzymes for enteric protection of the organisms against the harsh gastric environment, which may also provide the probiotic

organisms with a source of nutrient. A study by Charalampopoulos et al., (2003) has revealed protective effects of barley on viability of *L. plantarum*, *L. acidophilus* and *L. reuteri* under acidic conditions. About 3-log and 1.5-log cycles of improvement of viability was noted for *L. plantarum* and *L. acidophilus* (which are also component species of Symprove^{TM}) in the presence of barley (Charalampopoulos et al., 2003). Actimel® and Yakult® on the other hand are fermented milk products which may depend on the milk proteins for protection and nourishment (Livney, 2010). In addition, the fat content of these products may protect the organisms in passage through the gastrointestinal tract (Ranadheera et al., 2012); a small study has highlighted this by showing significant reductions of *L. casei* DN-114 001 within Actimel® Fat Free (0.428 ± 0.059-log CFU mL^{-1}), after 5 h of simulated gastrointestinal transit (P<0.05), compared with insignificant reductions for Actimel® Original (Wills, 2012). Furthermore, as already pointed out, the pH of these liquid products were acidic, likely due to the metabolic activities of the probiotic strains themselves or other starter cultures hence, they may have developed the necessary factors for acid stress (Cotter and Hill, 2003) and may have therefore coped better than the lyophilised products when placed into systems akin to their natural milieu. The results suggest that even among the liquid products, there may have been less acid shock for products with lower product pH especially within the first few minutes in simulated gastric environment.

The lyophilised products on the other hand may not have had robust systems for enteric protection besides their possible lack of intrinsic or relevant acid stress adaptation mechanism or factors. Besides, their dispersion in the simulated fluids did not significantly alter pH except VSL#3®. However it was also observed that although most of these lyophilised products demonstrated poor tolerance in the porcine and simulated gastric fluids relative to the liquid products, when rehydrated prior tolerance, these products generally showed appreciable viability. This may be due to the improved integrity or repair (through hydration) of the cells (which were possibly damaged during lyophilisation), which made the cells perhaps more tolerant to the harsh treatment in the simulated gastric fluid.

Generally, consumers often prefer solid-dose products as they are less bulky, convenient and require less specific storage conditions (Aulton, 2013). When compared to solid formulations, liquid dosage forms are often quite unstable once opened (Bansal and Garg, 2008, Aulton, 2013). Solid dosage forms of probiotic may therefore present an

advantage of stability and long shelf life; the lyophilised products evaluated however did not particularly exhibit this. Means of ensuring their stability and gastrointestinal survival need to be explored.

Thirdly, the findings suggest that the PGF provided a harsher environment than the other simulated systems: SGF* and FaSSGF at similar pH. Even when these simulated systems were used at pH more consistent with the human stomach, results with some of the products were sometimes not as poor as in PGF. PGF may have represented a harsher environment as a result of the enzymatic and digestive substance composition of the fluid; these are important variables of the human gastric fluid that are often ignored in viability testing. The measurements of surface tension, osmolality and buffer capacity of the gastric fluids also corroborate the relevance of using a more bio-relevant fluid for gastric tolerance testing. The results from the PGF system may therefore be more representative of how the products evaluated will perform *in vivo* and are also important in terms of relevant results for decisions about which product consumers should use and how to use them.

5.4.5 Microcalorimetric gastric tolerance assay mimicking the fasted state

The power-time curves of Actimel[®], Symprove[TM], VSL#3[®] and Yakult[®] in PGF simulating the ratio of fluid to product in the fasted stomach are shown in Figure 5.6. The power-time curves show the abrupt decay of the products in PGF after very brief period of growth. Amongst the products, VSL#3[®] was associated with the most heat output during its metabolism in the simulated fluid.
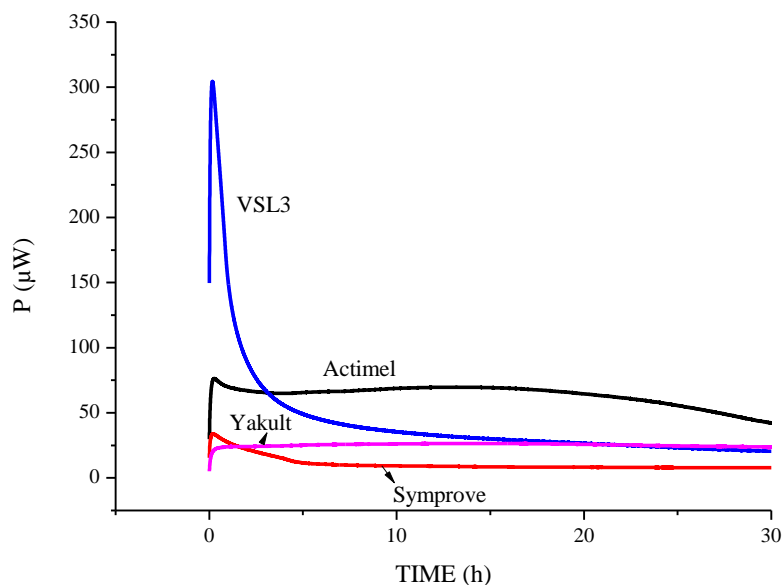
Figure 5.6. Power-time curves of Actimel®, Symprove™, VSL#3® and Yakult® in PGF mimicking the ratio of fluid to product in the fasted stomach

The cumulative heat plots of the different products in MRSc after 30 min gastric tolerances in the different simulated fluids mimicking the fasted state are also shown in Figures 5.7a-5.7c and 5.8a-5.8b. The results show very good correlation with the plate count. The liquid products showed better tolerance to the simulated gastric fluids as they showed less lag period compared to the lyophilised products. The differences in the simulated fluids were also highlighted with the microcalorimeter; showing again that the PGF was harsher and feasibly, a more realistic simulant of the human gastric fluid. The effect of hydration of the products was also demonstrated as some of the lyophilised products (especially Biokult® and Biobalance support) showed better performances when hydrated prior in SGF*(3.4) and FaSSGF (3.4), which were comparable to the liquid products (Figure 5.7b and 5.7c) even though the inocula of the hydrated lyophilised products were more dilute relative to their unhydrated counterparts. However, the measured characteristics of the simulated fluids suggested SGF* and FaSSGF at pH 3.4 do not adequately mimic the human gastric fluid, hence survival of the cells in these fluids were not biologically relevant for predicting *in vivo* tolerance.

The simulated fluids at lower pH were also in agreement with PGF for some products than at their equivalent pH. The better survival of the products in SGF* at pH 3.4

relative to 1.2 for both the microcalorimetric assay and the plate method is in accordance with findings from Sahadeva et al., (2011) who also reported tremendous reductions in viability at pH 1.5 for some probiotics but unchanged viability when the products were assessed in the simulated fluid at pH 3 relative to the viability at 0 h.
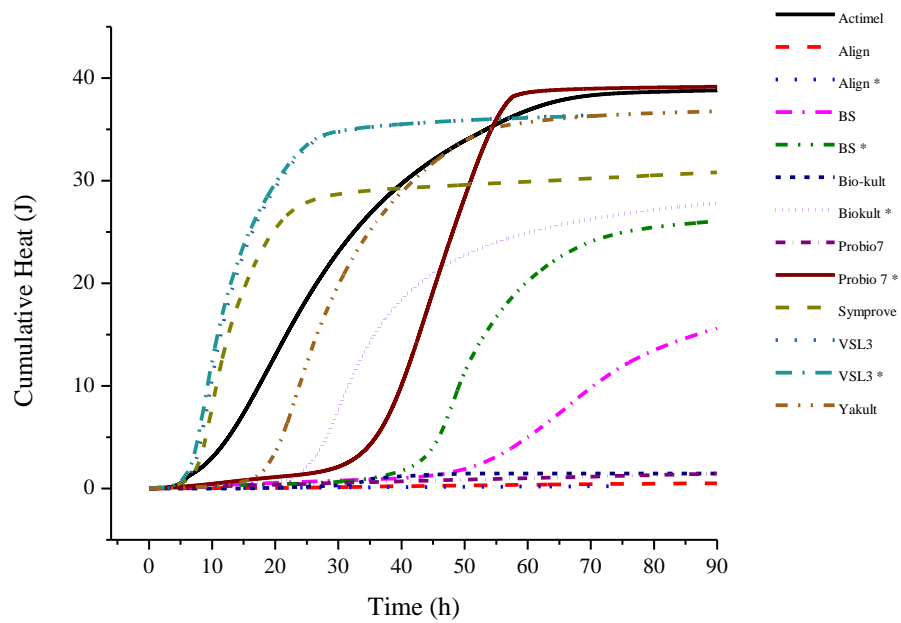
Figure 5.7a. Heat curves of commercial products inoculated into MRSc broth after 30 min of gastric tolerance in PGF pH 3.4 representing fasted state tolerance
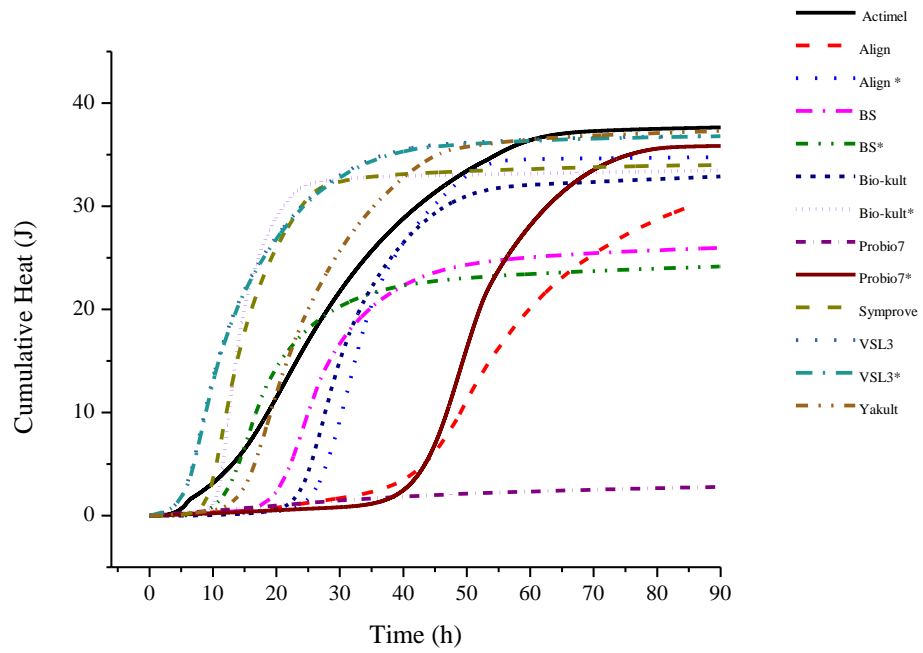
Figure 5.7b. Heat curves of commercial products inoculated into MRSc broth after 30 min of gastric tolerance in SGF* pH 3.4 representing fasted state tolerance
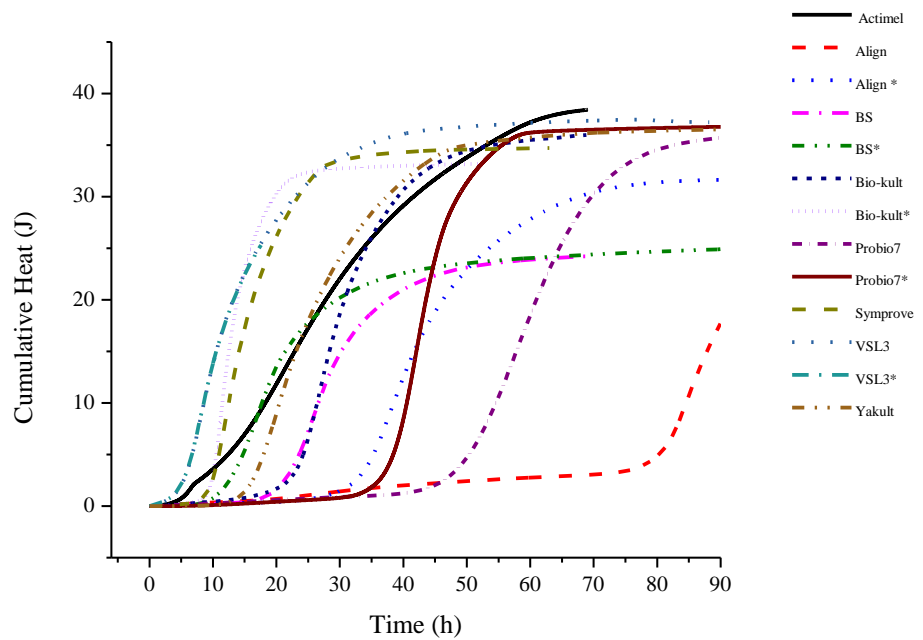
Figure 5.7c. Heat curves of commercial products inoculated into MRSc broth after 30 min of gastric tolerance in FaSSGF pH 3.4 representing fasted state tolerance
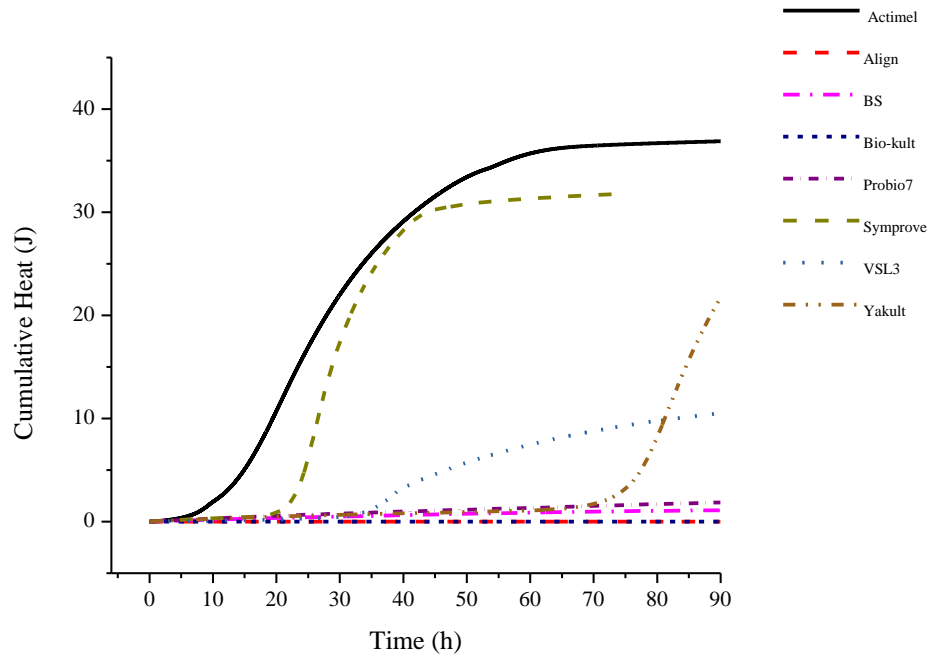
Figure 5.8a. Heat curves of commercial products inoculated into MRSc broth after 30 min of gastric tolerance in SGF* pH 1.2 representing fasted state tolerance
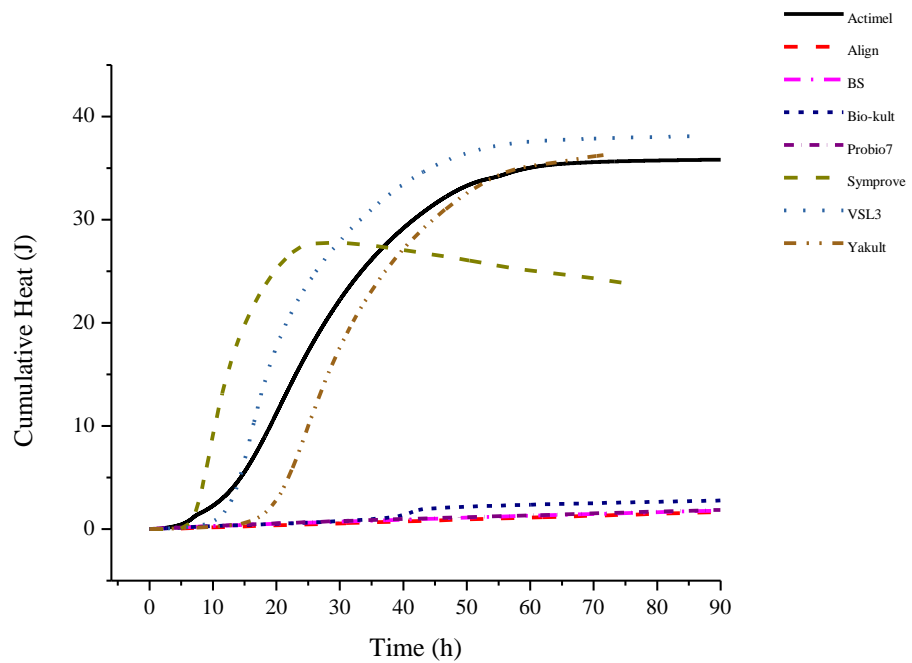
Figure 5.8b. Heat curves of commercial products inoculated into MRSc broth after 30 min of gastric tolerance in FaSSGF 1.6 representing fasted state tolerance

The results from the microcalorimeter are important in that they simulate the survival of the probiotics in the gastric fluid and possible growth thereafter. Hence, for solid products that may have delivery systems to prevent acid release, the microcalorimetric data could have provided evidence of release in non-acidic environment. Also, even though the microcalorimetric data were collected over periods of 90 h, (which was for the sake of comparative analysis with the plate technique) data could have been gathered over a much shorter duration and inoculations could have also been made to higher concentrations. For some of the multistrain products, the microcalorimeter additionally was able to differentiate different cells that had survived hence in some instances, some multistrain products assumed the same power-time curve (differentiated data not shown) inferring that some species in some of these products were possibly killed in the simulated fluids and the species remaining were similar to the species contained in other products.

When the product-simulated gastric fluid mixtures were directly placed in the microcalorimeter and studied, the power-time curves generated showed decay unlike the classic growth curves possibly due to the harsh environment of the fluid. The curves suggested mere survival of the cells in the harsh environment of the gastric fluid. However because the instrument was measuring heat output derived from respiring cells in the product-gastric fluid mixture, it is likely the simulated ratio of gastric fluid to product skewed the results to favour the highest volume of gastric fluid (due to extra nutrient available to the few cells) or the lowest volume of gastric fluid (due to the extra volume of nutrient available in the product itself). The ampoule microcalorimeter was therefore not useful for such study design.

Overall, this gastric tolerance study is the first to provide comparative data on actual products in situations that most closely mimic the *in vivo* condition. Other studies only test tolerance of individual strains of bacteria rather than products and often look at these strains or product in isolation (Sun and Griffiths, 2000, Huang and Adams, 2004, Corcoran et al., 2004, Chandramouli et al., 2004, Iyer and Kailasapathy, 2005, Sabikhi et al., 2008, Ding and Shah, 2009, Mokarram et al., 2009). The few studies that have compared products (Lin et al., 2006, Sahadeva et al., 2011) like most other studies used test systems that may also have little relevance for the *in vivo* situation.

5.4.6 Pharmaceutical consideration: Freeze-drying and storage of LA-5[®]

The survival of LA-5[®] at each stage of the freeze-drying process is shown in Figure 5.9. The viability loss was greatest during the drying process than the controlled freezing with significant loss (P<0.05) occurring when cells were freeze dried in 15% v/v glycerol.
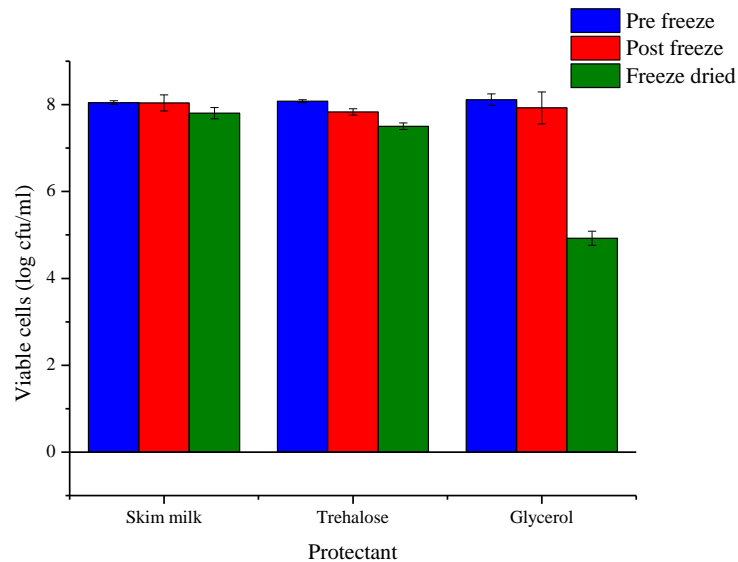
Figure 5.9. Viability of probiotic strain, LA-5[®] after freezing and freezing-drying using different protectants: 10% w/v skim, 10% w/v trehalose and 15% v/v glycerol. n=3. There was significant loss of the cells when freeze-dried

The viability of the cells freeze-dried in the different protectants during storage at room temperature and at 4°C is also shown in Figure 5.10a and 5.10b. Differences were observed among the protectants and the two storage conditions. For 15% v/v glycerol, total loss of viability was observed within 3 days after freeze-drying. During storage at 4°C, viability was generally maintained for 1 month with no significant reduction (P>0.05) in viability observed for cells freeze dried in skim milk or trehalose.

226
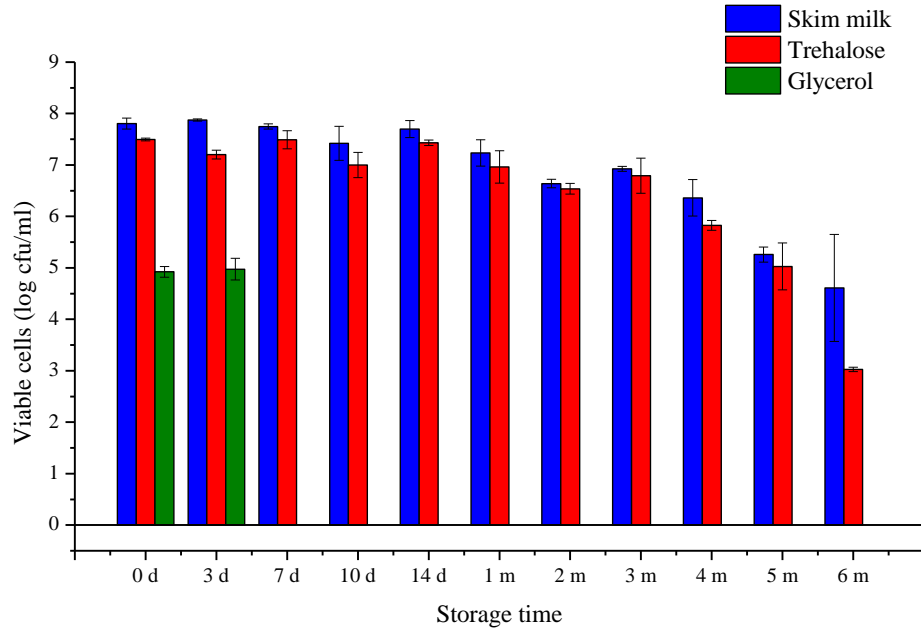
Figure 5.10a. Viability of freeze-dried *L. acidophilus* LA-5® using the protective agents during storage at 4°C for 6 months. Data are expressed as mean ± SD (n=3). d = days, m = months
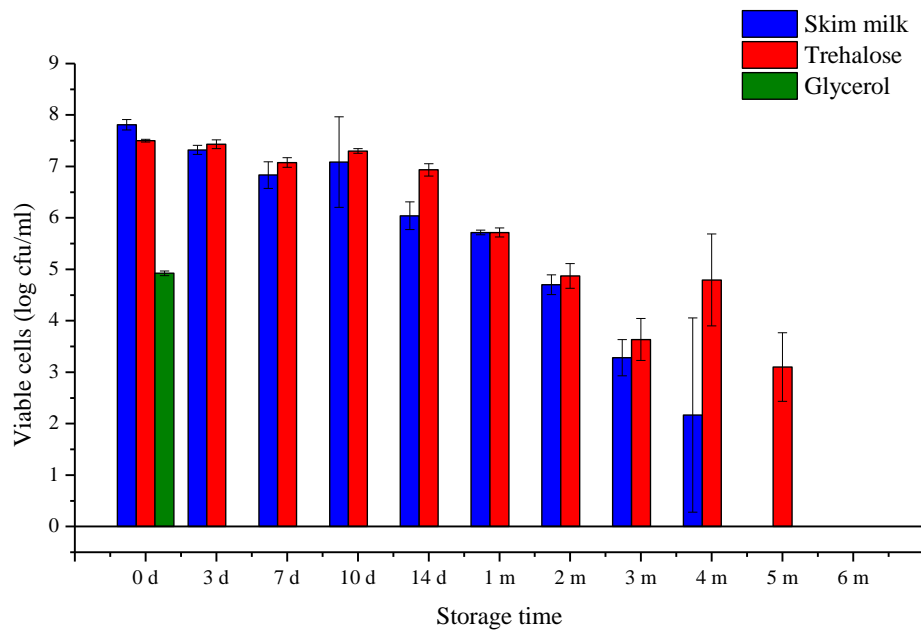
Figure 5.10b. Viability of freeze-dried *L. acidophilus* LA-5® using the protective agents during storage in room temperature for 6 months. Data are expressed as mean ± SD (n=3). d = days, m = months

For the room temperature storage, the viability of the cells in both skim milk and

trehalose decreased over time but the losses that occurred were not significantly different (P>0.05) from day 0 up to day 14 day for each protectant. Trehalose performed better in protecting the cells at this storage condition.

Even though freeze-drying is the favourite method of producing bacterial cultures (Morgan et al., 2006), there are losses in cell viability during the process. One of the main causes of the loss of viability is the need of freezing the samples before dessication (Morgan et al., 2006). The freezing process and especially the cooling rate could damage the cells as discussed in Chapter 2. Our experience with freezing bacteria indicated that freezing of bacteria over liquid nitrogen vapour produced better recoveries than uncontrolled freezing in liquid nitrogen or in the freezer where larger ice crystals may form which can damage the cell membrane leading to death of the culture. In the study, when LA-5® was frozen over liquid nitrogen vapour, the results showed insignificant losses of cell viability during the freezing process. The results showed that the major losses occurred during drying. As previously described, freeze-drying process involves two key drying stages: primary and secondary drying (Tang and Pikal, 2004, Morgan et al., 2006). During the primary drying, samples are held at low temperatures and the water in the frozen sample sublimed by the reduction of the chamber pressure below the ice pressure within the sample (Tang and Pikal, 2004). During the secondary drying, the bound water within a sample is sublimed by slowly increasing the chamber temperature. The removal of water can cause loss of the structure and function of these cells and this can be prevented when protective agents are added (Costa et al., 2000, Abadias et al., 2001a, Carvalho et al., 2004a, Morgan et al., 2006). These protectants either increase the viscosity inside or outside the cells to reduce the molecular mobility to a minimum value (Morgan et al., 2006) or form hydrogen bonds, which help to maintain the tertiary protein structure (Leslie et al., 1995).

The results show that even though 15% v/v glycerol was a good cryoprotectant for freezing of bacteria, it was not a good protectant for freeze-drying. During drying, the aqueous medium in which glycerol was dissolved may have been initially evaporated, leaving a concentrated glycerol solution which might have caused an osmotic shock in the cells. Between the other two protectants, trehalose performed better in protecting the cells when they were stored at room temperature. This is not surprising because trehalose has a high glass transition temperature, T_g, of 110^oC (Hernandez Garcia, 2011) in the anhydrous form hence will enable an amorphous trehalose obtained as a result of

freeze-drying to remain stable at elevated temperatures, for example, at 25°C (room temperature). Apart from this, trehalose is also capable of forming crystalline hydrates and it has been suggested that formation of some crystalline hydrate results in keeping the remaining amorphous sugar dry, thereby maintaining a high T_g and stability of the freeze dried matrix (Crowe et al., 1998). Skim milk, on the other hand has a glass transition of 92°C (Roos, 2002) and is also known to be stable above its glass transition with acknoweledged extra positive factors such as providing protective coating for the cells through its proteins and also creating porous structure in freeze-dried samples hence making rehydration easier (Carvalho et al., 2004b). However, presence of moisture has been found to significantly alter its glass transition. For example a moisture content of 4.52 g/100 g of dry powder has been shown to lower the glass transition of skim milk to 46.7°C (Ozmen and Langrish, 2002). Trehalose may therefore be better as a protectant for storage in humid environments. Both protectants were nevertheless better when stored at 4°C than at room temperature even though there were still significant losses in viability over the duration of storage at 4°C, which could be attributed to factors such as the water activity in the freeze-dried bacteria and also the absence of antioxidants. These factors are evinced in Kurtmann et al., (2009) study in which they showed the combined effect of water activity, oxygen level and the concentration of ascorbate on the 12 weeks storage stability of LA-5® freeze–dried using maltodextrin or sucrose as protectants with or without sodium ascorbate. These authors demonstrated that increasing water activities decreased the survival of LA-5® during storage whilst reduced oxygen level significantly improved the storage stability of the cells. The authors also showed that the detrimental effect of oxygen was eliminated by including ascorbate in the freeze-drying medium (Kurtmann et al., 2009).

Almost all of the lyophilised commercial products stated the product did not require refrigeration. Most were instructed to be stored in cool and dry places. Only one of products stated it should be stored in the fridge. The results clearly demonstrate the advantage of storing these products in the fridge. Other studies (Abadias et al., 2001b, Costa et al., 2002, Savini et al., 2010) have also shown the advantage of storing freeze-dried probiotics or bacteria at 4°C than at room temperature storage. For instance, Costa et al., (2002) reported a 3-log reduction of viability of freeze-dried *Pantoea agglomerans* after storage in room temperature for 90 days compared to 0.5-log reductions at 4°C.

5.5 Chapter summary

The use of probiotics has gained worldwide recognition and has a huge market. Proper product quality control needs to be conducted to ensure that the content claims of labels are actually present at the time the product reaches a consumer. There also needs to be stringent assays nonetheless simulating the human gut to determine if products could potentially survive and colonize the intestine for therapeutic benefit. The results from this study have shown that the probiotic products evaluated contained at least one of the labelled species, but generally, some did not meet their label claim, which agrees with previous studies (Temmerman et al., 2001, Weese, 2002, Elliot and Teversham, 2004, Drago et al., 2004, Drago et al., 2010, Aureli et al., 2010, Weese and Martin, 2011).

For the gastric tolerance assays, the study differentiated between the fed and fasted state. It was assumed that in the fasted state the fluid volume of the stomach was 60 mL and in the fed state, 1500 mL. The ratios of simulated gastric fluid to product volume used to complete the tolerance assays were determined according to the stated dose on the product. The assays were conducted for 2 h to mimic the transit through the stomach in the fasted state and in excess to mimic the longer transit time in the fed state. The results suggest that the probiotic products survived better in the fasted state hence it would be appropriate if they are taken on an empty stomach as this will ensure that they are exposed to less fluid volume and transit faster to the intestine where the cells could be more stable. The results also highlighted important differences between the bio-relevant porcine gastric fluid and the other simulated gastric fluids showing that the other fluids at lower pH were in agreement with PGF at pH 3.4 than at equivalent pH with PGF.

Although food-based probiotics account for majority of commercial products, probiotics are also frequently sold as supplements- dried form; freeze-dried powders packed into capsules or sachet (Saxelin, 2008) as this offers a convenient means of storage and transport for both the manufacturer and the consumer and also reduces the overall cost to both. Findings from this study were that most of the lyophilised probiotics did not survive well in the products. A small study investigated their survival at different storage conditions by freeze-drying LA-5® and storing at room temperature and at 4°C. The different stages of the freeze-drying and storage showed that significant losses occurred during the drying stage than the controlled freezing. Storage of the freeze-dried cells at 4°C was better than at room temperature.

The limitations of these experiments were that for the identification of the species contained in the commercial products, morphological identification of the colonies, Gram stain appearance and biochemical tests were used to identity the strains without any genetic method. Misidentification of the species sometimes occurred and bifidobacteria could also be only identified at the genus level. The results may have also been influenced by the culturing condition used. Still, given this limitation, the results gave useful information about the quality of the products.

The tolerance assays did not simulate the processes of digestion, gastric secretion and transit however the test systems that were used mimicked the gastric composition, volume and emptying rate. Also, while PGF appeared to be the most bio-relevant fluid tested, the pH of this fluid was higher than that generally seen in the fasted human stomach since this was taken from a freely fed animal. In the PGF test systems, volume was therefore the only variable factor between the fed and fasted state. Also, other crucial factors that could affect viability in the gut such as intestinal transit or the adhesive properties of the bacteria in the large intestine, which are important for survival and functionality of probiotics apart from gastric tolerance, were not tested.

6 Prebiotic effect

6.1 Introduction

The *in vitro* assessment of efficacy of prebiotics usually involves elaborate microbiological techniques or a combination of microbiological techniques and molecular methods (Sghir et al., 1998, Venema et al., 2003, Probert et al., 2004, Van de Wiele et al., 2004, Van den Abbeele et al., 2013). In this small study, the microcalorimeter was applied to investigate the effect of a potential prebiotic.

Prebiotics are non-digestible food ingredients that stimulate the growth of beneficial microorganisms of the microbiota. Its concept was first introduced by Gibson and Roberfroid, (1995) who defined them as "non-digestible food ingredients that beneficially affects the host by selectively stimulating the growth and or activity of one or a limited number of bacteria in the colon and thus, improves health" (Gibson and Roberfroid, 1995). In 2004, the definition of a prebiotic was amended as "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host wellbeing and health" (Gibson et al., 2004). At the 6th Meeting of the ISAPP (London, Ontario), prebiotics were redefined as "selectively fermented dietary ingredients that result in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health". The concept of prebiotics was based on the fact that establishment of exogenous bacteria (probiotics) could be limited with their manipulative effect on the endogenous microbiota only short-lived (Gibson and Roberfroid, 1995). Hence prebiotics were introduced to selectively stimulate the growth and or activity of bacterial species already resident in the colon by providing a specific substrate for their growth and metabolism (Gibson and Roberfroid, 1995). The microbiota derives substrates for growth from the diet, such as substrates that have escaped digestion in the upper gastrointestinal tract and endogenous sources such as mucins (Rowland et al., 1985, Cummings et al., 1989). Most of the bacteria found in the colon (the most heavily colonized part of the gut) are strict anaerobes and these achieve nutrition basically by fermentation. The major substrate of fermentation which these bacteria use for growth are dietary non-digestible carbohydrates which comprise resistant starches, resistant dextrins, dietary fibres, non-digestible oligosaccharides (eg. raffinose, stachyose, inulin-type fructans, galactans and mannnans), non-starch

polysaccharides (NSP eg. pectins, arabinogalactans, gum Arabic, guar gum and hemicellulose), undigested disaccharides (eg. lactose) and sugar alcohols (eg. lactitol and isomalt) (Bingham et al., 1990, Cummings and Macfarlane, 1991, Roberfroid et al., 2010). These are part of the diet, which are not digested in the upper part of the digestive system. It must be noted that while the microbiota is distributed throughout the gut, transit of food through the stomach and small intestine is usually rapid for the microbiota to exert a significant effect until the available food substrates get to the colon, where movement is markedly slow, hence the colonic microbiota have ample time to degrade (Flint et al., 2008, Roberfroid et al., 2010). It is known that although an extensive variety of colonic microbiota ferment resistant starch, NSP, most dietary fibres and some non-digestible oligosaccharides, their respective degree of degradation may vary (Englyst and Macfarlane, 1986). Some non-digestible oligosaccharides (for example raffinose, inulin-type fructans and galactans) arriving at the colon are known to be rapidly and selectively fermented by only smaller number of bacteria for example, bifidobacteria and lactobacilli (Roberfroid et al., 2010).

The main bacteria of the colonic microbiota that are saccharolytic and contribute to most of the fermentative activity in the gut are members of the genera *Bacteroides, Bifidobacterium, Ruminococcus, Eubacterium, Lactobacillus* and *Clostridium* (Roberfroid et al., 2010). Apart from the dietary non-digestible carbohydrates, other substrates used by the colonic microbiota for growth include proteins, peptides, amino acids, lysed bacteria and sloughed epithelial cells. The proteolytic members of the colonic microbiota are members of the genera *Bacteroides* and *Clostridium* (Roberfroid et al., 2010). The degradation of proteins by the colonic microbiota may lead to toxic metabolites such as ammonia, phenolic compounds and amines and has been linked to diseases such as colon cancer and IBD when the protein fermentation occurs in excess in the distal colon (Macfarlane et al., 1986, Smith and Macfarlane, 1996, Roberfroid et al., 2010). But saccharolytic fermentations leads to end products such as SCFA, lactate, pyruvate, ethanol, succinate and gases such as H_2, CO_2, CH_4 and H_2S (Cummings, 1981, Macfarlane and Macfarlane, 1997). The usefulness of the products of carbohydrate fermentation of these bacteria has already been discussed in Chapter 1. Consequently, an important aspect of prebiotics is their selectivity for the growth of some saccharolytic members (mostly the *Bifidobacterium* and *Lactobacillus*) of the microbiota hence their ingestion would cause the population of specific species or genera of bacteria to be increased in a system and therefore contribute to an enhanced beneficial effect of these

bacteria (Gibson and Roberfroid, 1995, Roberfroid et al., 2010). Some of these beneficial effects include the change of the gut microbiota towards a healthier composition; improvement of the intestinal functions such as prevention of constipation, and managing lactose intolerance; increasing of mineral absorption and improvement of bone health; modulation of the immune system; prevention of intestinal infection; reduction in risk of colon cancer, type 2 diabetes, intestinal inflammation, obesity and metabolic syndrome (Roberfroid et al., 2010). Clinical evidence of prebiotic effects of some substrates are reviewed by Macfarlane et al., (2006) and Roberfroid et al., (2010).

Substrates used as prebiotics are expected to be neither hydrolysed nor absorbed in the upper part of the GI tract (Gibson and Roberfroid, 1995, Gibson et al., 2004, Roberfroid et al., 2010). They are also expected to be resistant to gastric acidity (Gibson et al., 2004, Roberfroid et al., 2010). They must be selectively fermented by potentially beneficial bacteria in the colon; a very high degree of selective fermentation by bifidobacteria and lactobacilli is usually desirable (Roberfroid et al., 2010). They must also alter the composition of the colonic microbiota towards a healthier composition and preferably induce effects, which are beneficial to the host health (Gibson and Roberfroid, 1995, Roberfroid et al., 2010).

The most commonly used prebiotics are fructo-oligosaccharides and galacto-oligosaccharides which are known to enhance the growth of bifidobacteria (Macfarlane et al., 2008). Also, other dietary carbohydrates such as soybean oligosaccharides (Lan et al., 2007), resistant starch (Lesmes et al., 2008, Giuberti et al., 2013), xylo-oligosaccharides (Moura et al., 2007, Zhang et al., 2008), lactose, lactulose and lactitol (Saarela et al., 2003) etc. have shown *in vitro* prebiotic effects.

Traditionally, *in vitro* prebiotic potential of substrates is investigated by incubating potential substrates with either pure culture of selected bacteria or faecal samples in simple batch cultures (Rycroft et al., 2001, Ghoddusi et al., 2007) or single and multi-stage chemostats to mimic the complexity of the human large intestine (Molly et al., 1994, Probert et al., 2004, Van de Wiele et al., 2004, Van den Abbeele et al., 2013). Microbial changes are analysed using either traditional microbiological techniques or molecular techniques or both to show the selectivity of fermentation by the bacterial species (for example selective utilization of the substrate by bifidobacteria and not *E. coli* or *Streptococcus faecalis* or *Lactobacillus acidophilus*, Minami et al., 1983); to

show changes in faecal microbiota (for example increase in bifidobacteria but not clostridia, Rycroft et al., 2001) and comparison of the efficacy of different substrates (Rycroft et al., 2001, Probert and Gibson, 2002, Ghoddusi et al., 2007, Van den Abbeele et al., 2013). Some of the *in vitro* studies also aim to measure and compare the production of SCFA and gases as a result of fermentation of different substrates (Rycroft et al., 2001, Cardelle-Cobas et al., 2012).

A small study was designed by including a potential prebiotic in the growth media of pure cultures of the probiotic species or faecal slurry to observe growth in the presence of the substrate in the microcalorimeter.

6.2 Objectives

The objective of this study was:

- To investigate whether a potential prebiotic substrate would increase the growth of pure probiotic species or specific members of colonic bacteria in the microcalorimeter

6.3 Materials and Methods

6.3.1 Microbiological media and chemicals

Peptone water and yeast extract were obtained from Oxoid Ltd, UK. KH_2PO_4, K_2HPO_4, $NaHCO_3$, sodium taurocholate, haemin, vitamin K, resazurin, Tween 80 and inulin were purchased from Sigma-Aldrich, UK. NaCl, $MgSO_4.7H_2O$, $CaCl_2.6H_2O$ were obtained from VWR International, UK. L-cysteine hydrochloride was purchased from Fisher Scientific, UK.

6.3.2 Preparation of faecal slurry

Faecal slurry was prepared by obtaining freshly voided human faeces from three healthy volunteers who had not been on any medications or antibiotics for a period of at least 6

months. The faecal material was transferred into an Anaerobic Workstation kept at 37°C and was diluted with PBS solution (in per litre quantity: KH_2PO_4 1g; K_2HPO_4 2 g; NaCl 7.5 g), to obtain a 40% w/w slurry (eg. 60 g of faecal material was diluted with 90 g of PBS). This mixture was homogenized using an Ultra Turrax (IKA T18 Basic) at a speed of 18000 rpm until no large solid agglomerates could be observed. This was sieved through an open mesh fabric (Sefar Nitex^{TM}) with pore size of 350 μm to remove any unhomogenised fibrous material. A sterile basal medium prepared with composition described by Hughes et al., (2008) (in per litre quantity: peptone water 2 g; yeast extract 2 g; NaCl 0.1 g; K_2HPO_4 0.04 g; $MgSO_4.7H_2O$ 0.01 g; $CaCl_2.6H_2O$ 0.01 g; $NaHCO_3$ 2 g; haemin 0.005 g; L-cysteine hydrochloride 0.5 g; bile salts 0.5 g; Tween 80 2 mL; vitamin K 10 μL; resazurin solution 0.025% 4 mL) was added to the sieved homogenised faecal slurry to achieve a 1:1 dilution (Yadav et al., 2013). The faecal slurry obtained was added to a sterile 50% v/v glycerol in ¼ strength Ringer's solution in a 3:1 dilution (eg. 35 mL of faecal slurry was diluted with 15 mL of 50% v/v glycerol in ¼ Ringer's). The attained faecal slurry was dispensed into 3 mL aliquots, sealed and frozen at -80°C.

6.3.3 Defrosting of frozen faecal slurry

Defrosting of frozen faecal slurry was done in the same way as defrosting of frozen bacterial stocks from liquid nitrogen described in section 2.3.8.

6.3.4 Microcalorimetric fermentations

3 mL of fresh faecal slurry was filled into a sterile 3 mL calorimetric ampoule, aseptically sealed and placed in the TAM. Data were recorded as described previously. This was repeated for the frozen faecal samples after defrosting. Defrosted faecal slurry was also inoculated in a 3 mL 50% v/v BHI in CMM (CMM-BHI) in a sterile calorimetric ampoule to a 1 in 100 dilution and studied in the microcalorimeter. The fermentation of the faecal slurry in the presence of a potential prebiotic was studied by inoculating faecal slurry (1 in 100) into CMM-BHI supplemented with 1% w/v inulin in a calorimetric ampoule. Data were recorded as described previously.

Pure cultures of LA-5® and BB-12® were also inoculated into CMM-BHI supplemented with 1or 3% w/v inulin and studied in the microcalorimeter.

6.4 Results and Discussion

Faecal samples are crude; hence their composition cannot be standardized in replicate experiments. To standardize the inoculum and control reproducibility, slurries made from the faecal material were frozen down so that instead of obtaining fresh faecal material to make fresh slurry for every experiment, which could introduce variability, the frozen samples from one batch of slurry was used. Frozen samples were comparable to the fresh sample as indicated by Figure 6.1. The power-time curves showed organisms possibly in a stationary phase; there was probably not enough energy source for growth as a steady decay of the growth curve was observed. When the slurry was inoculated into fresh medium (CMM-BHI), there was exponential growth of the organisms (Figure 6.2), with a reduction of pH of the culture from an initial 7.24 ± 0.01 to 6.39 ± 0.03 after microcalorimetric measurement. Greater growth was observed when 1% w/v inulin was added to the medium evident with the increase of the AUC or heat output (representing growth, Braissant et al., 2010a, Figure 6.3) and further decrease of the pH of the medium to 5.86 ± 0.02 from an initial value of 7.24 ± 0.01 (Table 6.1). When 1% w/v inulin was supplemented into the growth media of LA-5® and BB-12®, the power-time curves indicated that the growth of these species were not significantly enhanced (Figures 6.4 and 6.5) but significant change of the pH of the medium occurred (Table 6.1). It must be noted that the pH of the medium with and without inulin supplementation preinoculation was not different. Supplementation with 3% w/v inulin caused a slight increase in growth of the species especially that of LA-5® at ca. 6 h. Also whilst the unsupplemented culture returned to baseline ca. 13 h and remained there after, inulin supplemented cultures of LA-5® showed further metabolic activity after ca. 30 h which continued for the duration tested.
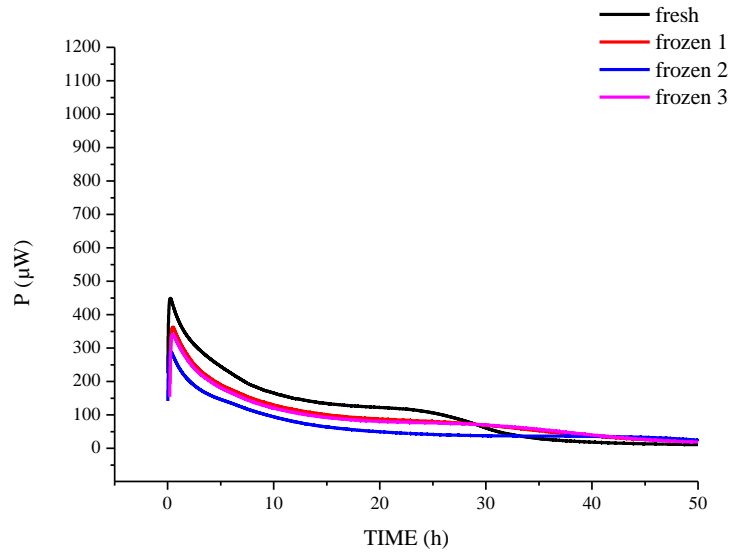
Figure 6.1. Power-time curves of cryopreserved faecal slurry: fresh and frozen samples.
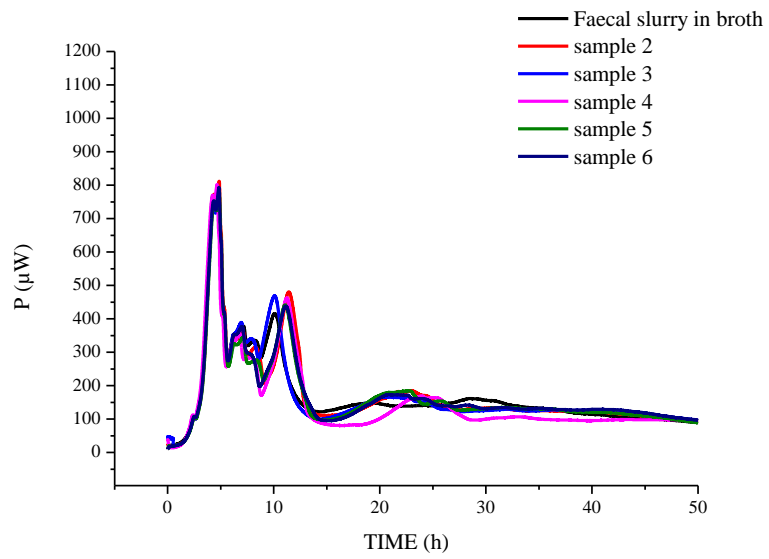
Figure 6.2. Power-time curve of faecal slurry inoculated into fresh medium (CMM-BHI).
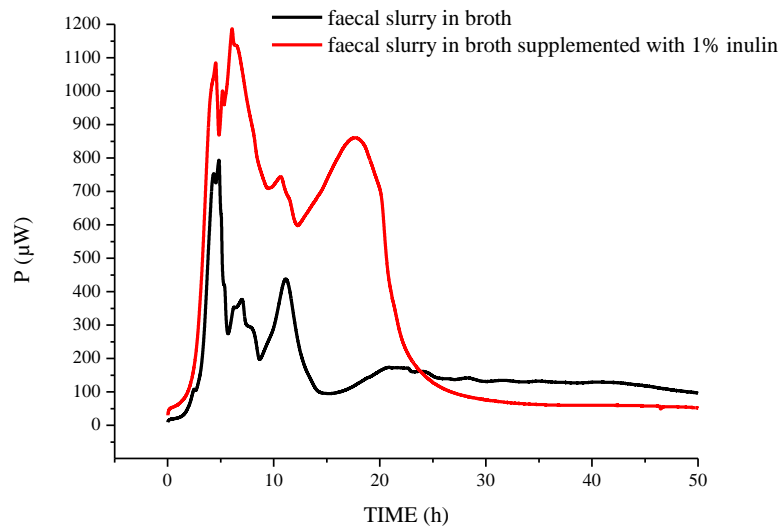
Figure 6.3. Comparison of power-time curve of faecal slurry in CMM-BHI and medium supplemented with 1% w/v inulin.
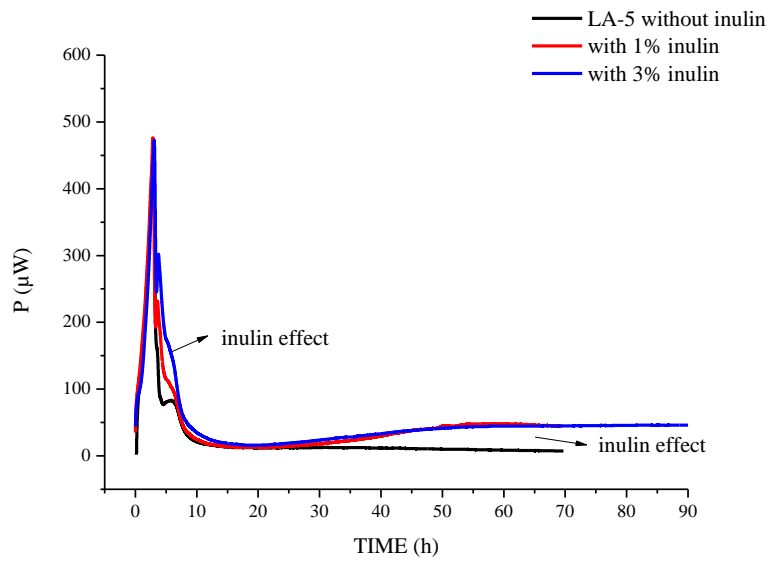
Figure 6.4. Power-time curves of LA-5® with and without inulin (1% and 3% w/v)
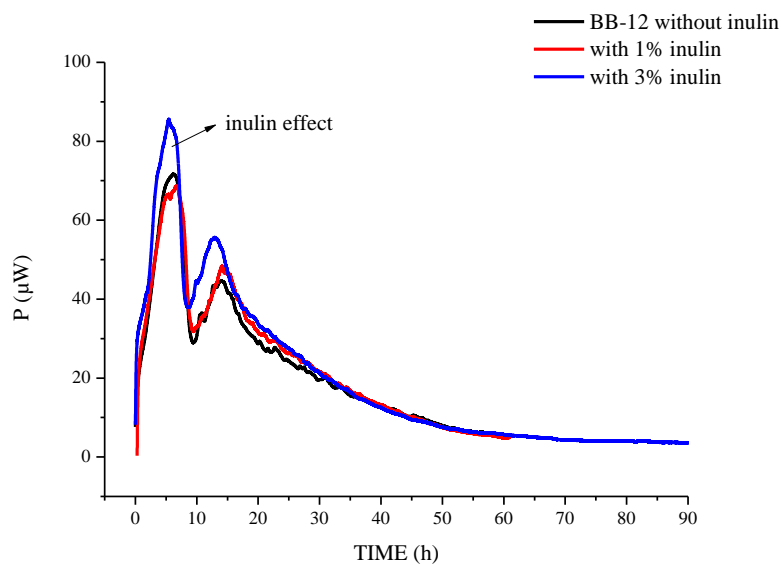
Figure 6.5. Power-time curves of BB-12® with and without inulin (1% and 3% w/v)

Table 6.1. Final pH after experiments with and without inulin supplementation of the culture

Culture	Final pH
Faecal slurry in CMM-BHI without inulin	6.39 ± 0.03
Faecal slurry in CMM-BHI with 1% w/v inulin	5.86 ± 0.02
LA-5® in CMM-BHI without inulin	5.70 ± 0.10
LA-5® in CMM-BHI with 1% w/v inulin	5.40 ± 0.04
LA-5® in CMM-BHI with 3% w/v inulin	4.57 ± 0.15
BB-12® in CMM-BHI without inulin	5.51 ± 0.01
BB-12® in CMM-BHI with 1% w/v inulin	5.36 ± 0.04
BB-12® in CMM-BHI with 3% w/v inulin	5.12 ± 0.11

[A]   [B]

Figure 6.6. Images of ampoules of cultures of LA-5® without [A] and with 3% w/v inulin [B] supplementation

Post TAM assessment of the ampoules also revealed a very cloudy culture of inulin supplemented LA-5® compared to an unsupplemented culture (Figure 6.6).

Inulin is a known prebiotic and its prebiotic potential has been investigated extensively. For instance, Van den Abbeele et al., (2013) comparing inulin and long-chain arabinoxylan (a wheat derived fiber) and also two elaborate chemostats, simulator of the human intestinal microbial ecosystem model (SHIME) and dynamic TNO *in vitro* model of the colon (TIM-2) combined with conventional plate count, DGGE (to monitor shifts within the SHIME microbiota) and the intestinal-chip (I-chip, a phylogenetic microarray, applied standardly to monitor microbial shifts in the TIM-2), reported increased abundance of bifidobacteria (including *B. catenulatum*, *B. adolescentis*, *B. angulatum* and *B. bifidum*) whilst members of the Bacteroidetes were decreased. Also a significant reduction of *Ruminococcus bromii* was reported with inulin. Another study by Wang and Gibson (1993) demonstrated the prebiotic effects of inulin and oligofructose. Their bacterial growth data showed that bifidobacteria were preferentially stimulated in growth whilst *E. coli* and *Clostridium* were maintained at low levels (Wang and Gibson, 1993). It is not surprising that microcalorimetry was able to detect increased growth of the faecal culture with inulin supplementation. What is unusual is the similarity in shape of power-time curve produced with and without supplementation with inulin. Faecal slurry could contain different groups of bacteria: *Bacteroides*, *Bifidobacterium*, *Lactobacillus*, *C. leptum*, *C. sporogenes, Enterobacteria* etc (Van den Abbeele et al., 2013). While some groups (as exemplified by previous studies) may be able to effectively metabolise

241

inulin, other groups may not. Even amongst groups, some species, and strains may not be able to metabolise inulin. For example, *Bifidobacterium breve* Yakult and *B. adolescentis* LMG 10734 were shown not to be able to degrade inulin whereas *B. longum* LMG 11047 and *B. angulatum* LMG 11039T could partially degrade inulin and also degrade oligofructose when grown in coculture fermenations with *Bacteroides thetaiotaomicron* LMG 11262, a strain able to metabolize both inulin and oligofructose (Falony et al., 2009). So it is expected that, upon addition of inulin to the medium, certain groups or species of bacteria multiply preferentially than other groups and this should reflect in the power-time curve that is generated. The power-time curve would be expected to be different from that which was produced from the unsupplemented medium.

From our experiences from the other studies in the previous chapters, the power-time curve that was generated could mean that a group of bacteria dominated the ampoule (given the medium and medium condition) and this group again metabolised inulin, dominating the others in the ampoule. When inulin was supplemented into the growth media of LA-5® and BB-12®, although the final pH and appearance of the cultures after microcalorimetric measurements implied inulin may have been fermented by the pure species especially when it was supplemented at 3% w/v for LA-5®, the prebiotic effect of inulin for the pure species shown in the microcalorimeter was not as pronounced as the faecal culture. It is likely the pure species may have partially degraded inulin or possibly degraded it when the other nutrient sources in the medium were exhausting. For instance, for LA-5®, the "inulin effect" was more noticeable after 30 h of microcalorimetric incubation (Figure 6.4). The reason for this may be that the pure species may have adapted on the other sources of nutrients supplied in the medium and may have therefore preferentially metabolised the other substrate (with possibly accumulated toxic waste) before inulin. Another observation was that BB-12® may not have been favoured in growth in the medium and condition in the ampoule since the heat output was significantly less than the other cultures.

In summary, the data shows that inulin was degraded by the faecal culture; possibly by a specific group, which dominated the culture. Inulin might have been degraded by the pure species: BB-12® and LA-5® but was more obvious at higher concentration. It is also likely degradation of inulin occurred after other carbohydrate sources in the medium were exhausting. Overall, the data show that the prebiotic potential of substrates could

242

be studied in the microcalorimeter. As mentioned previously, the methods currently in use to determine microbial changes when studying the prebiotic potential of substrates are based on conventional microbiological techniques (colony counts on selective agars) and molecular techniques. The plate technique, apart from being laborious and time consuming is also unable to account for unculturable organisms (Liesack and Stackebrandt, 1992) hence differences exists between microbiological approaches and molecular tools. For example, Sghir et al., (1998) using fructo-oligosaccharide discovered that analysing the microbiota using traditional plate technique gives a different population profile compared to the use of 16S ribosomal nucleic acid genus specific probes demonstrating that the probe method was more consistent with the SCFA profile of the population than the plate technique (Sghir et al., 1998). Van de Wiele et al., (2004) were also able to show that PCR was a more precise technique to detect differences in bifidobacterial populations than conventional culturing techniques (Van de Wiele et al., 2004) when they used the SHIME to study the prebiotic effect of chicory inulin. The results obtained from this study suggest that microcalorimetry offers a potential to study the prebiotic potential of substrates and could complement the current methods. However, it has its own limitations for instance, the influence of interspecies effect (thus the possibility of competition between species even pre-supplementation with a potential substrate since the microcalorimeter measures the overall activity of a culture embracing interspecies effect). But it could still be explored further for prebiotic studies.

The current study had several limitations in its design. For instance, the medium used for the study contained other carbohydrates sources (eg. glucose), which could have been preferentially utilised by the pure species. A more effective design was to use inulin as the sole carbohydrate source so that the organisms would have been limited by the sole substrate for metabolism. Also, pure culture of selected bacteria could have also been used to have control over the microcalorimetric data and to enable easier post microcalorimetric analyses of the culture, for example plate counting.

Chapter 7 Conclusions and future work

It has been shown through the work presented in the different chapters of this thesis and also by many different authors (Beezer, 1980, Vine and Bishop, 2005, Wadso 2009, Braissant et al., 2010a, Braissant et al., 2010c, Ning et al., 2013) that microcalorimetry is a useful and versatile technique for the study of many aspects of bacteria. As stated by Vine and Bishop (2005), several features of bacteria also make them very suitable for study by microcalorimetry. For instance, they have high metabolic rates hence even small numbers are easy to detect in a heterogenous system. To date, it has been shown that heat output is a dependable and characteristic feature of bacterial cells. It is clear and established that with pure cultures of bacteria, one could possibly identify and determine the effects of substrates on an organism on the basis of heat production (Beezer, 1980, Li et al., 2000, O'Neill et al., 2003, Bonkat et al., 2012, Lago Rivero et al., 2012, Lago Rivero et al., 2013), but the possibility of achieving the same with mixed cultures, acknowledging possible and relevant interspecies interaction and effect was the central subject of investigation of this thesis.

The work in this thesis has shown that microcalorimetry is undoubtedly, very useful for the difficult task of detection of growth of bacteria in mixed cultures. The results from Chapter 2 demonstrated interspecies interaction: competition and coexistence between mixed cultures of *P. aeruginosa*, *S. aureus* and *E. coli* in the microcalorimeter. Chapters 3, 4 and 5 also demonstrated that microcalorimetry could be used to study some functional characteristics of probiotics, surmounting some of the issues associated with the conventional plate enumeration or plate diffusion assay. Chapter 6 also demonstrated that the prebiotic potential of substrates could potentially be studied in the microcalorimeter.

However, it is also apparent from the experiments conducted and the results that microcalorimetry also has some severe limitations when used for these applications. For instance, growth differentiation in mixed cultures were purely based on microcalorimetric identification, hence differentiation was limited when identification was reduced. The results from Chapter 2 and 3 showed that power-time curves of individual species that were distinct under a particular condition, were varied at ease with minor changes in medium composition and oxygen level in the ampoule and also depended on the metabolic properties of the organism being studied. Hence as the

ampoule condition moved towards an anaerobic environment, loss of detailed structure of the power-time curve occurred unless perhaps there was varied energy sources available to the organism which could utilize different metabolic pathways. Consequently the utilization of the microcalorimeter to differentiate between species was restricted and became time dependent when loss of structure or thermal detail of the power-time curve occurred. In those instances, inocula concentration was very critical, as any shift in the power-time curve along the abscissa could have complicated the identification of a species. This finding is consistent with previous reports (Monk and Wadso, 1975, Newell, 1980 (and references therein)). For instance Monk and Wadso (1975) also demonstrated in their study that the characteristic profiles of bacterial growth were strongly related to the oxygen status of the growth medium. They demonstrated that in anaerobic conditions, *Streptococcus faecalis* showed the same simple bell shaped curve as *Streptococcus faecium* and *Streptococcus agalactiae* (a β-haemolytic *Streptococcus* sp.) although a different curve (with more detail) was obtained for *Streptococcus faecalis* under aerobic condition or partially aerobic conditions (Monk and Wadso, 1975). Since the detail/structure is required for differentiating between organisms, a biphasic growth with respect to oxygen, substrates (Schaarschmidt and Lamprecht, 1978, Newell, 1980) or the metabolic complexity of a species may be needed to maximize identification or differentiation in mixed culture. Whilst this implies that strict control of oxygen in the calorimetric ampoule is not needed for differentiation and consequent applications, the desired condition may also not be necessarily optimal for growth and hence characterization of some species in mixed culture in the microcalorimeter. Also, for meaningful interpretation of metabolic data, it has often been suggested that strictly defined medium and condition need to be simulated in the calorimetric ampoule to allow mathematical expression of the data (Beezer, 1980). Both purposes: growth differentiation and quantitative data (eg. metabolic activity contributing to a heat peak or relevant substrate/metabolite concentration) could be difficult to satisfy at the same time unless perhaps "strictly defined complexity" is used eg. limiting 3 or more defined substrates in an anaerobic environment or defined partially aerobic environment.

The results also demonstrated that microcalorimetric characterisation is a growth dependent process and therefore is subject to all the constraints operating upon all growth processes for example nutrient depletion, pH shifts, accumulation of waste etc. when operated in a batch of medium. As discussed in Chapter 1, there are two main

types of designs of the microcalorimeter applicable to microbiological studies: the ampoule, representing the simple batch and the flow-system. For the later design, a reaction medium is held in a reservoir outside the microcalorimeter, usually in a thermostatic bath and is pumped through a calorimetric cell. Used media can be collected as waste through channels or returned into the media reservoir. Although the ampoule is commonly used for characterization (O' Neill et al., 2003, Lago Rivero et al., 2012, Bonkat et al., 2012, Lago Rivero et al., 2013) and was employed for all the studies in this thesis, the flow design has also been used for characterisation (Beezer et al., 1979, Beaubien et al., 1987) but to a limited extent due to its disadvantages: time-consuming sterilizations, possible contamination and safety concerns during experiments. As has been argued for growth experiments in batch cultures, extrapolation or transfer of findings from these systems to describe growth in natural systems is not in accord as these systems are oversimplified (Monod, 1950, Novick and Szilard, 1950, Hoskisson and Hobbs, 2005). The chemostats may therefore be "the best laboratory idealization" of natural systems (Novick and Szilard, 1950, Veldkamp and Jannasch, 1972, Hoskisson and Hobbs, 2005, Schmidt et al., 2011) as they involve pH controlled single and multiple component continuous culture systems which could more represent natural environments and could control factors such as nutrient depletion (a major limitation of the study in Chapter 3) and accumulation of metabolic waste products (which although useful for studying interspecies interaction, may not be always necessary). As already stated in this thesis, the mechanism for probiosis, although not elucidated in detail, has been proposed to involve several components: bacteria-host interaction and bacteria-bacteria interactions (O'Toole and Cooney, 2008, Bermudez-Brito et al., 2012). The antagonistic activities of probiotics could also involve several mechanisms: from production of antimicrobial substances (organic acids, peroxides, antimicrobial peptides including bacteriocins, deconjugated bile acids) to competitive adhesiveness and interference (Servin, 2004, Bermudez-Brito et al., 2012, Hassan et al., 2012). If full appreciation of these mechanisms is sought after, or comprehensive interpretation of the microcalorimetric data is required, nutrients may need to be constantly supplied or accumulated waste or acids removed from the environment and this calls for other components and analytical sensors, for instance pH electrodes, sensors for measuring metabolites formed and consumed, sensors for measuring available energy source, entry ports for buffers, etc. incorporated into the calorimetric design. Presently its design limits its relevant application. There have been attempts by some authors, for instance, Johansson and Wadso, (1999b) who constructed an isothermal microcalorimetric

titration vessel equipped with pH and oxygen electrodes and a spectrophotometer as a means of controlling growth processes and to reduce the unspecificity of the data obtained (Johansson and Wadso, 1999b). Though their design was promising, such designs are still not commercially available and needs further research.

Also, the comparison of the power-time curves between pure species and mixed cultures for the assessment of interspecies interactions (antagonism) or effects necessitates more than a subjective judgment. An objective assessment needs to employ the use of mathematical analysis programmes or by deriving parameters such as growth rate, lag phase duration or maximum growth (Xie et al., 1988, Yang et al., 2008, Kong et al., 2009). Such parameters are achieved by the use of growth models. The use of these growth models has been reviewed by Braissant et al., (2013) who has highlighted some advantages, limitations and assumptions when they are compared with conventional methods such as plate methods or OD. There is still room for further research in that area.

The use of microcalorimetry for growth detection in mixed cultures or polymicrobial systems with its associated applications may always require followed up analysis with other analytical methods or incorporated analytical support components or sensors to interpret microcalorimetric data, which could be nonspecific. But from the results, it is also apparent that a lot can be accomplished within the ampoule. Its clear advantage is that unlike the conventional plate technique, it does not record mere endpoint process but gives the kinetics of the process and also overcomes the tedium associated with the conventional method. There are now multichannel instruments, which offer high throughput screening (eg. TAM III, TAM 48, TA Instruments; calScreener^{TM}, SymCel) but these instruments, which are batch microcalorimeters, still suffer from the fundamental shortcomings of the batch systems discussed.

In summary, the work in this thesis has demonstrated clearly that microcalorimetry is a valuable tool for detection of bacteria growth in mixed cultures and assessing functional characteristics of probiotics and potential prebiotics. It has huge potential in microbiological assays, and could potentially address some microbiological problems.

Other areas, which need further research, are the prebiotic effect of potential substrates in the microcalorimeter. Discussed in Chapter 6 is the asserted effect of prebiotics to selectively stimulate the growth of beneficial members of the microbiota (Roberfroid et

al., 2010). To demonstrate the prebiotic potential of a substrate, a defined medium need to be constructed with the prebiotic as the sole energy and carbon source. In that way, a species, which had previously adapted to other carbohydrate sources, may need to solely degrade the prebiotic for growth.

Also, for the *C. difficile* work, a gut model of *Clostrium difficile* infection can be constructed with the flow design or by simulating the *in vivo* complexity with multi-stage fermenters alongside other analytical sensors or methods incorporated into the microcalorimetric design. *C. difficile* spores can be infected into the system and monitored for dominance in the system. Potential probiotics could be added to the system to investigate if there is any shift towards dominance of the probiotics or reduction in *C. difficile* toxins using the combined effort of microcalorimetry and other conventional or non-coventional methods. However this model will still not simulate immunological events, which are proposed to occur in the human gut (Sekirov et al., 2010, Bermudez-Brito et al., 2012). Adherence and interference could be simulated with added human cell lines (Chan et al., 1985, Reid et al., 1987, Bernet et al., 1994, Gopal et al., 2001) but overly complex design will risk the relevant interpretation of the microcalorimetric data.

Additionally for the *C. difficile* work, other *C. difficile* strains should be tested and further animal studies need to be done to determine if the commercial probiotics that demonstrated *in vitro* anticlostridial activities can indeed play evidential role in prophylaxis or treatment of the infection in animal models. Also, the specificity of action of the antimicrobial compounds contained in the supernatant of the probiotic cultures needs further investigation.

Also, during the studies conducted in Chapter 5, it was realized that compared to the solid probiotic products, which performed poorly in terms of survival in the product and during gastric tolerance assay, the liquid products showed better gastric tolerance and stability (within product shelf life) but they may involve higher cost for transport and storage and have a shorter shelf life. Means of ensuring the stability and gastrointestinal survival of solid probiotics need further research since they present an advantage of cost and convenience. There are delivery strategies such as microencapsulation, which has potential for improving survival of solid probiotics (Chandramouli et al., 2004, Ding and Shah, 2009, Islam et al., 2010, Cook et al., 2012). Probiotics are also marketed as tablets,

granules, pellets etc. and there have been attempts to enteric coat some probiotic tablets and pellets (Brachkova et al., 2009). But significant losses occur from tableting or pelletization process alone (Brachkova et al., 2009). Capsules have the advantage of not requiring a compression step, hence the heat exposure which could be associated with significant viability loss during tableting (Bansal and Garg, 2008). Lyophilised probiotics filled into capsule could therefore be explored for enteric coating to control release of the capsule content. Also, other colonic or enteric delivery strategies could be explored as a future work.

In vitro susceptibility testings are usually conducted in pure culture, which is not representative of many of the infections in human, which occur as consortia of two or more bacterial species. As discussed in Chapter 2, and shown by other authors (Hoffman et al., 2006, Biswas et al., 2009, Riedele and Reichl, 2011, Kluge et al., 2012), bacterial interactions might affect the pathogenicity of the co-infecting species and could have consequences on the therapeutic efficacy of a selected antibiotic. Co-pathogenicity of species in mixed infection could arise due to the possible protective effect of the species over each other or the likelihood that a species could adapt relevant virulence strategies to resist a dominating species (Riedele and Reichl, 2011). It will be an interesting investigation to find out how the population distribution could change when there is an antibiotic challenge with the mixed cultures in Chapter 2 or when an effective antibiotic of one of the species in pure culture is used against it in mixed cultures in the microcalorimeter.

Presentations and Publications

The work reported in this thesis has been presented at the following conferences:

- A real-time study of defined mixed cultures of bacteria, XVIII International Society of Biological Calorimetry, Lund, Sweden, Podium Presentation, June, 2014
- *In vitro* inhibition of *Clostridium difficile* by commercial probiotics: a real time study, PhD Research Day, UCL School of Pharmacy, Podium Presentation, April, 2014
- A microcalorimetric assay of probiotic viability after exposure to gastric fluids, APS UKPharmSci, Hertfordshire, Hartfield, UK, Podium Presentation, September, 2014
- *In vitro* demonstration of inhibition of *Clostridium difficile* by commercial probiotics: a real time study, 24th ECCMID Conference, Barcelona, Spain, Poster Presentation, May, 2014
- A microcalorimetric study of mixed cultures of *P. aeruginosa*, *S. aureus* and *E. coli,* Poster Presentation AAPS Annual Meeting and Exposition, San Antonio, USA, November, 2013; APS UKPharmSci, Edinburgh, UK, September, 2013
- Continuous real time observation of inhibition of *Clostridium difficile* by probiotics Poster Presentation, AAPS Annual Meeting and Exposition, San Antonio, USA, November, 2013; APS UKPharmSci, Edinburgh, UK, September 2013; UKICRS, Reading, UK, April, 2013
- Assessment of commercial probiotic viability in pig and simulated gastric fluids Poster Presentation, AAPS Annual Meeting and Exposition, San Antonio, USA, November, 2013
- Probiotics: are they effective? Thermal Methods Group Meeting, Ware, UK, November, 2012

The following manuscripts have been accepted or are under preparation from the work reported in this thesis:

- Comparative survival of commercial probiotic formulations: Tests in biorelevant gastric fluids and real-time measurements using microcalorimetry (Beneficial Microbes).
- A microcalorimetric study of mixed cultures of *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli*
- A microcalorimetric study of mixed cultures of *Clostridium difficile* and commercial probiotics

References

AAS, J. A., PASTER, B. J., STOKES, L. N., OLSEN, I. & DEWHIRST, F. E. 2005. Defining the normal bacterial flora of the oral cavity. *Journal of Clinical Microbiology*, 43, 5721-5732.

ABADIAS, M., BENABARRE, A., TEIXIDO, N., USALL, J. & VINAS, I. 2001a. Effect of freeze drying and protectants on viability of the biocontrol yeast Candida sake. *International Journal of Food Microbiology,* 65**,** 173-182.

ABADIAS, M., TEIXIDO, N., USALL, J., BENABARRE, A. & VINAS, I. 2001b. Viability, efficacy, and storage stability of freeze-dried biocontrol agent Candida sake using different protective and rehydration media. *Journal of Food Protection,* 64**,** 856-861.

ABD EL-SALAM, M. H., SALEH, F. A., KHOLIF, A. M., EL-SAYED, E. M., ABDOU, S. M. & EL-SHIBINY, S., 2004. Isolation and characterization of bacteriocins produced by Bifidobacterium lactis BB-12 and Bifidobacterium longum BB-46. *9th Egyptian Conference for Dairy Science and Technology, Cairo, Egypt*. 9–11 October.

ABRAHAMSSON, T. R., JAKOBSSON, T., BOTTCHER, M. F., FREDRIKSON, M., JENMALM, M. C., BJORKSTEN, B. & OLDAEUS, G. 2007. Probiotics in prevention of IgE-associated eczema: a double-blind, randomized, placebo-controlled trial. *Journal of Allergy Clinical Immunology*, 119, 1174-1180.

ABURUB, A., RISLEY, D. S. & MISHRA, D. 2008. A critical evaluation of fasted state simulating gastric fluid (FaSSGF) that contains sodium lauryl sulfate and proposal of a modified recipe. *International Journal of Pharmaceutics*, 347, 16-22.

ADLERBERTH, I., LINDBERG, E., ABERG, N., HESSELMAR, B., SAALMAN, R., STRANNEGARD, I. L. & WOLD, A. E. 2006. Reduced enterobacterial and increased staphylococcal colonization of the infantile bowel: An effect of hygienic lifestyle? *Pediatric Research*, 59, 96-101.

AGERHOLM-LARSEN, L., BELL, M. L., GRUNWALD, G. K. & ASTRUP, A. 2000. The effect of a probiotic milk product on plasma cholesterol: a meta-analysis of short-term intervention studies. *European Journal of Clinical Nutrition,* 54**,** 856-860.

ALEXANDER, M., DAGGETT, P. M., GHERNA, R., JONG, J. & SIMIONE, F. 1980. American Type Collection Methods. Laboratory manual on preservation, freezing, and freeze-drying as applied to algae, bacteria, fungi and protozoa. American Type Culture Collection, Rockville, MD. 1–46.

ALKLINT, C., WADSO, L. & SJOHOLM, I. 2005. Accelerated storage and isothermal microcalorimetry as methods of predicting carrot juice shelf-life. *Journal of the Science of Food and Agriculture,* 85**,** 281-285.

ALLEN, S. J., WAREHAM, K., WANG, D. L., BRADLEY, C., HUTCHINGS, H., HARRIS, W., DHAR, A., BROWN, H., FODEN, A., GRAVENOR, M. B. & MACK, D. 2013. Lactobacilli and bifidobacteria in the prevention of antibiotic-associated diarrhoea and Clostridium difficile diarrhoea in older inpatients (PLACIDE): a randomised, double-blind, placebo-controlled, multicentre trial. *Lancet*, 382, 1249-1257.

ALVAREZ-OLMOS, M. I., BAROUSSE, M. M., RAJAN, L., VAN DER POL, B. J., FORTENBERRY, D., ORR, D. & FIDEL, P. L. 2004. Vaginal lactobacilli in adolescents - Presence and relationship to local and systemic immunity, and to bacterial vaginosis. *Sexually Transmitted Diseases*, 31, 393-400.

AMMOR, S., TAUVERON, G., DUFOUR, E. & CHEVALLIER, I. 2006. Antibacterial activity of lactic acid bacteria against spoilage and pathogenic bacteria isolated from the same meat small-scale facility - 1 - Screening and characterization of the antibacterial compounds. *Food Control,* 17**,** 454-461.

ANAND, S. K., SRINIVASAN, R. A. & RAO, L. K. 1984. Antimicrobial activity associated with Bifidobacterium bifidum-I. *Cultured Dairy Products Journal* 2, 6–7.

ANAND, S. K., SRINIVASAN, R. A. & RAO, L. K. 1985. Antimicrobial activity associated with Bifidobacterium bifidum-II. *Cultured Dairy Products Journal* 2, 21–23.

ANNUK, H., SHCHEPETOVA, J., KULLISAAR, T., SONGISEPP, E., ZILMER, M. & MIKELSAAR, M. 2003. Characterization of intestinal lactobacilli as putative probiotic candidates. *Journal of Applied Microbiology*, 94, 403-412.

APELLA, M. C., GONZALEZ, S. N., DEMACIAS, M. E. N., ROMERO, N. & OLIVER, G. 1992. In vitro studies on the inhibition of the growth of Shigella-sonnei by Lactobacillus-casei and Lact-acidophilus. *Journal of Applied Bacteriology,* 73**,** 480-483.

ARMAND, M., BOREL, P., PASQUIER, B., DUBOIS, C., SENFT, M., ANDRE, M., PEYROT, J., SALDUCCI, J. & LAIRON, D. 1996. Physicochemical characteristics of emulsions during fat digestion in human stomach and duodenum. *American Journal of Physiology-Gastrointestinal and Liver Physiology,* 271**,** G172-G183.

ARUNACHALAM, K. D. 1999. Role of bifidobacteria in nutrition, medicine and technology. *Nutrition Research*, 19, 1559-1597.

ASHBY, L. J., BEEZER, A. E. & BUCKTON, G. 1989. In vitro dissolution testing of oral controlled release preparations in the presence of artificial foodstuffs .1. Exploration of alternative methodology - microcalorimetry. *International Journal of Pharmaceutics,* 51**,** 245-251.

ASHRAF, R. & SHAH, N. P. 2011. Selective and differential enumerations of Lactobacillus delbrueckii subsp bulgaricus, Streptococcus thermophilus, Lactobacillus acidophilus, Lactobacillus casei and Bifidobacterium spp. in yoghurt - A review. *International Journal of Food Microbiology,* 149**,** 194-208.

AULTON, M. E. 2013. Aulton's Pharmaceutics: The Design and manufacture of medicines, Edinburgh, Churchill Livingstone.

AURELI, P., FIORE, A., SCALFARO, C., CASALE, M. & FRANCIOSA, G. 2010. National survey outcomes on commercial probiotic food supplements in Italy. *International Journal of Food Microbiology,* 137**,** 265-273.

BAINES, S. D., O'CONNOR, R., FREEMAN, J., FAWLEY, W. N., HARMANUS, C., MASTRANTONIO, P., KUIJPER, E. J. & WILCOX, M. H. 2008. Emergence of reduced susceptibility to metronidazole in Clostridium difficile. *Journal of Antimicrobial Chemotherapy,* 62**,** 1046-1052.

BALDAN, R., CIGANA, C., TESTA, F., BIANCONI, I., DE SIMONE, M., PELLIN, D., DI SERIO, C., BRAGONZI, A. & CIRILLO, D. M. 2014. Adaptation of Pseudomonas aeruginosa in cystic fibrosis airways influences virulence of Staphylococcus aureus in vitro and murine models of co-infection. *Plos One*, 9, e89614.

BALDONI, D., HERMANN, H., FREI, R., TRAMPUZ, A. & STEINHUBER, A. 2009. Performance of microcalorimetry for early detection of methicillin resistance in clinical isolates of Staphylococcus aureus. *Journal of Clinical Microbiology,* 47**,** 774-776.

BANSAL, T. & GARG, S. 2008. Probiotics: From functional foods to pharmaceutical products. *Current Pharmaceutical Biotechnology,* 9**,** 267-287.

BAO, Y., ZHANG, Y., ZHANG, Y., LIU, Y., WANG, S., DONG, X., WANG, Y. & ZHANG, H. 2010. Screening of potential probiotic properties of Lactobacillus fermentum isolated from traditional dairy products. *Food Control*, 21, 695-701.

BARBOSA, T. M., SERRA, C. R., LA RAGIONE, R. M., WOODWARD, M. J. & HENRIQUES, A. O. 2005. Screening for Bacillus isolates in the broiler gastrointestinal tract. *Applied and Environmental Microbiology,* 71**,** 968-978.

BAREFOOT, S. F. & KLAENHAMMER, T. R.1983. Detection and activity of Lactacin B, a bacteriocin produced by Lactobacillus acidophilus *Applied and Environmental Microbiology*, 45,1808–1815.

BARRETT, E., MATTARELLI, P., SIMPSON, P. J., O'TOOLE, P. W., FITZGERALD, G. F., ROSS, R. P. & STANTON, C. 2012. Culture Media for the detection and enumeration of bifidobacteria in food production. In*:* CORRY, J. E. L., CURTIS, G. D. W. & BAIRD, R. M. (eds.) *Handbook of culture media for food and water microbiology, 3rd Edition.* Cambridge: Royal Society of Chemistry.

BARTLETT, J. G. 2002. Clinical practice. Antibiotic-associated diarrhea. *New England Journal of Medicine,* 346**,** 334-9.

BARTLETT, J. G. 2010. Clostridium difficile: progress and challenges. *Antimicrobial Therapeutics Reviews*, 1213, 62-69.

BARTLETT, J. G., CHANG, T. W., GURWITH, M., GORBACH, S. L. & ONDERDONK, A. B. 1978. Antibiotic-associated pseudomembranous colitis due to toxin-producing clostridia. *The New England Journal of Medicine,* 298**,** 531-4.

BARTLETT, J. G., ONDERDONK, A. B., CISNEROS, R. L. & KASPER, D. L. 1977. Clindamycin-associated colitis due to a toxin-producing species of Clostridium in hamsters. *The Journal of Infectious Diseases,* 136**,** 701-5.

BARTOLONI, A., MANTELLA, A., GOLDSTEIN, B. P., DEI, R., BENEDETTI, M., SBARAGLI, S. & PARADISI, F. 2004. In-vitro activity of nisin against clinical isolates of Clostridium difficile. *Journal of Chemotherapy,* 16**,** 119-121.

BARTSCH, S. M., UMSCHEID, C. A., FISHMAN, N. & LEE, B. Y. 2013. Is Fidaxomicin Worth the Cost? An Economic Analysis. *Clinical Infectious Diseases,* 57**,** 555-561.

BATTLEY, E. H. 1960. Growth-reaction equations for saccharomyces-cerevisiae. *Physiologia Plantarum*, 13, 192-203.

BAUER, M. P., NOTERMANS, D. W., VAN BENTHEM, B. H. B., BRAZIER, J. S., WILCOX, M. H., RUPNIK, M., MONNET, D. L., VAN DISSEL, J. T., KUIJPER, E. J. & GRP, E. S. 2011. Clostridium difficile infection in Europe: a hospital-based survey. *Lancet*, 377, 63-73.

BAUMANN, D. P. & REINBOLD, G. W. 1966. Freezing of lactic cultures. *Journal of Dairy Science,* 49**,** 259-264.

BAYNE-JONES, S. 1929. Bacterial calorimetry I. General considerations. Description of differential microcalorimeter. *Journal of Bacteriology*, 17, 105-122.

BE'ER, A., ZHANG, H. P., FLORIN, E. L., PAYNE, S. M., BEN-JACOB, E. & SWINNEY, H. L. 2009. Deadly competition between sibling bacterial colonies. *Proceedings of the National Academy of Sciences of the United States of America,* 106**,** 428-433.

BEAUBIEN, A., KEITA, L. & JOLICOEUR, C. 1987. Flow microcalorimetry investigation of the influence of surfactants on a heterogeneous aerobic culture. *Applied and Environmental Microbiology*, 53, 2567-2573.

BEEZER, A. E. 1980. Biological microcalorimetry. London: Academic press.

BEEZER, A. E., NEWELL, R. D. & TYRRELL, H. J. V. 1976. Application of flow microcalorimetry to analytical problems - preparation, storage and assay of frozen inocula of Saccharomyces-cerevisiae. *Journal of Applied Bacteriology*, 41, 197-207.

BEEZER, A. E., NEWELL, R. D. & TYRRELL, H. J. V. 1979. Characterization and metabolic studies of Saccharomyces-cerevisiae and Kluyveromyces-fragilis by flow micro-calorimetry. *Antonie Van Leeuwenhoek Journal of Microbiology,* 45**,** 55-63.

BEGLEY, M., GAHAN, C. G. M. & HILL, C. 2005. The interaction between bacteria and bile. *Fems Microbiology Reviews,* 29**,** 625-651.

BELAICH, A. & BELAICH, J. P. 1976. Microcalorimetric study of the anaerobic growth of Escherichia coli: measurements of the affinity of whole cells for various energy substrates. *Journal of Bacteriology*, 125, 19-24.

BELAICH, J. P. & PRAT, H. 1963. Thermogenese et croissance de Pseudomonas lindneri en glucose limitant. *Comptes Rendus Des Seances De La Societe De Biologie Et De Ses Filiales*, 157, 316-322.

BELAICH, J. P. 1962. These de specialite, University of Marseilles.

BELAY, N. & RASOOLY, A. 2002. Staphylococcus aureus growth and enterotoxin A production in an anaerobic environment. *Journal of Food Protection*, 65, 199-204.

BEN-AMOR, K., VERBAARSCHOT, P., BREEUWER, P., AKKEREMANS, A. D. L., DE VOS, W. M., ROMBOUTS, F. M. & ABEE, T. 2001. Use of flow cytometry and cell sorting for the assessment of the viability of bifidobacteria during bile salts stress. *Abstracts of the General Meeting of the American Society for Microbiology*, 101, 567-567.

BENDALI, F., DURAND, A., HEBRAUD, M. & SADOUN, D. 2011. Lactobacillus paracasei subsp paracasei: an Algerian isolate with antibacterial activity against enteric pathogens and probiotic fitness. *Journal of Food and Nutrition Research,* 50**,** 139-149.

BERMUDEZ-BRITO, M., PLAZA-DIAZ, J., MUNOZ-QUEZADA, S., GOMEZ-LLORENTE, C. & GIL, A. 2012. Probiotic mechanisms of action. *Annals of Nutrition and Metabolism,* 61**,** 160-174.

BERNET, M. F., BRASSART, D., NEESER, J. R. & SERVIN, A. L. 1994. Lactobacillus-acidophilus la-1 binds to cultured human intestinal-cell lines and inhibits cell attachment and cell invasion by enterovirulent bacteria. *Gut,* 35**,** 483-489.

BEST, E. L., FREEMAN, J. & WILCOX, M. H. 2012. Models for the study of Clostridium difficile infection. *Gut Microbes*, 3, 145-167.

BHUNIA, A. K., JOHNSON, M. C. & RAY, B. 1988. Purification, characterization and antimicrobial spectrum of a bacteriocin produced by Pediococcus-acidilactici. *Journal of Applied Bacteriology,* 65**,** 261-268.

BIK, E. M., ECKBURG, P. B., GILL, S. R., NELSON, K. E., PURDOM, E. A., FRANCOIS, F., PEREZ-PEREZ, G., BLASER, M. J. & RELMAN, D. A. 2006. Molecular analysis of the bacterial microbiota in the human stomach. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 732-737.

BINGHAM, S. A., PETT, S. & DAY, K. C. 1990. Non-starch polysaccharide intake of a representative sample of british adults. *Journal of Human Nutrition and Dietetics,* 3**,** 333-338.

BISWAS, L., BISWAS, R., SCHLAG, M., BERTRAM, R. & GOTZ, F. 2009. Small-colony variant selection as a survival strategy for Staphylococcus aureus in the presence of Pseudomonas aeruginosa. *Applied and Environmental Microbiology,* 75**,** 6910-6912.

BJORKSTEN, B. 2006. The gut microbiota: a complex ecosystem. *Clinical and Experimental Allergy*, 36, 1215-1217.

BLOSSOM, D. B. & MCDONALD, L. C. 2007. The challenges posed by reemerging Clostridium difficile infection. *Clinical Infectious Diseases,* 45**,** 222-227.

BOHNHOFF, M., DRAKE, B. L. & MILLER, C. P. 1955. The effect of an antibiotic on the susceptibility of the mouse's intestinal tract to Salmonella infection. *Antibiotics annual,* 3**,** 453-5.

BOIVINET, P. 1964. Thesis, University Aix-Marseille.

BOLING, E. A., BLANCHAR.GC & RUSSELL, W. J. 1973. Bacterial identification by microcalorimetry. *Nature,* 241**,** 472-472.

BONKAT, G., BRAISSANT, O., WIDMER, A. F., FREI, R., RIEKEN, M., WYLER, S., GASSER, T. C., WIRZ, D., DANIELS, A. U. & BACHMANN, A. 2012. Rapid detection of urinary tract pathogens using microcalorimetry: principle, technique and first results. *Bju International,* 110**,** 892-897.

BOUCHEMAL, K., AGNELY, F., KOFFI, A. & PONCHEL, G. 2009. A concise analysis of the effect of temperature and propanediol-1, 2 on Pluronic F127 micellization using isothermal titration microcalorimetry. *Journal of Colloid and Interface Science,* 338**,** 169-176.

BOUFFARD, A. 1895. *C. R. Hebdomadaires Seances Acad Sci*, Paris, 121, 357.

BOUZA, E. 2012. Consequences of Clostridium difficile infection: understanding the healthcare burden. *Clinical Microbiology and Infection*, 18, 5-12.

BOYD, M. A., ANTONIO, M. A. D. & HILLIER, S. L. 2005. Comparison of API 50 CH strips to whole-chromosomal DNA probes for identification of Lactobacillus species. *Journal of Clinical Microbiology*, 43, 5309-5311.

BRACHKOVA, M. I., DUARTE, A. & PINTO, J. F. 2009. Evaluation of the viability of Lactobacillus spp. after the production of different solid dosage forms. *Journal of Pharmaceutical Sciences,* 98**,** 3329-3339.

BRAISSANT, O., BINDSCHEDLER, S., CAILLEAU, G., AUBRECHT, R., KOVACIC, L. & LEE, N. 2010b. Isothermal Microcalorimetry a Novel Tool for Non-Disruptive Analysis of Microbial Heat Production in Subsurface Ecosystems. *Origins of Life and Evolution of Biospheres*, 40, 561-562.

BRAISSANT, O., BONKAT, G., WIRZ, D. & BACHMANN, A. 2013. Microbial growth and isothermal microcalorimetry: Growth models and their application to microcalorimetric data. *Thermochimica Acta*, 555, 64-71.

BRAISSANT, O., WIRZ, D., GOEPFERT, B. & DANIELS, A. U. 2010c. Biomedical use of isothermal microcalorimeters. *Sensors,* 10**,** 9369-9383.

BRAISSANT, O., WIRZ, D., GOEPFERT, B. & DANIELS, A. U. 2010d. "The heat is on": Rapid microcalorimetric detection of mycobacteria in culture. *Tuberculosis,* 90**,** 57-59.

BRAISSANT, O., WIRZ, D., GOPFERT, B. & DANIELS, A. U. 2010a. Use of isothermal microcalorimetry to monitor microbial activities. *Fems Microbiology Letters*, 303, 1-8.

BRANDL, K., PLITAS, G., MIHU, C. N., UBEDA, C., JIA, T., FLEISHER, M., SCHNABL, B., DEMATTEO, R. P. & PAMER, E. G. 2008. Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. *Nature,* 455**,** 804-U8.

BRAVO, D., BRAISSANT, O., SOLOKHINA, A., CLERC, M., DANIELS, A. U., VERRECCHIA, E. & JUNIER, P. 2011. Use of an isothermal microcalorimetry assay to characterize microbial oxalotrophic activity. *Fems Microbiology Ecology,* 78**,** 266-274.

BRAZIER, J. S., RAYBOULD, R., PATEL, B., DUCKWORTH, G., PEARSON, A., CHARLETT, A., DUERDEN, B. I. & NETWORK, H. P. A. R. M. 2008. Distribution and antimicrobial susceptibility patterns of Clostridium difficile PCR ribotypes in English hospitals, 2007-08. *Euro surveillance : European communicable disease bulletin,* 13.

BRIOUKHANOV, A. L., THAUER, R. K. & NETRUSOV, A. I. 2002. Catalase and superoxide dismutase in the cells of strictly anaerobic microorganisms. *Mikrobiologiya*, 71, 330-335.

BRITISH PHARMACOPOEIA. 2013. Volume V, Appendix XII B. Dissolution ANNEX: Recommendations on dissolution testing; Appendix I A. General regeants: gastric juice, artificial.

BRITTON, R. A. & YOUNG, V. B. 2012. Interaction between the intestinal microbiota and host in Clostridium difficile colonization resistance. *Trends in Microbiology,* 20**,** 313-319.

BRITTON, R. A. & YOUNG, V. B. 2014. Role of the Intestinal Microbiota in Resistance to Colonization by Clostridium difficile. *Gastroenterology,* 146**,** 1547-1553.

BROWN, P. O., PALMER, C., BIK, E. M., DIGIULIO, D. B. & RELMAN, D. A. 2007. Development of the human infant intestinal microbiota. *Plos Biology*, 5, 1556-1573.

BSAC. 2013. BSAC methods for antimicrobial susceptibility testing. 1-87.

BUJALANCE, C., JIMENEZ-VALERA, M., MORENO, E. & RUIZ-BRAVO, A. 2006. A selective differential medium for Lactobacillus plantarum. *Journal of Microbiological Methods*, 66, 572-575.

BURKE, K. E. & LAMONT, J. T. 2013. Fecal transplantation for recurrent Clostridium difficile infection in older adults: A Review. *Journal of the American Geriatrics Society*, 61, 1394-1398.

BURKE, K. E. & LAMONT, J. T. 2014. Clostridium difficile infection: A worldwide disease. *Gut and Liver*, 8, 1-6.

BURNS, D. A. & MINTON, N. P. 2011. Sporulation studies in Clostridium difficile. *Journal of Microbiological Methods,* 87**,** 133-138.

BURNS, D. A., HEAP, J. T. & MINTON, N. P. 2010. Clostridium difficile spore germination: an update. *Research in Microbiology*, 161, 730-734.

BUTLER, R. N., ZACHARAKIS, B., MOORE, D., CRAWFORD, K. & DAVIDSON, G. 2001. Non-invasive measurement of a fermentation pattern shift in infants during the first three months of life. *Gastroenterology,* 120**,** A266-A266.

CABALLERO-FRANCO, C., KELLER, K., DE SIMONE, C. & CHADEE, K. 2007. The VSL#3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 292, G315-G322.

CAMASCHELLA, P., MIGNOT, O., PIROVANO, F. & SOZZI, T. 1998. Method for differentiated enumeration of mixed cultures of thermophilic lactic acid bacteria and bifidobacteria by using only one culture medium. *Lait,* 78**,** 461-467.

CARDELLE-COBAS, A., OLANO, A., CORZO, N., VILLAMIEL, M., COLLINS, M., KOLIDA, S. & RASTALL, R. A. 2012. In vitro fermentation of lactulose-derived oligosaccharides by mixed fecal microbiota. *Journal of Agricultural and Food Chemistry,* 60**,** 2024-2032.

CARMEN COLLADO, M. & HERNANDEZ, M. 2007. Identification and differentiation of Lactobacillus, Streptococcus and Bifidobacterium species in fermented milk products with bifidobacteria. *Microbiological Research*, 162, 86-92.

CARROLL, C. K. 2013. Laboratory technical advances in the diagnosis of *Clostridium difficile*. In Tang, Y.W., Stratton, C. W. (eds.). Advanced Techniques in Diagnostic Microbiology. Springer Science+Business Media New York. 769-783.

CARVALHO, A. S., SILVA, J., HO, P., TEIXEIRA, P., MALCATA, F. X. & GIBBS, P. 2004b. Relevant factors for the preparation of freeze-dried lactic acid bacteria. *International Dairy Journal,* 14, 835-847.

CARVALHO, A. S., SILVA, J., HO, P., TEIXEIRA, P., MALCATA, F. X. & GIBBS, P. 2004a. Effects of various sugars added to growth and drying media upon thermotolerance and survival throughout storage of freeze-dried lactobacillus delbrueckii ssp bulgaricus. *Biotechnology Progress,* 20, 248-254.

CHAMPAGNE, C. P., GARDNER, N., BROCHU, E. & BEAULIEU, Y. 1991. The freeze-drying of lactic-acid bacteria - a review. *Canadian Institute of Food Science and Technology Journal-Journal De L Institut Canadien De Science Et Technologie Alimentaires*, 24, 118-128.

CHAMPAGNE, C. P., RAYMOND, Y., GONTHIER, J. & AUDET, P. 2009. Enumeration of the contaminating bacterial microbiota in unfermented pasteurized milks enriched with probiotic bacteria. *Canadian Journal of Microbiology*, 55, 410-418.

CHAMPAGNE, C. P., ROSS, R. P., SAARELA, M., HANSEN, K. F. & CHARALAMPOPOULOS, D. 2011. Recommendations for the viability assessment of probiotics as concentrated cultures and in food matrices. *International Journal of Food Microbiology*, 149, 185-193.

CHAN, R. C. Y., REID, G., IRVIN, R. T., BRUCE, A. W. & COSTERTON, J. W. 1985. Competitive-exclusion of uropathogens from human uroepithelial cells by lactobacillus whole cells and cell-wall fragments. *Infection and Immunity,* 47, 84-89.

CHANDRAMOULI, V., KAILASAPATHY, K., PEIRIS, P. & JONES, M. 2004. An improved method of microencapsulation and its evaluation to protect Lactobacillus spp. in simulated gastric conditions. *Journal of Microbiological Methods*, 56, 27-35.

CHANDRAMOULI, V., KAILASAPATHY, K., PEIRIS, P. & JONES, M. 2004. An improved method of microencapsulation and its evaluation to protect Lactobacillus spp. in simulated gastric conditions. *Journal of Microbiological Methods,* 56, 27-35.

CHAPMAN, C. M. C., GIBSON, G. R. & ROWLAND, I. 2011. Health benefits of probiotics: are mixtures more effective than single strains? *European Journal of Nutrition,* 50, 1-17.

CHAPMAN, C. M. C., GIBSON, G. R. & ROWLAND, I. 2012. In vitro evaluation of single- and multi-strain probiotics: Inter-species inhibition between probiotic strains, and inhibition of pathogens. *Anaerobe,* 18, 405-413.

CHAPMAN, C. M., GIBSON, G. R., TODD, S. & ROWLAND, I. 2013. Comparative in vitro inhibition of urinary tract pathogens by single- and multi-strain probiotics. *European Journal of Nutrition,* 52, 1669-77.

CHARALAMPOPOULOS, D., PANDIELLA, S. S. & WEBB, C. 2003. Evaluation of the effect of malt, wheat and barley extracts on the viability of potentially probiotic lactic acid bacteria under acidic conditions. *International Journal of Food Microbiology,* 82, 133-41.

CHARTERIS, W. P., KELLY, P. M., MORELLI, L. & COLLINS, J. K. 1997. Selective detection, enumeration and identification of potentially probiotic Lactobacillus and Bifidobacterium species in mixed bacterial populations. *International Journal of Food Microbiology,* 35, 1-27.

CHARTERIS, W. P., KELLY, P. M., MORELLI, L. & COLLINS, J. K. 1998. Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic Lactobacillus and Bifidobacterium species in the upper human gastrointestinal tract. *Journal of Applied Microbiology,* 84, 759-768.

CHEIKHYOUSSEF, A., POGORI, N., CHEN, W. & ZHANG, H. 2008. Antimicrobial proteinaceous compounds obtained from bifidobacteria: from production to their application. *International Journal of Food Microbiology*, 125, 215-222.

CHEN, H.-Y., YAO, J., ZHOU, Y., CHEN, H.-L., WANG, F., GAI, N., ZHUANG, R.-S., CECCANTI, B., MASKOW, T. & ZARAY, G. 2008. Investigation of the toxic effect of cadmium on Candida humicola and Bacillus subtilis using a microcalorimetric method. *Journal of Hazardous Materials,* 159, 465-470.

CHEN, J. J., CAI, W. & FENG, Y. 2007a. Development of intestinal bifidobacteria and lactobacilli in breast-fed neonates. *Clinical Nutrition*, 26, 559-566.

CHEN, X. Y., XU, J. J., SHUAI, J. B., CHEN, J. S., ZHANG, Z. F. & FANG, W. H. 2007b. The S-layer proteins of Lactobacillus crispatus strain ZJ001 is responsible for competitive exclusion against Escherichia coli O157: H7 and Salmonella typhimurium. *International Journal of Food Microbiology*, 115, 307-312.

CHUNG, H. J., BANG, W. & DRAKE, M. A. 2006. Stress response of Escherichia coli. *Comprehensive Reviews in Food Science and Food Safety*, 5, 52-64.

CLINICAL AND LABORATORY STANDARDS INSTITUTE. 2009. Performance standards for antimicrobial disk susceptibility tests. Wayne, PA: Clinical and Laboratory Standards Institute, Approved standard M2–A10.

CLINICAL AND LABORATORY STANDARDS INSTITUTE. 2009. Performance standards for antimicrobial susceptibility testing. Wayne, PA: Clinical and Laboratory Standards Institute. Nineteenth informational supplement M100–S19.

CLOSTRIDIUM DIFFICILE RIBOTYPING NETWORK (CDRN) for England and Northern Ireland 2011-13. [online]. Available from: https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/329156/C_difficile_ribotyping_network_CDRN_report.pdf. [20 June 2014].

CLOUD, J. & KELLY, C. P. 2007. Update on Clostridium difficile associated disease. *Current Opinion in Gastroenterology,* 23**,** 4-9.

COEURET, V., GUEGUEN, M. & VERNOUX, J. P. 2004a. Numbers and strains of lactobacilli in some probiotic products. *International Journal of Food Microbiology,* 97**,** 147-156.

COHEN, L. E., SMITH, C. J., PISTER, J. D. & WELLS, R. F. 1974. Clindamycin (cleocin) colitis. *American Journal of Roentgenology,* 121**,** 301-304.

COLEMAN, N. S., MARCIANI, L., BLACKSHAW, E., WRIGHT, J., PARKER, M., YANO, T., YAMAZAKI, S., CHAN, P. Q., WILDE, K., GOWLAND, P. A., PERKINS, A. C. & SPILLER, R. C. 2003. Effect of a novel 5-HT3 receptor agonist MKC-733 on upper gastrointestinal motility in humans. *Alimentary Pharmacology & Therapeutics*, 18, 1039-1048.

COLLADO, M. C., MORENO, Y., HERNANDEZ, E., COBO, J. M. & HERNANDEZ, M. 2005. Note. In vitro viability of Bifidobacterium strains isolated from commercial dairy products exposed to human gastrointestinal conditions. *Food Science and Technology International*, 11, 307-314.

COLLINS, F. M. & CARTER, P. B. 1978. Growth of salmonellae in orally infected germfree mice. *Infection and Immunity,* 21**,** 41-47.

COLLINS, M. D. & GIBSON, G. R. 1999. Probiotics, prebiotics, and synbiotics: approaches for modulating the microbial ecology of the gut. *American Journal of Clinical Nutrition*, 69, 1052S-1057S.

CONROY, M. E., SHI, H. N. & WALKER, W. A. 2009. The long-term health effects of neonatal microbial flora. *Current Opinion in Allergy and Clinical Immunology*, 9, 197-201.

CONWAY, P. L., GORBACH, S. L. & GOLDIN, B. R. 1987. Survival of lactic-acid bacteria in the human stomach and adhesion to intestinal-cells. *Journal of Dairy Science,* 70**,** 1-12.

COOK, M. T., TZORTZIS, G., CHARALAMPOPOULOS, D. & KHUTORYANSKIY, V. V. 2011. Production and evaluation of dry alginate-chitosan microcapsules as an enteric delivery vehicle for probiotic bacteria. *Biomacromolecules*, 12, 2834-2840.

COOK, M. T., TZORTZIS, G., CHARALAMPOPOULOS, D. & KHUTORYANSKIY, V. V. 2012. Microencapsulation of probiotics for gastrointestinal delivery. *Journal of Control Release,* 162**,** 56-67.

CORCORAN, B. M., ROSS, R. P., FITZGERALD, G. F. & STANTON, C. 2004. Comparative survival of probiotic lactobacilli spray-dried in the presence of prebiotic substances. *Journal of Applied Microbiology*, 96, 1024-1039.

COSTA, E., USALL, J., TEIXIDO, N., GARCIA, N. & VINAS, I. 2000. Effect of protective agents, rehydration media and initial cell concentration on viability of Pantoea agglomerans strain CPA-2 subjected to freeze-drying. *Journal of Applied Microbiology,* 89**,** 793-800.

COSTA, E., USALL, J., TEIXIDO, N., TORRES, R. & VINAS, I. 2002. Effect of package and storage conditions on viability and efficacy of the freeze-dried biocontrol agent Pantoea agglomerans strain CPA-2. *Journal of Applied Microbiology,* 92**,** 873-878.

COTTER, P. D. & HILL, C. 2003. Surviving the acid test: Responses of gram-positive bacteria to low pH. *Microbiology and Molecular Biology Reviews*, 67, 429-453.

COUPE, A. J., DAVIS, S. S. & WILDING, I. R. 1991. Variation in gastrointestinal transit of pharmaceutical dosage forms in healthy-subjects. *Pharmaceutical Research*, 8, 360-364.

COWMAN, R. A. & SPECK, M. L. 1965. Ultra-low temperature storage of lactic streptococci. *Journal of Dairy Science*, 48, 1531-1532.

CROWE, J. H., CARPENTER, J. F. & CROWE, L. M. 1998. The role of vitrification in anhydrobiosis. *Annual Review of Physiology*, 60, 73-103.

CRUZ-GUERRERO, A., HERNANDEZ-SANCHEZ, H., RODRIGUEZ-SERRANO, G., GOMEZ-RUIZ, L., GARCIA-GARIBAY, M. & FIGUEROA-GONZALEZ, I. 2014. Commercial probiotic bacteria and prebiotic carbohydrates: a fundamental study on prebiotics uptake, antimicrobials production and inhibition of pathogens. *Journal of the Science of Food and Agriculture,* 94**,** 2246-52.

CUI, J. H., GOH, J. S., KIM, P. H., CHOI, S. H. & LEE, B. J. 2000. Survival and stability of bifidobacteria loaded in alginate poly-l-lysine microparticles. *International Journal of Pharmaceutics*, 210, 51-59.

CULLIGAN, E. P., HILL, C. & SLEATOR, R. D. 2009. Probiotics and gastrointestinal disease: successes, problems and future prospects. *Gut Pathogens*, 1,1-12.

CUMMINGS, J. H. & MACFARLANE, G. T. 1991. The control and consequences of bacterial fermentation in the human colon. *Journal of Applied Bacteriology,* 70**,** 443-459.

CUMMINGS, J. H. 1981. Short chain fatty acids in the human colon. *Gut,* 22**,** 763-79.

CUMMINGS, J. H., GIBSON, G. R. & MACFARLANE, G. T. 1989. Quantitative estimates of fermentation in the hind gut of man. *Acta Veterinaria Scandinavica, Supplements* 86, 1989: Proceedings of the International Symposium on Comparative Aspects of the Physiology of Digestion in Ruminants and Hindgut Fermenters, 76-82.

CYSTIC FIBROSIS FOUNDATION PATIENT REGISTRY: Annual Data Report 2012. [online]. Available from: http://www.cff.org. [21 August 2014].

DAHAN, A. S. & AMIDON, G. L. 2008 Gastrointestinal Dissolution and Absorption of Class II Drugs, in Drug Bioavailability: Estimation of Solubility, Permeability, Absorption and Bioavailability, Volume 40, Second Edition (eds H. van de Waterbeemd and B. Testa), Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany. doi: 10.1002/9783527623860.ch3

DASS, N. B., JOHN, A. K., BASSIL, A. K., CRUMBLEY, C. W., SHEHEE, W. R., MAURIO, F. P., MOORE, G. B. T., TAYLOR, C. M. & SANGER, G. J. 2007. The relationship between the effects of short-chain fatty acids on intestinal motility in vitro and GPR43 receptor activation. *Neurogastroenterology and Motility*, 19, 66-74.

DAVEY, H. M. 2011. Life, death, and in-between: meanings and methods in microbiology. *Applied and Environmental Microbiology, 77*, 5571-5576.

DE KRAKER, M. E. A., WOLKEWITZ, M., DAVEY, P. G., GRUNDMANN, H. & GRP, B. S. 2011. Clinical impact of antimicrobial resistance in European hospitals: Excess mortality and length of hospital stay related to methicillin-resistant Staphylococcus aureus bloodstream infections. *Antimicrobial Agents and Chemotherapy*, 55, 1598-1605.

DE LA COCHETIERE, M. F., DURAND, T., LEPAGE, P., BOURREILLE, A., GALMICHE, J. P. & DORE, J. 2005. Resilience of the dominant human fecal microbiota upon short-course antibiotic challenge. *Journal of Clinical Microbiology*, 43, 5588-5592.

DE MAN, J. C., ROGOSA, M. & SHARPE, M. E. 1960. A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology, 23*, 130-135.

DE PALMA, G., NADAL, I., MEDINA, M., DONAT, E., RIBES-KONINCKX, C., CALABUIG, M. & SANZ, Y. 2010. Intestinal dysbiosis and reduced immunoglobulin-coated bacteria associated with coeliac disease in children. *Bmc Microbiology*, 10, 63.

DE VALDEZ, G. F., DE GIORI, G. S., DE RUIZ HOLGADO, A. P. & OLIVER, G. 1985a. Effect of the rehydration medium on the recovery of freeze-dried lactic-acid bacteria. *Applied and Environmental Microbiology*, 50, 1339-1341.

DE VALDEZ, G. F., DE GIORI, G. S., HOLGADO, A. P. D. & OLIVER, G. 1985b. Rehydration conditions and viability of freeze-dried lactic-acid bacteria. *Cryobiology, 22*, 574-577.

DE VRESE, M. & SCHREZENMEIR, J. 2008. Probiotics, prebiotics, and synbiotics. *Advances in Biochemical Engeneering/Biotechnology*, 111, 1-66.

DEBAST, S. B., BAUER, M. P., KUIJPER, E. J. & COMM 2014. European society of clinical microbiology and infectious diseases: Update of the treatment guidance document for Clostridium difficile infection. *Clinical Microbiology and Infection*, 20, 1-26.

DETHLEFSEN, L., ECKBURG, P. B., BIK, E. M. & RELMAN, D. A. 2006. Assembly of the human intestinal microbiota. *Trends in Ecology & Evolution*, 21, 517-523.

DETHLEFSEN, L., HUSE, S., SOGIN, M. L. & RELMAN, D. A. 2008a. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA Sequencing. *Plos Biology*, 6, 2383-2400.

DETHLEFSEN, L., HUSE, S., SOGIN, M. L. & RELMAN, D. A. 2008b. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. *Plos Biology, 6*, e280.

DIAL, S., ALRASADI, K., MANOUKIAN, C., HUANG, A. & MENZIES, D. 2004. Risk of Clostridium difficile diarrhea among hospital inpatients prescribed proton pump inhibitors: cohort and case-control studies. *Canadian Medical Association Journal*, 171, 33-38.

DING, W. K. & SHAH, N. P. 2009. An improved method of microencapsulation of probiotic bacteria for their stability in acidic and bile conditions during storage. *Journal of Food Science, 74*, M53-M61.

DORE, J. & CORTHIER, G. 2010. The human intestinal microbiota. *Gastroentérologie clinique et biologique*, 34, S7-S15.

DRAGO, L., DE VECCHI, E., NICOLA, L., COLOMBO, A. & GISMONDO, M. R. 2004. Microbiological evaluation of commercial probiotic products available in Italy. *Journal of Chemotherapy*, 16, 463-467.

DRAGO, L., GISMONDO, M. R., LOMBARDI, A., DEHAEN, C. & GOZZINI, L. 1997. Inhibition of in vitro growth of enteropathogens by new Lactobacillus isolates of human intestinal origin. *Fems Microbiology Letters, 153*, 455-463.

DRAGO, L., RODIGHIERO, V., CELESTE, T., ROVETTO, L. & DE VECCHI, E. 2010. Microbiological evaluation of commercial probiotic products available in the USA in 2009. *Journal of Chemotherapy*, 22, 373-377.

DRASAR, B. S. & SHINER, M. 1969b. Studies on the intestinal flora. II. Bacterial flora of the small intestine in patients with gastrointestinal disorders. *Gut*, 10, 812-819.

DRASAR, B. S., SHINER, M. & MCLEOD, G. M. 1969a. Studies on the intestinal flora. I. The bacterial flora of the gastrointestinal tract in healthy and achlorhydric persons. *Gastroenterology*, 56, 71-9.

DRESSMAN, J. B. 1986. Comparison of canine and human gastrointestinal physiology. *Pharmaceutical Research*, 3, 123-131.

DRESSMAN, J. B., AMIDON, G. L., REPPAS, C. & SHAH, V. P. 1998. Dissolution testing as a prognostic tool for oral drug absorption: Immediate release dosage forms. *Pharmaceutical Research,* 15**,** 11-22.

DRESSMAN, J. B., BERARDI, R. R., DERMENTZOGLOU, L. C., RUSSELL, T. L., SCHMALTZ, S. P., BARNETT, J. L. & JARVENPAA, K. M. 1990. Upper gastrointestinal (GI) pH in young, healthy-men and women. *Pharmaceutical Research*, 7, 756-761.

DRUDY, D., FANNING, S. & KYNE, L. 2007. Toxin A-negative, toxin B-positive Clostridium difficile. *International Journal of Infectious Diseases,* 11**,** 5-10.

DUBBERKE, E. R. & WERTHEIMER, A. I. 2009. Review of current literature on the economic burden of Clostridium difficile infection. *Infection Control and Hospital Epidemiology*, 30, 57-66.

DUBEY, A. P., RAJESHWARI, K., CHAKRAVARTY, A. & FAMULARO, G. 2008. Use of VSL#3 in the treatment of rotavirus diarrhea in children. *Journal of Clinical Gastroenterology*, 42, S126-S129.

DUBRUNFAUT, M. 1856. Note sur la chaleur at le travail méchanique produits par la fermentation vineuse. *C. R. Hebdomadaires Seances Acad*. 42, 945-948.

DUERDEN, B. I. 2011. Contribution of a government target to controlling Clostridium difficile in the NHS in England. *Anaerobe,* 17**,** 175-179.

DUNNE, C., MURPHY, L., FLYNN, S., O'MAHONY, L., O'HALLORAN, S., FEENEY, M., MORRISSEY, D., THORNTON, G., FITZGERALD, G., DALY, C., KIELY, B., QUIGLEY, E. M. M., O'SULLIVAN, G. C., SHANAHAN, F. & COLLINS, J. K. 1999. Probiotics: from myth to reality. Demonstration of functionality in animal models of disease and in human clinical trials. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology*, 76, 279-292.

DUTEAU, N. M., ROGERS, J. D., BARTHOLOMAY, C. T. & REARDON, K. F. 1998. Species-specific oligonucleotides for enumeration of Pseudomonas putida F1, Burkholderia sp. strain JS150, and Bacillus subtilis ATCC 7003 in biodegradation experiments. *Applied and Environmental Microbiology,* 64**,** 4994-4999.

EFENTAKIS, M. & DRESSMAN, J. B. 1998. Gastric juice as a dissolution medium: surface tension and ph. *European Journal of Drug Metabolism and Pharmacokinetics*, 23, 97– 102.

EIDTMANN, A. & SCHAUZ, K. 1992. Cryopreservation of protoplasts from sporidia of ustilago-maydis. *Mycological Research,* 96**,** 318-320.

EL-NEZAMI, H., MYKKANEN, H., KANKAANPAA, P., SALMINEN, S. & AHOKAS, J. 2000. Ability of Lactobacillus and Propionibacterium strains to remove aflatoxin B-1 from the chicken duodenum. *Journal of Food Protection,* 63**,** 549-552.

ELLIOT, E. & TEVERSHAM, K. 2004. An evaluation of nine probiotics available in South Africa, August 2003. *South African Medical Journal*, 94, 121-124.

ELMER, G. W., SURAWICZ, C. M. & MCFARLAND, L. V. 1996. Biotherapeutic agents - A neglected modality for the treatment and prevention of selected intestinal and vaginal infections. *Jama-Journal of the American Medical Association*, 275, 870-876.

ENGLYST, H. N. & MACFARLANE, G. T. 1986. Breakdown of resistant and readily digestible starch by human gut bacteria. *Journal of the Science of Food and Agriculture,* 37**,** 699-706.

ESCHBACH, M., SCHREIBER, K., TRUNK, K., BUER, J., JAHN, D. & SCHOBERT, M. 2004. Long-term anaerobic survival of the opportunistic pathogen Pseudomonas aeruginosa via pyruvate fermentation. *Journal of Bacteriology*, 186, 4596-604.

EVALDSON, G., HEIMDAHL, A., KAGER, L. & NORD, C. E. 1982. The normal human anaerobic microflora. *Scandinavian journal of infectious diseases. Supplementum,* 35**,** 9-15.

EVANS, D. F., PYE, G., BRAMLEY, R., CLARK, A. G., DYSON, T. J. & HARDCASTLE, J. D. 1988. Measurement of gastrointestinal pH profiles in normal ambulant human-subjects. *Gut*, 29, 1035-1041.

EWASCHUK, J. B., DIAZ, H., MEDDINGS, L., DIEDERICHS, B., DMYTRASH, A., BACKER, J., LANGEN, M. L. V. & MADSEN, K. L. 2008. Secreted bioactive factors from Bifidobacterium infantis enhance epithelial cell barrier function. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 295, G1025-G1034.

FALLANI, M., YOUNG, D., SCOTT, J., NORIN, E., AMARRI, S., ADAM, R., AGUILERA, M., KHANNA, S., GIL, A., EDWARDS, C. A., DORE, J. & TEAM, I. 2010. Intestinal microbiota of 6-week-old infants across Europe: Geographic influence beyond delivery mode, breast-feeding, and antibiotics. *Journal of Pediatric Gastroenterology and Nutrition*, 51, 77-84.

FALONY, G., CALMEYN, T., LEROY, F. & DE VUYST, L. 2009. Coculture Fermentations of Bifidobacterium species and Bacteroides thetaiotaomicron reveal a mechanistic insight into the prebiotic effect of inulin-type fructans. *Applied and Environmental Microbiology*, 75, 2312-2319.

FAMULARO, G., DE SIMONE, C., MATTEUZI, D. & PIROVANO, F. 1999. Traditional and high potency probiotic preparations for oral bacteriotherapy. *Biodrugs*, 12, 455-470.

FAN, D. D., WANG, L. H., SHANG, L. A., SHI, H. J., MA, X. X., MI, Y. & XU, K. Z. 2008. A microcalorimetric method for studying the biological effects of Mg2+ ion on recombinant Escherichia coli. *Chemical and Biochemical Engineering Quarterly,* 22**,** 363-368.

FAO/WHO, 2001. Joint FAO/WHO expert consultation and report of a joint evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria on food additives. Córdoba, Argentina, 1-4 October 2001. [online]. Available from: http://www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf. [12 November 2013]

FDA, Guidance for Industry. 2000. Waiver of the in vivo bioavailability and bioequivalence studies for immediate-release solid oral dosage forms based on a biopharmaceutics classification system; U.S. Department of Health and Human, Food and Drug Administration (FDA), Center for Drug Evaluation and Research: Washington, DC, 1−13.

FEDORKA-CRAY, P. J., CRAY, W. C., ANDERSON, G. A., NICKERSON, K. W. 1988. Bacterial tolerance to 100% dimethylsulfoxide, *Canadian Journal of Microbiology*. 34, 688–689.

FIELD, D., QUIGLEY, L., O'CONNOR, P. M., REA, M. C., DALY, K., COTTER, P. D., HILL, C. & ROSS, R. P. 2010. Studies with bioengineered Nisin peptides highlight the broad-spectrum potency of Nisin V. *Microbial Biotechnology,* 3**,** 473-486.

FINEGOLD, S. M. 1969. Intestinal bacteria - role they play in normal physiology, pathologic physiology, and infection. *California Medicine,* 110**,** 455-459.

FLINT, H. J., BAYER, E. A., RINCON, M. T., LAMED, R. & WHITE, B. A. 2008. Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. *Nature Reviews Microbiology,* 6**,** 121-131.

FOLKERS, B. L., SCHURING, C., ESSMANN, M. & LARSEN, B. 2010. Quantitative real time PCR detection of Clostridium difficile growth inhibition by probiotic organisms. *North American Journal of Medical Sciences,* 2**,** 5-10.

FOMIN, D. C., ALYCHEVA, I. S., VESELOVSKAYA, L. I., TATCHIN, S. M. 1973. Antibacterial properties of dimethylsulphoxide. *Khimioterapia infektsiy i lekarstvennoy ustoychivosti patogennych mikroorganizmov, Moskva*, 63–64.

FONSECA, B. B., BELETTI, M. E., DA SILVA, M. S., DA SILVA, P. L., DUARTE, I. N. & ROSSI, D. A. 2010. Microbiota of the cecum, ileum morphometry, pH of the crop and performance of broiler chickens supplemented with probiotics. *Revista Brasileira De Zootecnia-Brazilian Journal of Animal Science,* 39**,** 1756-1760.

FORDTRAN, J. S. & WALSH, J. H. 1973. Gastric-acid secretion rate and buffer content of stomach after eating - results in normal subjects and in patients with duodenal-ulcer. *Journal of Clinical Investigation*, 52, 645-657.

FORREST, W. W., WALKER, D. J. & HOPGOOD, M. F. 1961. Enthalpy changes associated with lactic fermentation of glucose. *Journal of Bacteriology*, 82, 685-690.

FUJITA, T., MONK, P. R. & WADSO, I. 1978. Calorimetric identification of several strains of lactic-acid bacteria. *Journal of Dairy Research*, 45, 457-463.

FULLER, R. 1989. Probiotics in man and animals. *Journal of Applied Bacteriology*, 66, 365-378.

FULLER, R. 1991. Probiotics in human medicine. *Gut*, 32, 439-442.

FURRIE, E., MACFARLANE, S., KENNEDY, A., CUMMINGS, J. H., WALSH, S. V., O'NEIL D, A. & MACFARLANE, G. T. 2005. Synbiotic therapy (Bifidobacterium longum/Synergy 1) initiates resolution of inflammation in patients with active ulcerative colitis: a randomised controlled pilot trial. *Gut*, 54, 242-9.

GAISFORD, S. & O'NEILL, M. A.A. 2006. Pharmaceutical isothermal calorimetry. New York; London : Informa Healthcare.

GAISFORD, S. 2012. Isothermal microcalorimetry for quantifying amorphous content in processed pharmaceuticals. *Advanced Drug Delivery Reviews,* 64**,** 431-439.

GAISFORD, S., BEEZER, A. E., BISHOP, A. H., WALKER, M. & PARSONS, D. 2009. An in vitro method for the quantitative determination of the antimicrobial efficacy of silver-containing wound dressings. *International Journal of Pharmaceutics*, 366, 111-116.

GARRITY, M. G., BELL, J. A. & LILBURN, T. 2005. In Bergey's manual of systemic bacteriology, 2nd ed. Bergey's manual trust, Springer Science+Business Media. Inc., 233 Spring Street: New York 2, 323-359.

GERDING, D. N., MUTO, C. A. & OWENS, R. C. 2008a. Measures to control and prevent Clostridium difficile infection. *Clinical Infectious Diseases,* 46**,** S43-S49.

GERDING, D. N., MUTO, C. A. & OWENS, R. C. 2008b. Treatment of Clostridium difficile infection. *Clinical Infectious Diseases,* 46**,** S32-S42.

GERRITSEN, J., SMIDT, H., RIJKERS, G. T. & DE VOS, W. M. 2011. Intestinal microbiota in human health and disease: the impact of probiotics. *Genes and Nutrition,* 6**,** 209-240.

GHERNA, R. L. 1981. Preservation. In: GERHARDT, P., MURRAY, R.G.E., COSTILOW, R.N., NESTER, E.W., WOOD, W.A., KRIEG, N.R. and PHILLIPS, G.B. 1980. Manual of Methods for General Bacteriology. ASM Press, Washington, DC. 208–217.

GHODDUSI, H. B., GRANDISON, M. A., GRANDISON, A. S. & TUOHY, K. M. 2007. In vitro study on gas generation and prebiotic effects of some carbohydrates and their mixtures. *Anaerobe,* 13**,** 193-199.

GIANNELL, R. A., ZAMCHECK, N. & BROITMAN, S. A. 1972. Gastric-acid barrier to ingested microorganisms in man - studies in-vivo and in-vitro. *Gut*, 13, 251-256.

GIBSON, C. A., LANDERKI.GB & MORSE, P. M. 1966. Effects of additives on survival of lactic streptococci in frozen storage. *Applied Microbiology,* 14**,** 665-&.

GIBSON, G. R. & ROBERFROID, M. B. 1995. Dietary modulation of the human colonic microbiota - introducing the concept of prebiotics. *Journal of Nutrition,* 125**,** 1401-1412.

GIBSON, G. R. & WILLIAMS, C. M. 1999. Gut fermentation and health advantages: Myth or reality? *British Journal of Nutrition,* 81**,** 83-84.

GIBSON, G. R., MACFARLANE, G.T. 1995. Human colonic bacteria: role in nutrition, physiology and pathology. Boca Raton, FL: CRC Press.

GIBSON, G. R., PROBERT, H. M., VAN LOO, J., RASTALL, R. A. & ROBERFROID, M. B. 2004. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutrition Research Reviews,* 17**,** 259-275.

GIEL, J. L., SORG, J. A., SONENSHEIN, A. L. & ZHU, J. 2010. Metabolism of bile salts in mice influences spore germination in Clostridium difficile. *Plos One*, 5.

GILLOR, O., ETZION, A. & RILEY, M. A. 2008. The dual role of bacteriocins as anti- and probiotics. *Applied Microbiology and Biotechnology*, 81, 591-606.

GISMONDO, M. R., DRAGO, L. & LOMBARDI, A. 1999. Review of probiotics available to modify gastrointestinal flora. *International Journal of Antimicrobial Agents*, 12, 287-292.

GIUBERTI, G., GALLO, A., MOSCHINI, M. & MASOERO, F. 2013. In vitro production of short-chain fatty acids from resistant starch by pig faecal inoculum. *Animal,* 7**,** 1446-1453.

GOETZE, O., TREIER, R., FOX, M., STEINGOETTER, A., FRIED, M., BOESIGER, P. & SCHWIZER, W. 2009. The effect of gastric secretion on gastric physiology and emptying in the fasted and fed state assessed by magnetic resonance imaging. *Neurogastroenterology and Motility*, 21, 725-e42.

GOLDENBERG, J. Z., MA, S. S., SAXTON, J. D., MARTZEN, M. R., VANDVIK, P. O., THORLUND, K., GUYATT, G. H. & JOHNSTON, B. C. 2013. Probiotics for the prevention of Clostridium difficile-associated diarrhea in adults and children. *Cochrane Database of Systematic Reviews*, 5, CD006095.

GOLDIN, B. R. & GORBACH, S. L. 1984. The effect of milk and Lactobacillus feeding on human intestinal bacterial enzyme-activity. *American Journal of Clinical Nutrition,* 39**,** 756-761.

GOLDIN, B. R. 1998. Health benefits of probiotics. *British Journal of Nutrition,* 80**,** S203-S207.

GOPAL, P. K., PRASAD, J., SMART, J. & GILL, H. S. 2001. In vitro adherence properties of Lactobacillus rhamnosus DR20 and Bifidobacterium lactis DR10 strains and their antagonistic activity against an enterotoxigenic Escherichia coli. *International Journal of Food Microbiology,* 67**,** 207-216.

GORBACH, S. L. 1971. Intestinal microflora. *Gastroenterology*, 60, 1110-1129.

GORBACH, S. L., PLAUT, A. G., NAHAS, L., WEINSTEIN, L., SPANKNEBEL, G. & LEVITAN, R. 1967. Studies of intestinal microflora. II. Microorganisms of the small intestine and their relations to oral and fecal flora. *Gastroenterology*, 53, 856-67.

GORDON, D. M. & O'BRIEN, C. L. 2006. Bacteriocin diversity and the frequency of multiple bacteriocin production in Escherichia coli. *Microbiology*, 152, 3239-3244.

GORDON, H. A. & PESTI, L. 1971. The gnotobiotic animal as a tool in the study of host microbial relationships. *Bacteriological reviews,* 35**,** 390-429.

GORDON, J. I., TURNBAUGH, P. J., HAMADY, M., YATSUNENKO, T., CANTAREL, B. L., DUNCAN, A., LEY, R. E., SOGIN, M. L., JONES, W. J., ROE, B. A., AFFOURTIT, J. P., EGHOLM, M., HENRISSAT, B., HEATH, A. C. & KNIGHT, R. 2009. A core gut microbiome in obese and lean twins. *Nature*, 457, 480-484.

GRACIAS, K. S. & MCKILLIP, J. L. 2004. A review of conventional detection and enumeration methods for pathogenic bacteria in food. *Canadian Journal of Microbiology,* 50**,** 883-890.

GRANDY, G., MEDINA, M., SORIA, R., TERAN, C. G. & ARAYA, M. 2010. Probiotics in the treatment of acute rotavirus diarrhoea. A randomized, double-blind, controlled trial using two different probiotic preparations in Bolivian children. *Bmc Infectious Diseases,* 10.

GRANGER, D.N., BARROWMAN, J.A. & KVIETYS, P.R. 1985. Clinical gastrointestinal physiology. WB. Saunders: Philadelphia.

GRATTEPANCHE, F., LACROIX, C., AUDET, P. & LAPOINTE, G. 2005. Quantification by real-time PCR of Lactococcus lactis subsp cremoris in milk fermented by a mixed culture. *Applied Microbiology and Biotechnology,* 66**,** 414-421.

GREEN, P. N. & WOODFORD, S. K. 1992. Preservation studies on some obligately methanotrophic bacteria. *Letters in Applied Microbiology,* 14**,** 158-162.

GUARNER, F. & MALAGELADA, J. R. 2003a. Gut flora in health and disease. *Lancet,* 361**,** 512-519.

GUARNER, F. & SCHAAFSMA, G. J. 1998. Probiotics. *International Journal of Food Microbiology*, 39, 237-238.

GUINANE, C. M. & COTTER, P. D. 2013. Role of the gut microbiota in health and chronic gastrointestinal disease: understanding a hidden metabolic organ. *Therapeutic Advances in Gastroenterology,* 6**,** 295-308.

GUO, B., HARSTALL, C., LOUIE, T., VAN ZANTEN, S. V. & DIELEMAN, L. A. 2012. Systematic review: faecal transplantation for the treatment of Clostridium difficile-associated disease. *Alimentary Pharmacology & Therapeutics*, 35, 865-875.

GUO, H., YAO, J., CAI, M., QIAN, Y., GUO, Y., RICHNOW, H. H., BLAKE, R. E., DONI, S. & CECCANTI, B. 2012. Effects of petroleum contamination on soil microbial numbers, metabolic activity and urease activity. *Chemosphere,* 87**,** 1273-1280.

GUSLANDI, M. 2007. Probiotic agents in the treatment of irritable bowel syndrome. *Journal of International Medical Research*, 35, 583-589.

HARRIGAN, W.F. 1998. Laboratory methods in food microbiology. Gulf Professional Publishing, Medical, 92-98.

HARRISON, A. P. 1956. Causes of death of bacteria in frozen suspensions. *Antonie Van Leeu Wenhoek Journal of Microbiology and Serology,* 22**,** 407-418.

HARTUNG, J. 1986. On the use of flow microcalorimetry in estimating the biological effect of adverse substances on Escherichia-coli .1. flow microcalorimetric assays with microorganisms for antibiotics and chemicals (literature-review). *Zentralblatt Fur Bakteriologie Mikrobiologie Und Hygiene Serie B-Umwelthygiene Krankenhaushygiene Arbeitshygiene Praventive Medizin,* 183**,** 36-46.

HASLAM, S. C., KETLEY, J. M., MITCHELL, T. J., STEPHEN, J., BURDON, D. W. & CANDY, D. C. A. 1986. Growth of Clostridium-difficile and production of toxins A and B in complex and defined media. *Journal of Medical Microbiology*, 21, 293-297.

HASSAN, M., KJOS, M., NES, I. F., DIEP, D. B. & LOTFIPOUR, F. 2012. Natural antimicrobial peptides from bacteria: characteristics and potential applications to fight against antibiotic resistance. *Journal of Applied Microbiology,* 113**,** 723-736.

HATAKKA, K., HOLMA, R., EL-NEZAMI, H., SUOMALAINEN, T., KUISMA, M., SAXELIN, M., POUSSA, T., MYKKANEN, H. & KORPELA, R. 2008. The influence of Lactobacillus rhamnosus LC705 together with Propionibacterium freudenreichii ssp. shermanii JS on potentially carcinogenic bacterial activity in human colon. *International Journal of Food Microbiology*, 128, 406-10.

HATAKKA, K., SAVILAHTI, E., PONKA, A., MEURMAN, J. H., POUSSA, T., NASE, L., SAXELIN, M. & KORPELA, R. 2001. Effect of long term consumption of probiotic milk on infections in children attending day care centres: double blind, randomised trial. *British Medical Journal,* 322**,** 1327-1329.

HAVENAAR, R. & HUIS IN'T VELD, J. H. J. 1992. Probiotics: A general view. The Lactic Acid Bacteria, Vol. 1; The lactic acid bacteria in health and disease, 151-170.

HAYASHI, H., TAKAHASHI, R., NISHI, T., SAKAMOTO, M. & BENNO, Y. 2005. Molecular analysis of jejunal, ileal, caecal and recto-sigmoidal human colonic microbiota using 16S rRNA gene libraries and terminal restriction fragment length polymorphism. *Journal of Medical Microbiology*, 54, 1093-1101.

HEALTH CANADA. Accepted claims about the nature of probiotic microorganisms in food. Health Canada. [online]. Available from: http://www.hc-sc.gc.ca/fn-an/label-etiquet/claims-reclam/probiotics_claims-allegations_probiotiques-eng.php%20(2009). [30 August 2014]

HEALTH PROTECTION AGENCY. Results of the mandatory Clostridium difficile reporting scheme. [online]. Available from: http://www.hpa.org.uk/web/HPAweb&HPAwebStandard/HPAweb_C/ 1195733750761. [12 June 2014]

HEBUTERNE, X. 2003. Gut changes attributed to ageing: effects on intestinal microflora. *Current Opinion in Clinical Nutrition and Metabolic Care*, 6, 49-54.

HECKLY, R. J. 1978. Preservation of microorganisms. *Advances in Applied Microbiology*, 24, 1–53.

HEEG, D., BURNS, D. A., CARTMAN, S. T. & MINTON, N. P. 2012. Spores of Clostridium difficile clinical isolates display a diverse germination response to bile salts. *Plos One*, 7, e32381.

HEGLER, F. & KAPPLER, A. 2010. Cryopreservation of anoxygenic phototrophic Fe(II)-oxidizing bacteria. *Cryobiology,* 61**,** 158-160.

HEINE, W. 1996. The influence of different formulas on the intestinal microflora of infants. *Monatsschrift Kinderheilkunde,* 144**,** S169-S173.

HELL, M., BERNHOFER, C., STALZER, P., KERN, J. M. & CLAASSEN, E. 2013. Probiotics in Clostridium difficile infection: reviewing the need for a multistrain probiotic. *Beneficial Microbes,* 4**,** 39-51.

HELLMIG, S., VON SCHÖNING, F., GADOW, C., KATSOULIS, S., HEDDERICH, J., FÖLSCH, U. R. & STÜBER, E. 2006. Gastric emptying time of fluids and solids in healthy subjects determined by 13C breath tests: influence of age, sex and body mass index. *Journal of Gastroenterology and Hepatology*, 21, 1832–1838.

HENG, Z., CONGYI, Z., CUNXIN, W., JIBIN, W., CHAOJIANG, G., JIE, L. & YUWEN, L. 2005. Microcalorimetric study of virus infection - The effects of hyperthermia and alpha 1b recombinant homo interferon on the infection process of BHK-21 cells by foot and mouth virus. *Journal of Thermal Analysis and Calorimetry,* 79**,** 45-50.

HERBEL, S. R., VAHJEN, W., WIELER, L. H. & GUENTHER, S. 2013. Timely approaches to identify probiotic species of the genus Lactobacillus. *Gut Pathogens*, 5, 27.

HERNANDEZ GARCIA, A. 2011. Anhydrobiosis in bacteria: From physiology to applications. *Journal of Biosciences*, 36, 939-950.

HERNANDEZ, D., CARDELL, E. & ZARATE, V. 2005. Antimicrobial activity of lactic acid bacteria isolated from Tenerife cheese: initial characterization of plantaricin TF711, a bacteriocin-like substance produced by Lactobacillus plantarum TF711. *Journal of Applied Microbiology*, 99, 77-84.

HICKSON, M. 2011. Probiotics in the prevention of antibiotic-associated diarrhoea and Clostridium difficile infection. *Therapeutic Advances in Gastroenterology*, 4, 185-97.

HILL, C., GUARNER, F., REID, G., GIBSON, G. R., MERENSTEIN, D. J., POT, B., MORELLI, L., CANANI, R. B., FLINT, H. J., SALMINEN, S., CALDER, P. C. & SANDERS, M. E. 2014. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature reviews. Gastroenterology & Hepatology,* 11**,** 506-14.

HILL, M. J. & DRASAR, B. S. 1975. Normal colonic bacterial-flora. *Gut*, 16, 318-323.

HILL, M. J. 1997. Intestinal flora and endogenous vitamin synthesis. *European Journal of Cancer Prevention,* 6**,** S43-S45.

HOFFMAN, L. R., DEZIEL, E., D'ARGENIO, D. A., LEPINE, F., EMERSON, J., MCNAMARA, S., GIBSON, R. L., RAMSEY, B. W. & MILLER, S. I. 2006. Selection for Staphylococcus aureus small-colony variants due to growth in the presence of Pseudomonas aeruginosa. *Proceedings of the National Academy of Sciences of the United States of America,* 103**,** 19890-19895.

HOLDEMAN, L. V., GOOD, I. J. & MOORE, W. E. C. 1976. Human Fecal Flora - Variation in Bacterial Composition within Individuals and a Possible Effect of Emotional Stress. *Applied and Environmental Microbiology*, 31, 359-375.

HOOKMAN, P. & BARKIN, J. S. 2009. Clostridium difficile associated infection, diarrhea and colitis. *World Journal of Gastroenterology*, 15, 1554-1580.

HOOPER, L. V. & GORDON, J. I. 2001. Commensal host-bacterial relationships in the gut. *Science*, 292, 1115-1118.

HOOVER, D.G. & HARLANDER, S.K. 1993. Screening methods for detecting bacteriocin activity. In Hoover, D.G. & Steenscr, L.R. 1993. Bacteriocins of lactic acid bacteria. Academic press, California, USA, 23-39.

HOSKISSON, P. A. & HOBBS, G. 2005. Continuous culture - making a comeback? *Microbiology-Sgm,* 151**,** 3153-3159.

HOWELL, M., WIRZ, D., DANIELS, A. U. & BRAISSANT, O. 2012. Application of a microcalorimetric method for determining drug susceptibility in mycobacterium species. *Journal of Clinical Microbiology*, 50, 16-20.

HPA Summary points on Clostridium difficile Infection (CDI). 2011. [online]. Available from: http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1278944283388. [21 July 2014].

HUANG, H., WEINTRAUB, A., FANG, H. & NORD, C. E. 2009. Antimicrobial resistance in Clostridium difficile. *International Journal of Antimicrobial Agents,* 34**,** 516-522.

HUANG, Y. & ADAMS, M. C. 2004. In vitro assessment of the upper gastrointestinal tolerance of potential probiotic dairy propionibacteria. *International Journal of Food Microbiology*, 91, 253-260.

HUBALEK, Z. 2003. Protectants used in the cryopreservation of microorganisms. Cryobiology, 46, 205-29.

HUGHES, S. A., SHEWRY, P. R., GIBSON, G. R., MCCLEARY, B. V. & RASTALL, R. A. 2008. In vitro fermentation of oat and barley derived beta-glucans by human faecal microbiota. *Fems Microbiology Ecology,* 64**,** 482-493.

HURLEY, B. W. & NGUYEN, C. C. 2002. The spectrum of pseudomembranous enterocolitis and antibiotic-associated diarrhea. *Archives of Internal Medicine,* 162**,** 2177-2184.

HUTT, P., SHCHEPETOVA, J., LOIVUKENE, K., KULLISAAR, T. & MIKELSAAR, M. 2006. Antagonistic activity of probiotic lactobacilli and bifidobacteria against entero- and uropathogens. *Journal of Applied Microbiology*, 100, 1324-32.

HWANG, S. W. 1966. Long-term preservation of fungus cultures with liquid nitrogen refrigeration. *Applied Microbiology*, 14, 784-788.

IANNITTI, T. & PALMIERI, B. 2010. Therapeutical use of probiotic formulations in clinical practice. *Clinical Nutrition*, 29, 701-725.

ILETT, K. F., TEE, L. B. G., REEVES, P. T. & MINCHIN, R. F. 1990. Metabolism of drugs and other xenobiotics in the gut lumen and wall. *Pharmacology & Therapeutics,* 46**,** 67-93.

ISLAM, J., COHEN, J., RAJKUMAR, C. & LLEWELYN, M. J. 2012. Probiotics for the prevention and treatment of Clostridium difficile in older patients. *Age Ageing*, 41, 706-711.

ISLAM, M. A., YUN, C. H., CHOI, Y. J. & CHO, C. S. 2010. Microencapsulation of live probiotic bacteria. *Journal of Microbiology and Biotechnology*, 20, 1367-1377.

ISOLAURI, E., ARVOLA, T., SUTAS, Y., MOILANEN, E. & SALMINEN, S. 2000. Probiotics in the management of atopic eczema. *Clinical and Experimental Allergy,* 30**,** 1604-1610.

IYER, C. & KAILASAPATHY, K. 2005. Effect of co-encapsulation of probiotics with prebiotics on increasing the viability of encapsulated bacteria under in vitro acidic and bile salt conditions and in yogurt. *Journal of Food Science,* 70**,** M18-M23.

JACK, R. W., TAGG, J. R. & RAY, B. 1995. Bacteriocins of Gram-positive bacteria. *Microbiological Reviews*, 59, 171-200.

JANTRATID, E., JANSSEN, N., REPPAS, C. & DRESSMAN, J. B. 2008. Dissolution media simulating conditions in the proximal human gastrointestinal tract: An update. *Pharmaceutical Research*, 25, 1663-1676.

JARA, S., SANCHEZ, M., VERA, R., COFRE, J. & CASTRO, E. 2011. The inhibitory activity of Lactobacillus spp. isolated from breast milk on gastrointestinal pathogenic bacteria of nosocomial origin. *Anaerobe*, 17, 474-477.

JENSEN, H., GRIMMER, S., NATERSTAD, K. & AXELSSON, L. 2012. In vitro testing of commercial and potential probiotic lactic acid bacteria. *International Journal of Food Microbiology*, 153, 216-222.

JIMENEZDIAZ, R., RIOSSANCHEZ, R. M., DESMAZEAUD, M., RUIZBARBA, J. L. & PIARD, J. C. 1993. Plantaricins S and T, 2 new bacteriocins produced by Lactobacillus-plantarum lpco10 isolated from a green olive fermentation. *Applied and Environmental Microbiology,* 59**,** 1416-1424.

JOHANSSON, P. & WADSO, I. 1999a. Towards more specific information from isothermal microcalorimetric measurements on living systems. *Journal of Thermal Analysis and Calorimetry,* 57**,** 275-281.

JOHANSSON, P. & WADSO, I. 1999b. An isothermal microcalorimetric titration/perfusion vessel equipped with electrodes and spectrophotometer. *Thermochimica Acta*, 342, 19-29.

JOHNSON, A. P. & WILCOX, M. H. 2012. Fidaxomicin: a new option for the treatment of Clostridium difficile infection. *Journal of Antimicrobial Chemotherapy,* 67**,** 2788-2792.

JORGENSEN, J. H. & FERRARO, M. J. 2000. Antimicrobial susceptibility testing: Special needs for fastidious organisms and difficult-to-detect resistance mechanisms. *Clinical Infectious Diseases*, 30, 799-808.

JORGENSEN, J. H. & FERRARO, M. J. 2009. Antimicrobial Susceptibility Testing: A review of general principles and contemporary practices. *Clinical Infectious Diseases,* 49**,** 1749-1755.

JUMP, R. L. P., PULTZ, M. J. & DONSKEY, C. J. 2007. Vegetative Clostridium difficile survives in room air on moist surfaces and in gastric contents with reduced acidity: a potential mechanism to explain the association between proton pump inhibitors and C-difficile-associated diarrhea? *Antimicrobial Agents and Chemotherapy,* 51**,** 2883-2887.

KABANOVA, N., KAZARJAN, A., STULOVA, I. & VILU, R. 2009. Microcalorimetric study of growth of Lactococcus lactis IL1403 at different glucose concentrations in broth. *Thermochimica Acta,* 496**,** 87-92.

KAILASAPATHY, K. & CHIN, J. 2000. Survival and therapeutic potential of probiotic organisms with reference to Lactobacillus acidophilus and Bifidobacterium spp. *Immunology & Cell Biology*, 78, 80-88.

KALAKOUTSKIAI, L.V & POZHARITSKA, L.M 1968. Calorimetric studies on germinating and quiescent conidia of Actinomyces streptomycini B-6. *Journal of General and Applied Microbiology,* 14**,** 209-212.

KALANTZI, L., GOUMAS, K., KALIORAS, V., ABRAHAMSSON, B., DRESSMAN, J. B. & REPPAS, C. 2006. Characterization of the human upper gastrointestinal contents under conditions simulating bioavailability/bioequivalence studies. *Pharmaceutical Research*, 23, 165-176.

KALLIOMAKI, M., SALMINEN, S., ARVILOMMI, H., KERO, P., KOSKINEN, P. & ISOLAURI, E. 2001. Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. *Lancet,* 357**,** 1076-1079.

KANG, K. H., SHIN, H. J., PARK, Y. H., LEE, T. S., 1989. Studies on the antibacterial substances produced by lactic acid bacteria: purification and some properties of antibacterial substance 'Bifilong' produced by B. longum. *Korean Dairy Science* 1, 204–216.

KARLSSON, S., DUPUY, B., MUKHERJEE, K., NORIN, E., BURMAN, L. G. & AKERLUND, T. 2003. Expression of Clostridium difficile toxins A and B and their sigma factor TcdD is controlled by temperature. *Infection and Immunity*, 71, 1784-1793.

KAWAI, Y., SAITOH, B., TAKAHASHI, O., KITAZAWA, H., SAITO, T., NAKAJIMA, H. & ITOH, T. 2000. Primary amino acid and DNA sequences of gassericin T, a lactacin F-family bacteriocin produced by Lactobacillus gasseri SBT2055. *Bioscience Biotechnology and Biochemistry*, 64, 2201-2208.

KAWASE, K., SUZUKI, T., KIYOSAWA, I., OKONOGI, S., KAWASHIMA, T. & KUBOYAMA, M. (1983) Effects of composition of infant's formulas on the intestinal microflora of infants. *Bifidobacteria Microflora* 2: 25-31.

KEITH, S. C. 1913. Factors influencing the survival of bacteria at temperatures in the vicinity of the freezing point of water. *Science,* 37**,** 877-879.

KELLY, C. P. & LAMONT, J. T. 2008. Clostridium difficile - More difficult than ever. *New England Journal of Medicine,* 359.

KHAN, N. H., AHSAN, M., TAYLOR, W. D. & KOGURE, K. 2010. Culturability and survival of marine, freshwater and clinical Pseudomonas aeruginosa. *Microbes and Environments*, 25, 266-274.

KIM, M. N., KIM, N., LEE, S. H., PARK, Y. S., HWANG, J. H., KIM, J. W., JEONG, S. H., LEE, D. H., KIM, J. S., JUNG, H. C. & SONG, I. S. 2008. The effects of probiotics on PPI-triple therapy for Helicobacter pylori eradication. *Helicobacter*, 13, 261-268.

KLUGE, S., HOFFMANN, M., BENNDORF, D., RAPP, E. & REICHL, U. 2012. Proteomic tracking and analysis of a bacterial mixed culture. *Proteomics*, 12, 1893-1901.

KONG, W. J., WANG, J. B., JIN, C., ZHAO, Y. L., DAI, C. M., XIAO, X. H. & LI, Z. L. 2009. Effect of emodin on Candida albicans growth investigated by microcalorimetry combined with chemometric analysis. *Applied Microbiology and Biotechnology,* 83**,** 1183-1190.

KONG, W. J., XING, X. Y., XIAO, X. H., ZHAO, Y. L., WEI, J. H., WANG, J. B., YANG, R. C. & YANG, M. H. 2012. Effect of berberine on Escherichia coli, Bacillus subtilis, and their mixtures as determined by isothermal microcalorimetry. *Applied Microbiology and Biotechnology,* 96**,** 503-510.

KOS, B., SUSKOVIC, J., BEGANOVIC, J., GJURACIC, K., FRECE, J., IANNACCONE, C. & CANGANELLA, F. 2008. Characterization of the three selected probiotic strains for the application in food industry. *World Journal of Microbiology & Biotechnology,* 24**,** 699-707.

KOTSOU, M. G., MITSOU, E. K., OIKONOMOU, I. G. & KYRIACOU, A. A. 2008. In vitro assessment of probiotic properties of Lactobacillus strains from infant gut microflora. *Food Biotechnology*, 22, 1-17.

KRAMER, M., OBERMAJER, N., MATIJASIC, B. B., ROGELJ, I. & KMETEC, V. 2009. Quantification of live and dead probiotic bacteria in lyophilised product by real-time PCR and by flow cytometry. *Applied Microbiology and Biotechnology,* 84**,** 1137-1147.

KUEHNE, S. A., COLLERY, M. M., KELLY, M. L., CARTMAN, S. T., COCKAYNE, A. & MINTON, N. P. 2014. Importance of Toxin A, Toxin B, and CDT in virulence of an epidemic Clostridium difficile strain. *Journal of Infectious Diseases,* 209**,** 83-86.

KUIJPER, E. J., BARBUT, F., BRAZIER, J. S., KLEINKAUF, N., ECKMANNS, T., LAMBERT, M. L., DRUDY, D., FITZPATRICK, F., WIUFF, C., BROWN, D. J., COIA, J. E., PITUCH, H., REICHERT, P., EVEN, J., MOSSONG, J., WIDMER, A. F., OLSEN, K. E., ALLERBERGER, F., NOTERMANS, D. W., DELMEE, M., COIGNARD, B., WILCOX, M., PATEL, B., FREI, R., NAGY, E., BOUZA, E., MARIN, M., AKERLUND, T., VIROLAINEN-JULKUNEN, A., LYYTIKAINEN, O., KOTILA, S., INGEBRETSEN, A., SMYTH, B., ROONEY, P., POXTON, I. R. & MONNET, D. L. 2008. Update of Clostridium difficile infection due to PCR ribotype 027 in Europe, 2008. *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin,* 13.

KUIJPER, E. J., COIGNARD, B., TULL, P., ESGCD & ECDC 2006. Emergence of Clostridium difficile-associated disease in North America and Europe. *Clinical Microbiology and Infection*, 12, 2-18.

KUMAGAI, H., KUMAGAE, S., MITANI, K. & ENDO, T. 2004. Effects of supplementary probiotics to two different diets on dry matter intake, daily gain, digestibility, ruminal pH, and fecal microbial populations and metabolites in ewes. *Animal Science Journal,* 75**,** 219-224.

KURTMANN, L., CARLSEN, C. U., RISBO, J. & SKIBSTED, L. H. 2009. Storage stability of freeze-dried Lactobacillus acidophilus (La-5) in relation to water activity and presence of oxygen and ascorbate. *Cryobiology,* 58**,** 175-180.

LAGO RIVERO, N., LEGIDO SOTO, J. L., ARIAS SANTOS, I. & CASAS, L. M. 2013. Differentiation between Staphylococcus aureus and Staphylococcus epidermidis using microcalorimetry. *International Journal of Thermophysics,* 34**,** 1039-1048.

LAGO RIVERO, N., LEGIDO, J. L., ARIAS SANTOS, I. & CASAS, L. M. 2012. Comparative study of microcalorimetric behavior of Escherichia coli, Proteus mirabilis and Klebsiella pneumoniae. *Polish Journal of Microbiology,* 61**,** 199-204.

LAGO, N., LEGIDO, J. L., PAZ ANDRADE, M. I., ARIAS, I. & CASAS, L. M. 2011. Microcalorimetric study on the growth and metabolism of Pseudomonas aeruginosa. *Journal of Thermal Analysis and Calorimetry*, 105, 651-655.

LAMBERT, R., MARTIN, F. & VAGNE, M. 1968. Relationship between hydrogen ion and pepsin - concentration in human gastric secretion. *Digestion,* 1**,** 65-77.

LAMONT, J. T. 2002. Theodore E. Woodward Award. How bacterial enterotoxins work: insights from in vivo studies. *Transactions of the American Clinical and Climatological Association*, 113, 167-80; discussion 180-181.

LAN, Y., WILLIAMS, B. A., VERSTEGEN, M. W. A., PATTERSON, R. & TANIMINGA, S. 2007. Soy oligosaccharides in vitro fermentation characteristics and its effect on caecal microorganisms of young broiler chickens. *Animal Feed Science and Technology,* 133**,** 286-297.

LANGWORTHY, D. E., METZ, K. A. & DAWES, S. M. 2002. Flow cytometric methods for the study of bacteria and protozoan parasites in environmental matrices. *Abstracts of the General Meeting of the American Society for Microbiology*, 102, 386.

LARSEN, N., VOGENSEN, F. K., VAN DEN BERG, F. W. J., NIELSEN, D. S., ANDREASEN, A. S., PEDERSEN, B. K., ABU AL-SOUD, W., SORENSEN, S. J., HANSEN, L. H. & JAKOBSEN, M. 2010. Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. *Plos One*, 5.

LARSON, H. E., PARRY, J. V., PRICE, A. B., DAVIES, D. R., DOLBY, J. & TYRRELL, D. A. 1977. Undescribed toxin in pseudomembranous colitis. *British medical journal,* 1**,** 1246-8.

LAWLEY, T. D. & WALKER, A. W. 2013. Intestinal colonization resistance. *Immunology,* 138**,** 1-11.

LEE, H. M. & LEE, Y. 2008. A differential medium for lactic acid-producing bacteria in a mixed culture. *Letters in Applied Microbiology,* 46**,** 676-681.

LEE, Y. J., YU, W. K. & HEO, T. R. 2003. Identification and screening for antimicrobial activity against Clostridium difficile of Bifidobacterium and Lactobacillus species isolated from healthy infant faeces. *International Journal of Antimicrobial Agents,* 21**,** 340-346.

LEMA, M., WILLIAMS, L. & RAO, D. R. 2001. Reduction of fecal shedding of enterohemorrhagic Escherichia coli O157 : H7 in lambs by feeding microbial feed supplement. *Small Ruminant Research,* 39**,** 31-39.

LESLIE, S. B., ISRAELI, E., LIGHTHART, B., CROWE, J. H. & CROWE, L. M. 1995. Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. *Applied and Environmental Microbiology*, 61, 3592-3597.

LESMES, U., BEARDS, E. J., GIBSON, G. R., TUOHY, K. M. & SHIMONI, E. 2008. Effects of resistant starch type III polymorphs on human colon microbiota and short chain fatty acids in human gut models. *Journal of Agricultural and Food Chemistry,* 56**,** 5415-5421.

LEVISON, M. 1977. Factors influencing colonization of the gastrointestinal tract with Pseudomonas auruginosa. In Bergey's manual of systemic bacteriology, 2nd ed. Bergey's manual trust, Springer Science+Business Media. Inc., 233 Spring Street: New York 2, 323-359.

LEY, R. E., BACKHED, F., TURNBAUGH, P., LOZUPONE, C. A., KNIGHT, R. D. & GORDON, J. I. 2005. Obesity alters gut microbial ecology. *Proceedings of the National Academy of Sciences U S A*, 102, 11070-11075.

LEY, R. E., LOZUPONE, C. A., HAMADY, M., KNIGHT, R. & GORDON, J. I. 2008. Worlds within worlds: evolution of the vertebrate gut microbiota. *Nature Reviews Microbiology,* 6**,** 776-788.

LEY, R. E., PETERSON, D. A. & GORDON, J. I. 2006a. Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell*, 124, 837-48.

LEY, R. E., TURNBAUGH, P. J., KLEIN, S. & GORDON, J. I. 2006b. Microbial ecology - Human gut microbes associated with obesity. *Nature*, 444, 1022-1023.

LI, X., LIU, Y., WU, J. & QU, S. S. 2002. Action of the selenomorpholine compounds on the bacterium growth by microcalorimetry. *Chinese Journal of Chemistry*, 20, 829-833.

LI, X., LIU, Y., ZHAO, R. M., WU, J., SHEN, X. S. & QU, S. S. 2000. Microcalorimetric study of Escherichia coli growth inhibited by the selenomorpholine complexes. *Biological Trace Element Research,* 75**,** 167-175.

LIDDELL, J. 2014. Gut bacteria can prevent most diseases including autoimmune diseases. [online]. Available from: http://lifestyleclinic.co.za/auto-immune-diseases-2/ [18 September 2014]

LIESACK, W. & STACKEBRANDT, E. 1992. Unculturable microbes detected by molecular sequences and probes. *Biodiversity and Conservation,* 1**,** 250-262.

LILLY, D. M. & STILLWEL.RH 1965. Probiotics - Growth-promoting factors produced by microorganisms. *Science*, 147, 747-748.

LIM, K. S., HUH, C. S., BAEK, Y. J. & KIM, H. U. 1995. A selective enumeration medium for bifidobacteria in fermented dairy-products. *Journal of Dairy Science,* 78**,** 2108-2112.

LIN, W. H., HWANG, C. F., CHEN, L. W. & TSEN, H. Y. 2006. Viable counts, characteristic evaluation for commercial lactic acid bacteria products. *Food Microbiology,* 23**,** 74-81.

LINDAHL, A., UNGELL, A. L., KNUTSON, L. & LENNERNAS, H. 1997. Characterization of fluids from the stomach and proximal jejunum in men and women. *Pharmaceutical Research*, 14, 497-502.

LINDENEG, O., MELLEMGAARD, K., FABRICIUS, J. & LUNDQUIST, F. 1964. Myocardial utilization of acetate, lactate and free fatty acids after ingestion of ethanol. *Clinical Science,* 27**,** 427-35.

LINTON, A. H. 1983. Theory of antibiotic inhibition zone formation, disc sensitivity methods and MIC determinations. In Denver, A.D. & Quesnel, L.B. 1983. Antibiotic: Assessment of antimicrobial activity and resistance. Academic Press, Inc. London, 19-30.

LIVNEY, Y. D. 2010. Milk proteins as vehicles for bioactives. *Current Opinion in Colloid & Interface Science,* 15**,** 73-83.

LJUNGHOLM, K., WADSO, I. & MARDH, P. A. 1976. Microcalorimetric detection of growth of mycoplasmatales. *Journal of General Microbiology,* 96**,** 283-288.

LONG, S. S. & SWENSON, R. M. 1977. Development of anaerobic fecal flora in healthy newborn infants. *Journal of Pediatrics*, 91, 298-301.

LOVELOCK, J. E. & BISHOP, M. W. H. 1959. Prevention of freezing damage to living cells by dimethyl sulphoxide. *Nature,* 183**,** 1394-1395.

LOVELOCK, J. E. 1953. The mechanism of the protective action of glycerol against haemolysis by freezing and thawing. *Biochimica Et Biophysica Acta,* 11**,** 28-36.

LU, L. & WALKER, W. A. 2001. Pathologic and physiologic interactions of bacteria with the gastrointestinal epithelium. *American Journal of Clinical Nutrition*, 73, 1124S-1130S.

LYERLY, D. M., KRIVAN, H. C. & WILKINS, T. D. 1988. Clostridium difficile: its disease and toxins. *Clinical Microbiology Reviews*, 1, 1-18.

LYRAS, D., O'CONNOR, J. R., HOWARTH, P. M., SAMBOL, S. P., CARTER, G. P., PHUMOONNA, T., POON, R., ADAMS, V., VEDANTAM, G., JOHNSON, S., GERDING, D. N. & ROOD, J. I. 2009. Toxin B is essential for virulence of Clostridium difficile. *Nature,* 458**,** 1176-1181.

MACCONNACHIE, A. A., FOX, R., KENNEDY, D. R. & SEATON, R. A. 2009. Faecal transplant for recurrent Clostridium difficile-associated diarrhoea: a UK case series. *Qjm-an International Journal of Medicine*, 102, 781-784.

MACFARLANE, G. T. & MACFARLANE, S. 1997. Human colonic microbiota: ecology, physiology and metabolic potential of intestinal bacteria. *Scandinavian Journal of Gastroenterology Supplement*, 222, 3-9.

MACFARLANE, G. T., CUMMINGS, J. H. & ALLISON, C. 1986. Protein-degradation by human intestinal bacteria. *Journal of General Microbiology,* 132**,** 1647-1656.

MACFARLANE, G. T., STEED, H. & MACFARLANE, S. 2008. Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. *Journal of Applied Microbiology,* 104**,** 305-344.

MACFARLANE, S. & MACFARLANE, G. T. 2004. Bacterial diversity in the human gut. *Advances in Applied Microbiology,* 54**,** 261-289.

MACFARLANE, S., MACFARLANE, G. T. & CUMMINGS, J. H. 2006. Review article: prebiotics in the gastrointestinal tract. *Alimentary Pharmacology & Therapeutics,* 24**,** 701-714.

MACHAN, Z. A., TAYLOR, G. W., PITT, T. L., COLE, P. J. & WILSON, R. 1992. 2-Heptyl-4-hydroxyquinoline N-oxide, an antistaphylococcal agent produced by Pseudomonas aeruginosa. *Journal of Antimicrobial Chemotherapy*, 30, 615-623.

MACPHERSON, A. J. & HARRIS, N. L. 2004. Interactions between commensal intestinal bacteria and the immune system. *Nature Reviews Immunology,* 4**,** 478-485.

MACY, J. M. & PROBST, I. 1979. The biology of gastrointestinal bacteroides. *Annual Review of Microbiology*, 33, 561-594.

MADIGAN, M. T., MARTINKO, J. M. & PARKER J. 1997. Clinical and diagnostic microbiology and immunology, Brock biology of microorganisms. Prentice Hall, International, Inc., Englewood Cliffs, N. J., 876–877.

MAJAMAA, H. & ISOLAURI, E. 1997. Probiotics: A novel approach in the management of food allergy. *Journal of Allergy and Clinical Immunology,* 99**,** 179-185.

MAJHENIC, A. C., VENEMA, K., ALLISON, G. E., MATIJASIC, B. B., ROGELJ, I. & KLAENHAMMER, T. R. 2004. DNA analysis of the genes encoding acidocin LF221 A and acidocin LF221 B, two bacteriocins produced by Lactobacillus gasseri LF221. *Applied Microbiology and Biotechnology*, 63, 705-14.

MALAGELADA, J. R., LONGSTRETH, G. F., SUMMERSKILL, W. H. J. & GO, V. L. W. 1976. Measurement of gastric functions during digestion of ordinary solid meals in man. *Gastroenterology,* 70**,** 203-210.

MALIN, M., VERRONEN, P., KORHONEN, H., SYVAOJA, E. L., SALMINEN, S., MYKKANEN, H., ARVILOMMI, H., EEROLA, E. & ISOLAURI, E. 1997. Dietary therapy with Lactobacillus GG, bovine colostrum or bovine immune colostrum in patients with juvenile chronic arthritis: Evaluation of effect on gut defence mechanisms. *Inflammopharmacology,* 5**,** 219-236.
Mandatory MRSA, MSSA and *E. coli* bacteraemia and *C. difficile* infection data, 2013/14. [online]. Available from: https://www.gov.uk/government/collections/clostridium-difficile-guidance-data-and-analysis. [10 July 2014]

MARCIANI, L., GOWLAND, P. A., SPILLER, R. C., MANOJ, P., MOORE, R. J., YOUNG, P. & FILLERY-TRAVIS, A. J. 2001. Effect of meal viscosity and nutrients on satiety, intragastric dilution, and emptying assessed by MRI. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 280, G1227-G1233.

MARELLI, G., PAPALEO, E. & FERRARI, A. 2004. Lactobacilli for prevention of urogenital infections: a review. *European review for medical and pharmacological sciences,* 8**,** 87-95.

MAROO, S. & LAMONT, J. T. 2006. Recurrent Clostridium difficile. *Gastroenterology,* 130**,** 1311-1316.

MARTINEZ, R. C., FRANCESCHINI, S. A., PATTA, M. C., QUINTANA, S. M., GOMES, B. C., DE MARTINIS, E. C. & REID, G. 2009. Improved cure of bacterial vaginosis with single dose of tinidazole (2 g), Lactobacillus rhamnosus GR-1, and Lactobacillus reuteri RC-14: a randomized, double-blind, placebo-controlled trial. *Canadian Journal of Microbiology*, 55, 133-138.

MARY, P., OCHIN, D. & TAILLIEZ, R. 1986. Growth status of rhizobia in relation to their tolerance to low water activities and desiccation stresses. *Soil Biology & Biochemistry*, 18, 179-184.

MASCO, L., CROCKAERT, C., VAN HOORDE, K., SWINGS, J. & HUYS, G. 2007. In vitro assessment of the gastrointestinal transit tolerance of taxonomic reference strains from human origin and probiotic product isolates of Bifidobacterium. *Journal of Dairy Science,* 90**,** 3572-3578.

MASKOW, T., KEMP, R., BUCHHOLZ, F., SCHUBERT, T., KIESEL, B. & HARMS, H. 2010. What heat is telling us about microbial conversions in nature and technology: from chip- to megacalorimetry. *Microbial Biotechnology,* 3**,** 269-284.

MASOOD, M. I., QADIR, M. I., SHIRAZI, J. H. & KHAN, I. U. 2011. Beneficial effects of lactic acid bacteria on human beings. *Critical Reviews in Microbiology*, 37, 91-98.

MAUKONEN, J., ALAKOMI, H.-L., NOHYNEK, L., HALLAMAA, K., LEPPÄMÄKI, S., MÄTTÖ, J. & SAARELA, M. 2006. Suitability of the fluorescent techniques for the enumeration of probiotic bacteria in commercial non-dairy drinks and in pharmaceutical products. *Food Research International,* 39**,** 22-32.

MAZUR, P., LEIBO, S. P. & CHU, E. H. Y. 1972. A two factor hypothesis of freezing injury. *Experimental Cell Research,* 71, 345-355.

MCCONNELL, E. L., FADDA, H. M. & BASIT, A. W. 2008b. Gut instincts: explorations in intestinal physiology and drug delivery. *International Journal of Pharmaceutics*, 364, 213-226.

MCCONNELL, E. L., SHORT, M. D. & BASIT, A. W. 2008a. An in vivo comparison of intestinal pH and bacteria as physiological trigger mechanisms for colonic targeting in man. *Journal of Control Release,* 130**,** 154-160.

MCDANIEL, L. E. & BAILEY, E. G. 1968. Liquid nitrogen preservation of standard inoculum: gas-phase storage. *Applied Microbiology*, 16, 912-916.

MCDONALD, L. C., OWINGS, M. & JERNIGAN, D. B. 2006. Clostridium difficile infection in patients discharged from US short-stay hospitals, 1996-2003. *Emerging Infectious Diseases,* 12**,** 409-415.

MCFARLAND, L. V., SURAWICZ, C. M. & STAMM, W. E. 1990. Risk-factors for Clostridium-difficile carriage and C-difficile-associated diarrhea in a cohort of hospitalized-patients. *Journal of Infectious Diseases,* 162**,** 678-684.

MCFARLAND, L. V., SURAWICZ, C. M., RUBIN, M., FEKETY, R., ELMER, G. W. & GREENBERG, R. N. 1999. Recurrent Clostridium difficile disease: Epidemiology and clinical characteristics. *Infection Control and Hospital Epidemiology,* 20**,** 43-50.

MCMASTER-BAXTER, N. L. & MUSHER, D. M. 2007. Clostridium difficile: Recent epidemiologic findings and advances in therapy. *Pharmacotherapy*, 27, 1029-1039.

MEDSCAPE. March 2014. Clostridium difficile colitis. [online]. Available from: http://emedicine.medscape.com/article/186458-overview#aw2aab6b2b4 [Accessed on 7 May 2014]

MERYMAN, H. T. 1971. Cryoprotective Agents. *Cryobiology*, 8, 173-183.

MESSENS, W. & DE, V. L. 2002. Inhibitory substances produced by Lactobacilli isolated from sourdoughs--a review. *International Journal of Food Microbiology,* 72**,** 31-43.

METCHNIKOFF, E. 1908. The microbes of intestinal putrefaction. *Comptes Rendus Hebdomadaires Des Seances De L Academie Des Sciences*, 147, 579-582.

MEYERHOF, O. 1924. "Chemical dynamics in life phenomena". Monograph, 110.

MICHEL-BRIAND, Y. & BAYSSE, C. 2002. The pyocins of Pseudomonas aeruginosa. *Biochimie*, 84, 499-510.

MINAMI, Y., YAZAWA, K., TAMURA, Z., TANAKA, T. & YAMAMOTO, T. 1983. Selectivity of utilization of galactosyl-oligosaccharides by bifidobacteria. *Chemical & Pharmaceutical Bulletin,* 31**,** 1688-1691.

MINELLI, E. B. & BENINI, A. 2008. Relationship between number of bacteria and their probiotic effects. *Microbial Ecology in Health and Disease,* 20**,** 180-183.

MINISTERO DELLA SALUTE, Commissione unica per la nutrizione e la dietetica. Guidelines on probiotics and prebiotics. Ministero della Salute. [online]. Available from: http://www.salute.gov.it/imgs/C_17_pubblicazioni_1016_allegato.pdf. [30 August 2014]

MISHRA, C. & LAMBERT, J. 1996. Production of anti-microbial substances by probiotics. *Asia Pacific journal of clinical nutrition,* 5**,** 20-24.

MISHRA, V. & PRASAD, D. N. 2005. Application of in vitro methods for selection of Lactobacillus casei strains as potential probiotics. *International Journal of Food Microbiology*, 103, 109-115.

MITSUOKA, T. 1984. The effect of nutrition on intestinal flora. *Nahrung,* 28**,** 619-625.

MITTY, R. D. & LAMONT, J. T. 1994. Clostridium difficile diarrhea: pathogenesis, epidemiology, and treatment. *The Gastroenterologist,* 2**,** 61-69.

MOBERG, L. J. & SUGIYAMA, H. 1979. Microbial ecological basis of infant botulism as studied with germfree-mice. *Infection and Immunity,* 25**,** 653-657.

MODABER, I. 1975. Clostridium-difficile. *Acta Medica Iranica,* 18**,** 111-128.

MOKARRAM, R. R., MORTAZAVI, S. A., NAJAFI, M. B. H. & SHAHIDI, F. 2009. The influence of multi stage alginate coating on survivability of potential probiotic bacteria in simulated gastric and intestinal juice. *Food Research International*, 42, 1040-1045.

MOLL, W. M., UNGERECHTS, J., MARKLEIN, G. & SCHAAL, K. P. 1996. Comparison of BBL Crystal(R) ANR ID Kit and API rapid ID 32 A for identification of anaerobic bacteria. *Zentralblatt Fur Bakteriologie-International Journal of Medical Microbiology Virology Parasitology and Infectious Diseases,* 284**,** 329-347.

MOLLY, K., VANDEWOESTYNE, M., DESMET, I. & VERSTRAETE, W. 1994. Validation of the simulator of the human intestinal microbial ecosystem (shime) reactor using microorganism-associated activities. *Microbial Ecology in Health and Disease,* 7**,** 191-200.

MONK, P. & WADSO, I. 1968. A flow micro reaction calorimeter. *Acta Chemica Scandinavica*, 22, 1842-1852.

MONK, P. & WADSO, I. 1975. Use of microcalorimetry for bacterial classification. *Journal of Applied Bacteriology*, 38, 71-74.

MONK, P. & WADSO, I. 1975. Use of microcalorimetry for bacterial classification. *Journal of Applied Bacteriology,* 38**,** 71-74.

MONOD, J. 1949. The growth of bacterial cultures. *Annual Review of Microbiology*, 3, 371-394.

MONOD, J. 1950. La technique de culture continue theorie et applications. *Annales De L Institut Pasteur,* 79**,** 390-410.

MOORE, W. E. C. & HOLDEMAN, L. V. 1974. Human fecal flora - normal flora of 20 Japanese-Hawaiians. *Applied Microbiology*, 27, 961-979.

MOORE, W. E. C., CATO, E. P. & HOLDEMAN, L. V. 1969. Anaerobic bacteria of gastrointestinal flora and their occurrence in clinical infections. *Journal of Infectious Diseases,* 119**,** 641-649.

MOORE, W. E. C., RYSER, M. & HOLDEMAN, L. V. 1975. Predominant kinds of bacteria in gastro intestinal contents. *Abstracts of the Annual Meeting of the American Society for Microbiology,* 75**,** 61.

MORENO, Y., COLLADO, M. C., FERRUS, M. A., COBO, J. M., HERNANDEZ, E. & HERNANDEZ, M. 2006. Viability assessment of lactic acid bacteria in commercial dairy products stored at 4 degrees C using LIVE/DEAD (R) BacLight(TM) staining and conventional plate counts. *International Journal of Food Science and Technology*, 41, 275-280.

MORGAN, C. A., HERMAN, N., WHITE, P. A. & VESEY, G. 2006. Preservation of micro-organisms by drying; A review. *Journal of Microbiological Methods*, 66, 183-193.

MORGAN, T. D., BEEZER, A. E., MITCHELL, J. C. & BUNCH, A. W. 2001. A microcalorimetric comparison of the anti-Streptococcus mutans efficacy of plant extracts and antimicrobial agents in oral hygiene formulations. *Journal of Applied Microbiology,* 90**,** 53-58.

MOURA, P., BARATA, R., CARVALHEIRO, F., GIRIO, F., LOUREIRO-DIAS, M. C. & ESTEVES, M. P. 2007. In vitro fermentation of xylo-oligosaccharides from corn cobs autohydrolysis by Bifidobacterium and Lactobacillus strains. *Lwt-Food Science and Technology,* 40**,** 963-972.

MUDIE, D. M., AMIDON, G. L. & AMIDON, G. E. 2010. Physiological parameters for oral delivery and in vitro testing. *Molecular Pharmaceutics*, 7, 1388-1405.

MULLER, J. A., STANTON, C., SYBESMA, W., FITZGERALD, G. F. & ROSS, R. P. 2010. Reconstitution conditions for dried probiotic powders represent a critical step in determining cell viability. *Journal of Applied Microbiology,* 108**,** 1369-1379.

MUNOZ, P., GIANNELLA, M., ALCALA, L., SARMIENTO, E., YANEZ, J. F., PALOMO, J., CATALAN, P., CARBONE, J. & BOUZA, E. 2007. Clostridium diffidle-associated diarrhea in heart transplant recipients: Is hypogammaglobulinemia the answer? *Journal of Heart and Lung Transplantation,* 26**,** 907-914.

MYLLYLUOMA, E., AHONEN, A. M., KORPELA, R., VAPAATALO, H. & KANKURI, E. 2008. Effects of multispecies probiotic combination on Helicobacter pylori infection in vitro. *Clinical and Vaccine Immunology,* 15**,** 1472-1482.

NA, X. & KELLY, C. 2011. Probiotics in Clostridium difficile infection. *Journal of Clinical Gastroenterology*, 45 Suppl, S154-S158.

NAABER, P., SMIDT, I., STSEPETOVA, J., BRILENE, T., ANNUK, H. & MIKELSAAR, M. 2004. Inhibition of Clostridium difficile strains by intestinal Lactobacillus species. *Journal of Medical Microbiology,* 53**,** 551-554.

NAGARAJAN, K. & LOH, K. C. 2014. Molecular biology-based methods for quantification of bacteria in mixed culture: perspectives and limitations. *Applied microbiology and biotechnology,* 98**,** 6907-6919.

NAGY, E., PETTERSON, M. & MARDH, P. A. 1991. Antibiosis between bacteria isolated from the vagina of women with and without signs of bacterial vaginosis. *APMIS*, 99, 739-744.

NAJIM, H. N., MOHAMMED, A. Z., & SAAB KHUDHIR, Z. 2012. The antibacterial activity of bacteriocin produced by Lactobacillus acidophilus isolates against sensitive reference strain Lactobacillus acidophilus R0052 and its stability to different pH, heating and storage temperatures. *Proceeding of the Eleventh Veterinary Scientific Conference,* 274-279.

NARANG, A., KONOPKA, A. & RAMKRISHNA, D. 1997. New patterns of mixed-substrate utilization during batch growth of Escherichia coli K12. *Biotechnology and Bioengineering,* 55**,** 747-757.

NARAYANAPPA, D. 2008. Randomized double blinded controlled trial to evaluate the efficacy and safety of Bifilac in patients with acute viral diarrhea. *Indian Journal of Pediatrics*, 75, 709-713.

NARUSZEWICZ, M., JOHANSSON, M. L., ZAPOLSKA-DOWNAR, D. & BUKOWSKA, H. 2002. Effect of Lactobacillus plantarum 299v on cardiovascular disease risk factors in smokers. *American Journal of Clinical Nutrition,* 76**,** 1249-1255.

NASH, T., POSTGATE, J. R. & HUNTER, J. R. 1963. Similar effects of various neutral solutes on survival of Aerobacter aerogenes and of red blood cells after freezing and thawing. *Nature,* 199**,** 1113.

NEBRA, Y. & BLANCH, A. R. 1999. A new selective medium for Bifidobacterium spp. *Appl Environ Microbiol,* 65**,** 5173-5176.

NERANDZIC, M. M., PULTZ, M. J. & DONSKEY, C. J. 2009. Examination of potential mechanisms to explain the association between proton pump inhibitors and Clostridium difficile infection. *Antimicrobial Agents and Chemotherapy,* 53**,** 4133-4137.

NES, I. F. 2011. History, current knowledge, and future directions on bacteriocin research in lactic acid bacteria. *Prokaryotic Antimicrobial Peptides: from Genes to Applications*, 3-12.

NEWELL, R. D. 1975. Ph.D. Thesis, University of London.

NEWELL, R.D. 1980. The identification and characterization of microorganisms by microcalorimetry. In Beezer, A. E. 1980. Biological microcalorimetry. London: Academic press, 163-185.

NG, S. C., HART, A. L., KAMM, M. A., STAGG, A. J. & KNIGHT, S. C. 2009. Mechanisms of action of probiotics: recent advances. *Inflammatory Bowel Diseases,* 15**,** 300-310.

NICOLAIDES, E., GALIA, E., EFTHYMIOPOULOS, C., DRESSMAN, J. B. & REPPAS, C. 1999. Forecasting the in vivo performance of four low solubility drugs from their in vitro dissolution data. *Pharmaceutical Research*, 16, 1876-1882.

NIH. September 2013. Colonoscopy. [online]. Available from: http://catalog.niddk.nih.gov/ImageLibrary/detail.cfm?id=1450 [Accessed 7 May 2014]

NING, X. Q., QIAO, W. W., ZHANG, L. & GAO, X. 2013. Applications of microcalorimetry in environmental sciences. *Asian Journal of Chemistry,* 25**,** 8838-8842.

NOLETO, A. L. S., MALBURG, L. M. & BERGDOLL, M. S. 1987. Production of staphylococcal-enterotoxin in mixed cultures. *Applied and Environmental Microbiology*, 53, 2271-2274.

NOVICK, A. & SZILARD, L. 1950. Description of the chemostat. *Science,* 112**,** 715-716.

O'HARA, A. M. & SHANAHAN, F. 2006. The gut flora as a forgotten organ. *Embo Reports,* 7**,** 688-693.

O'MAHONY, F. C. & PAPKOVSKY, D. B. 2006. Rapid high-throughput assessment of aerobic bacteria in complex samples by fluorescence-based oxygen respirometry. *Applied and Environmental Microbiology*, 72, 1279-1287.

O'NEILL, M. A. A. & GAISFORD, S. 2011. Application and use of isothermal calorimetry in pharmaceutical development. *International Journal of Pharmaceutics,* 417**,** 83-93.

O'NEILL, M., VINE, G. J., BEEZER, A. E., BISHOP, A. H., HADGRAFT, J., LABETOULLE, C., WALKER, M., BOWLER, P. G. 2003. Antimicrobial properties of silver-containing wound dressings: a microcalorimetric study. *International Journal of Pharmaceutics,* 263**,** 61-68.

O'TOOLE, P. W. & COONEY, J. C. 2008. Probiotic bacteria influence the composition and function of the intestinal microbiota. *Interdisciplinary perspectives on infectious diseases,* 2008**,** 175285-175285.

OATLEY, J. T., RARICK, M. D., JI, G. E. & LINZ, J. E. 2000. Binding of aflatoxin B-1 to bifidobacteria in vitro. *Journal of Food Protection,* 63**,** 1133-1136.

OLIVARES, M., DIAZ-ROPERO, M. P., MARTIN, R., RODRIGUEZ, J. M. & XAUS, J. 2006. Antimicrobial potential of four Lactobacillus strains isolated from breast milk. *Journal of Applied Microbiology*, 101, 72-79.

OLIVEIRA, R. B. P., OLIVEIRA, A. D. L. & GLORIA, M. B. A. 2008. Screening of lactic acid bacteria from vacuum packaged beef for antimicrobial activity. *Brazilian Journal of Microbiology,* 39**,** 368-374.

OLSSON, C. & HOLMGREN, S. 2001. The control of gut motility. *Comparative Biochemistry and Physiology a-Molecular and Integrative Physiology*, 128, 481-503.

OSBORNE, J. A. & LEE, D. 1975. Studies on conditions required for optimum recovery of Tetrahymena-pyriformis strain s (phenoset a) after freezing to and thawing from -196 C. *Journal of Protozoology*, 22, 233-237.

OUWEHAND, A. C., SALMINEN, S. & ISOLAURI, E. 2002. Probiotics: an overview of beneficial effects. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology,* 82**,** 279-289.

OWENS, R. C. & AMBROSE, P. G. 2007. Amtimicrobial stewardship and the role of pharmacokinetics-pharmacodynamics in the modem antibiotic era. *Diagnostic Microbiology and Infectious Disease*, 57, 77S-83S.

OZMEN, L. & LANGRISH, T. A. G. 2002. Comparison of glass transition temperature and sticky point temperature for skim milk powder. *Drying Technology*, 20, 1177-1192.

PAIDHUNGAT, M. & SETLOW, P. 2000. Role of Ger proteins in nutrient and nonnutrient triggering of spore germination in Bacillus subtilis. *Journal of Bacteriology,* 182**,** 2513-2519.

PAREDES-SABJA, D., BOND, C., CARMAN, R. J., SETLOW, P. & SARKER, M. R. 2008. Germination of spores of Clostridium difficile strains, including isolates from a hospital outbreak of Clostridium difficile-associated disease (CDAD). *Microbiology-Sgm*, 154, 2241-2250.

PAREDES-SABJA, D., BOND, C., CARMAN, R. J., SETLOW, P. & SARKER, M. R. 2009. Germination of spores of Clostridium difficile strains, including isolates from a hospital outbreak of Clostridium difficile-associated disease (CDAD) (vol 154, pg 2241, 2008). *Microbiology-Sgm,* 155**,** 1376-1376.

PAREDES-SABJA, D., SETLOW, P. & SARKER, M. R. 2011. Germination of spores of Bacillales and Clostridiales species: mechanisms and proteins involved. *Trends in Microbiology*, 19, 85-94.

PAREDES-SABJA, D., SHEN, A. & SORG, J. A. 2014. Clostridium difficile spore biology: sporulation, germination, and spore structural proteins. *Trends in Microbiology*, xx, 1-11.

PARKES, G. C., SANDERSON, J. D. & WHELAN, K. 2009. The mechanisms and efficacy of probiotics in the prevention of Clostridium difficile-associated diarrhoea. *Lancet Infectious Diseases,* 9**,** 237-244.

PARVEZ, S., MALIK, K. A., KANG, S. A. & KIM, H. Y. 2006. Probiotics and their fermented food products are beneficial for health. *Journal of Applied Microbiology,* 100**,** 1171-1185.

PATTANI, R., PALDA, V. A., HWANG, S. W. & SHAH, P. S. 2013. Probiotics for the prevention of antibiotic-associated diarrhea and Clostridium difficile infection among hospitalized patients: systematic review and meta-analysis. *Open Med*, 7, e56-67.

PEDERSEN, P. B., VILMANN, P., BAR-SHALOM, D., MULLERTZ, A. & BALDURSDOTTIR, S. 2013. Characterization of fasted human gastric fluid for relevant rheological parameters and gastric lipase activities. *European Journal of Pharmaceutics and Biopharmaceutics,* 85**,** 958-965.

PEDRETTI, S. 2013. Probiotic market: up or down? *Nutrafoods*, 12, N18-N19.

PEI, Z. H., BINI, E. J., YANG, L. Y., ZHOU, M. S., FRANCOIS, F. & BLASER, M. J. 2004. Bacterial biota in the human distal esophagus. *Proceedings of the National Academy of Sciences of the United States of America*, 101, 4250-4255.

PEPIN, J., ALARY, M. E., VALIQUETTE, L., RAICHE, E., RUEL, J., FULOP, K., GODIN, D. & BOURASSA, C. 2005. Increasing risk of relapse after treatment of Clostridium difficile colitis in Quebec, Canada. *Clinical Infectious Diseases,* 40**,** 1591-1597.

PERESTELO, F. R., BLANCO, M. T., GUTIERREZ-NAVARRO, A. M. & FALCON, M. A. 1985. Growth inhibition of Staphylococcus-aureus by a staphylolytic enzyme from Pseudomonas-aeruginosa. *Microbios Letters*, 30, 85-94.

PEREZ, P. F., DORE, J., LECLERC, M., LEVENEZ, F., BENYACOUB, J., SERRANT, P., SEGURA-ROGGERO, I., SCHIFFRIN, E. J. & DONNET-HUGHES, A. 2007. Bacterial imprinting of the neonatal immune system: Lessons from maternal cells? *Pediatrics*, 119, E724-E732.

PETERSON, A. C., BLACK, J. J. & GUNDERSON, M. F. 1962. Staphylococci in competition .2. Effect of total numbers and proportion of Staphylococci in mixed cultures on growth in artificial culture medium. *Applied Microbiology*, 10, 23-30.

PIARD, J. C. & DESMAZEAUD, M. 1991. Inhibiting factors produced by lactic-acid bacteria .1. oxygen metabolites and catabolism end-products. *Lait*, 71, 525-541.

PIARD, J. C. & DESMAZEAUD, M. 1992. Inhibiting factors produced by lactic-acid bacteria .2. bacteriocins and other antibacterial substances. *Lait*, 72, 113-142.

PIDDOCK, L. J. V. 1990. Techniques used for the determination of antimicrobial resistance and sensitivity in bacteria. *Journal of Applied Bacteriology,* 68**,** 307-318.

PILLAI, A. & NELSON, R. 2008. Probiotics for treatment of Clostridium difficile-associated colitis in adults. *Cochrane Database of Systematic Reviews*, CD004611.

PLOWMAN, J. & PECK, M. W. 2002. Use of a novel method to characterize the response of spores of non-proteolytic Clostridium botulinum types B, E and F to a wide range of germinants and conditions. *Journal of Applied Microbiology,* 92**,** 681-694.

POCOCK, G., RICHARDS, C. D. & RICHARDS, D. A. 2006. The gastrointestinal system. In Human Physiology. Oxford University Press. 4th edition, 579-623.

POSTGATE, J. R. & HUNTER, J. R. 1961. Survival of frozen bacteria. *Journal of General Microbiology,* 26**,** 367-378.

POSTGATE, J. R. 1976. Death in microbes and macrobes. In GRAY T. R. G. & POSTGATE J. R. (ed.), The survival of vegetative microbes. Cambridge University Press, Cambridge, United Kingdom.

POXTON, I. R., MCCOUBREY, J. & BLAIR, G. 2001. The pathogenicity of Clostridium difficile. *Clinical microbiology and infection,* 7**,** 421-7.

PRAKASH, O., NIMONKAR, Y. & SHOUCHE, Y. S. 2013. Practice and prospects of microbial preservation. *Fems Microbiology Letters,* 339**,** 1-9.

PRAT, H. 1953. Observations on thermogenic bacteria. *Revue Canadienne De Biologie,* 12**,** 19-34.

PRAT, H., CALVET, E. & FRICKER, J. 1946. Etude microcalorimetriques du development des cultures bacteriennes et de la germination des graines. *Revue Canadienne de Biologie*, 5, 247-250.

PRESS, A. G., HAUPTMANN, I. A., HAUPTMANN, L., FUCHS, B., FUCHS, M., EWE, K. & RAMADORI, G. 1998. Gastrointestinal pH profiles in patients with inflammatory bowel disease. *Alimentary Pharmacology & Therapeutics,* 12**,** 673-678.

PROBERT, H. M. & GIBSON, G. R. 2002. Investigating the prebiotic and gas-generating effects of selected carbohydrates on the human colonic microflora. *Letters in Applied Microbiology,* 35**,** 473-480.

PROBERT, H. M., APAJALAHTI, J. H., RAUTONEN, N., STOWELL, J. & GIBSON, G. R. 2004. Polydextrose, lactitol, and fructo-oligosaccharide fermentation by colonic bacteria in a three-stage continuous culture system. *Applied and Environmental Microbiology,* 70**,** 4505-4511.

PUBLIC HEALTH ENGLAND, 2013. Update guidance on the management and treatment of Clostridium difficile infection. Gov.UK [online]. Available from: https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/321891/Clostridium_difficile_management_and_treatment.pdf [15 September 2014]

PUBLIC HEALTH ENGLAND, 2014. Annual Epidemiological Commentary: Mandatory MRSA, MSSA and *E. coli* bacteraemia and *C. difficile* infection data, 2013/14. [online]. Available from: https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/330529/HCAI_mandatory_surveillance_annual_epidemiological_commentary_2013_14.pdf [15 September 2014]

QUIGLEY, E. M. M. 2013. Gut bacteria in health and disease. *Gastroenterology & Hepatology,* 9**,** 560-569.

RAINKIE, D. & KOLBER, M. R. 2013. Probiotics for the prevention of Clostridium difficile. *Canadian Family Physician*, 59, 957.

RANADHEERA, C. S., EVANS, C. A., ADAMS, M. C. & BAINES, S. K. 2012. In vitro analysis of gastrointestinal tolerance and intestinal cell adhesion of probiotics in goat's milk ice cream and yogurt. *Food Research International*, 49, 619-625.

RAY, B., JEZESKI, J. J. & BUSTA, F. F. 1971. Effect of rehydration on recovery, repair, and growth of injured freeze-dried Salmonella-anatum. *Applied Microbiology*, 22, 184-189.

REA, M. C., ALEMAYEHU, D., ROSS, R. P. & HILL, C. 2013. Gut solutions to a gut problem: bacteriocins, probiotics and bacteriophage for control of Clostridium difficile infection. *Journal of Medical Microbiology,* 62**,** 1369-1378.

REA, M. C., DOBSON, A., O'SULLIVAN, O., CRISPIE, F., FOUHY, F., COTTER, P. D., SHANAHAN, F., KIELY, B., HILL, C. & ROSS, R. P. 2011. Effect of broad- and narrow-spectrum antimicrobials on Clostridium difficile and microbial diversity in a model of the distal colon. *Proceedings of the National Academy of Sciences of the United States of America,* 108**,** 4639-4644.

REA, M. C., SIT, C. S., CLAYTON, E., O'CONNOR, P. M., WHITTAL, R. M., ZHENG, J., VEDERAS, J. C., ROSS, R. P. & HILL, C. 2010. Thuricin CD, a posttranslationally modified bacteriocin with a narrow spectrum of activity against Clostridium difficile. *Proceedings of the National Academy of Sciences of the United States of America,* 107**,** 9352-9357.

REID, G. 2001b. Probiotic agents to protect the urogenital tract against infection. *American Journal of Clinical Nutrition,* 73**,** 437S-443S.

REID, G., BRUCE, A. W., MCGROARTY, J. A., CHENG, K. J. & COSTERTON, J. W. 1990. Is there a role for Lactobacilli in prevention of urogenital and intestinal infections. *Clinical Microbiology Reviews,* 3**,** 335-344.

REID, G., COOK, R. L. & BRUCE, A. W. 1987. Examination of strains of Lactobacilli for properties that may influence bacterial interference in the urinary-tract. *Journal of Urology,* 138**,** 330-335.

REID, G., HOWARD, J. & GAN, B. S. 2001a. Can bacterial interference prevent infection? *Trends in Microbiology*, 9, 424-428.

REIMER, L. & CARROLL, K. 2004. Procedure for the storage of microorganisms. In: Murray E., Baron, E., Pfaller, M., Tenover, F. & Yolken, R. Eds. Manual of Clinical Microbiology. ASM Press, Washington, DC 67–73.

RESHEF, L., NIV, J. & SHAPIRO, B. 1967. Effect of propionate on lipogenesis in adipose tissue. *Journal of Lipid Research*, 8, 682-687.

RHODES, J., BARNARDO, D. E., PHILLIPS, S. F., ROVELSTA.RA & HOFMANN, A. F. 1969. Increased reflux of bile into stomach in patients with gastric ulcer. *Gastroenterology*, 57, 241-252.

RIEDELE, C. & REICHL, U. 2011. Interspecies effects in a ceftazidime-treated mixed culture of Pseudomonas aeruginosa, Burkholderia cepacia and Staphylococcus aureus: analysis at the single-species level. *Journal of Antimicrobial Chemotherapy,* 66**,** 138-145.

RIFKIN, G. D., FEKETY, F. R. & SILVA, J., JR. 1977. Antibiotic-induced colitis implication of a toxin neutralised by Clostridium sordellii antitoxin. *Lancet,* 2**,** 1103-1106.

RILEY, M. A. & GORDON, D. M. 1992. A Survey of col plasmids in natural isolates of Escherichia-coli and an investigation into the stability of col-plasmid lineages. *Journal of General Microbiology*, 138, 1345-1352.

RIO, M. E., ZAGO, L. B., GARCIA, H. & WINTER, L. 2002. Undernutrition affect live lactobacillus effect on respiratory tract infections in children from social risk areas. *Archivos Latinoamericanos De Nutricion,* 52**,** 29-34.

RISHMAWI, N., GHNEIM, R., KATTAN, R., GHNEIM, R., ZOUGHBI, M., ABU-DIAB, A., TURKUMAN, S., DAUODI, R., SHOMALI, I., ISSA, A. E.-R., SIRIANI, I., MARZOUKA, H., SCHMID, I. & HINDIYEH, M. Y. 2007. Survival of fastidious and nonfastidious aerobic bacteria in three bacterial transport swab systems. *Journal of Clinical Microbiology*, 45, 1278-1283.

ROBERFROID, M., GIBSON, G. R., HOYLES, L., MCCARTNEY, A. L., RASTALL, R., ROWLAND, I., WOLVERS, D., WATZL, B., SZAJEWSKA, H., STAHL, B., GUARNER, F., RESPONDEK, F., WHELAN, K., COXAM, V., DAVICCO, M. J., LEOTOING, L., WITTRANT, Y., DELZENNE, N. M., CANI, P. D., NEYRINCK, A. M. & MEHEUST, A. 2010. Prebiotic effects: metabolic and health benefits. *British Journal of Nutrition*, 104 Suppl 2, S1-63.

ROEDIGER, W. E. & MOORE, A. 1981. Effect of short-chain fatty acid on sodium absorption in isolated human colon perfused through the vascular bed. *Digestive Diseases and Sciences,* 26**,** 100-106.

ROEDIGER, W. E. W. 1980. Anaerobic-bacteria, the colon and colitis. *Australian and New Zealand Journal of Surgery,* 50**,** 73-75.

ROGERS, A. M. & MONTVILLE, T. 1991. Improved agar diffusion assay for nisin quantification. *Food Biotechnology*, 5, 161-168.

RONG, X. M., HUANG, Q. Y., JIANG, D. H., CAI, P. & LIANG, W. 2007. Isothermal microcalorimetry: A review of applications in soil and environmental sciences. *Pedosphere,* 17**,** 137-145.

ROOS, Y. H. 2002. Importance of glass transition and water activity to spray drying and stability of dairy powders. *Lait,* 82, 475-484.

ROSENFELDT, V., BENFELDT, E., NIELSEN, S. D., MICHAELSEN, K. F., JEPPESEN, D. L., VALERIUS, N. H. & PAERREGAARD, A. 2003. Effect of probiotic Lactobacillus strains in children with atopic dermatitis. *Journal of Allergy and Clinical Immunology,* 111**,** 389-395.

ROSS, P. W. 1971. Quantitative studies on salivary flora. *Journal of Clinical Pathology,* 24**,** 717-720.

ROSSI, M., AMARETTI, A. & RAIMONDI, S. 2011. Folate production by probiotic bacteria. *Nutrients,* 3**,** 118-134.

ROUND, J. L. & MAZMANIAN, S. K. 2009. The gut microbiota shapes intestinal immune responses during health and disease. *Nature Reviews Immunology*, 9, 313-323.

ROWLAND, I. R., MALLETT, A. K. & WISE, A. 1985. The effect of diet on the mammalian gut flora and its metabolic-activities. *Crc Critical Reviews in Toxicology,* 16**,** 31-103.

RUBNER, M. 1903. *Hyg Rundsch* 13, 857.

RUBNER, M. 1904. *Archiv for Hygiene*, 48, 260.

RUBNER, M. 1906. *Archiv for Hygiene*, 57, 193-243.

RUEGER, M., BENSCH, G., TUENGLER, R. & REICHL, U. 2012. A flow cytometric method for viability assessment of Staphylococcus aureus and Burkholderia cepacia in mixed culture. *Cytometry* Part A, 81A, 1055-1066.

RUIZ, F. O., GERBALDO, G., ASURMENDI, P., PASCUAL, L. M., GIORDANO, W. & BARBERIS, I. L. 2009. Antimicrobial activity, inhibition of urogenital pathogens, and synergistic interactions between lactobacillus strains. *Current Microbiology,* 59**,** 497-501.

RUNGE, F. E. & HEGER, R. 2000. Use of microcalorimetry in monitoring stability studies. Example: Vitamin A esters. *Journal of Agricultural and Food Chemistry,* 48**,** 47-55.

RUPNIK, M., WILCOX, M. H. & GERDING, D. N. 2009. Clostridium difficile infection: new developments in epidemiology and pathogenesis. *Nature Reviews Microbiology,* 7**,** 526-536.

RUSSEL, M., YAO, J., CHEN, H., WANG, F., ZHOU, Y., ZHUANG, R., CHOI, M. M. F., CECCANTI, B., TREBSE, P. & GYULA, Z. 2009. Biological and microcalorimetric studies of the toxic effect of organoarsenic(v) compounds to wild strain of Bacillus thuringiensis. *Biological Trace Element Research,* 131**,** 192-203.

RUSSEL, W.J., FARLING, S.R., BLANCHARD, G.C. & BOLING, E.A. 1975. "Interim Review of Microbial Identification by Microcalorimetry in Microbiology-1975" (D. Schlessinger, ed.) American Society for Microbiology, Washington, D.C. 22-31.

RYCROFT, C. E., JONES, M. R., GIBSON, G. R. & RASTALL, R. A. 2001. A comparative in vitro evaluation of the fermentation properties of prebiotic oligosaccharides. *Journal of Applied Microbiology,* 91**,** 878-887.

SAARELA, M., HALLAMAA, K., MATTILA-SANDHOLM, T. & MATTO, J. 2003. The effect of lactose derivatives lactulose, lactitol and lactobionic acid on the functional and technological properties of potentially probiotic Lactobacillus strains. *International Dairy Journal,* 13**,** 291-302.

SAARELA, M., MOGENSEN, G., FONDEN, R., MATTO, J. & MATTILA-SANDHOLM, T. 2000. Probiotic bacteria: safety, functional and technological properties. *Journal of Biotechnology,* 84**,** 197-215.

SAAVEDRA, J. 2000. Probiotics and infectious diarrhea. *American Journal of Gastroenterology*, 95, S16-S18.

SABIKHI, L., BABU, R., THOMPKINSON, D. K. & KAPILA, S. 2008. Resistance of microencapsulated lactobacillus acidophilus la1 to processing treatments and simulated gut conditions. *Food and Bioprocess Technology*, 3, 586-593.

SACCARO, D. M., HIROTA, C. Y., TAMIME, A. & OLIVEIRA, M. N. 2011. Evaluation of different selective media for enumeration of probiotic micro-organisms in combination with yoghurt starter cultures in fermented milk. *African Journal of Microbiology Research,* 46, 3901-3906.

SAHADEVA, R.P.K., LEONG, S.F., CHUA, K. H., TAN, C.H., CHAN, H.Y., TONG, E.V., WONG, S.Y.W. & CHAN, H.K. 2011. Survival of commercial probiotic strains to pH and bile. *International Food Research Journal*, 18,1515-1522.

SAID, J., DODOO, C. C., WALKER, M., PARSONS, D., STAPLETON, P., BEEZER, A. E. & GAISFORD, S. 2014. An in vitro test of the efficacy of silver-containing wound dressings against Staphylococcus aureus and Pseudomonas aeruginosa in simulated wound fluid. *International Journal of Pharmaceutics*, 462, 123-128.

SALMINEN, S. J., GUEIMONDE, M. & ISOLAURI, E. 2005. Probiotics that modify disease risk. *Journal of Nutrition*, 135, 1294-1298.

SALMINEN, S., VON WRIGHT, A., MORELLI, L., MARTEAU, P., BRASSART, D., DE VOS, W. M., FONDEN, R., SAXELIN, M., COLLINS, K., MOGENSEN, G., BIRKELAND, S. E. & MATTILA-SANDHOLM, T. 1998. Demonstration of safety of probiotics -- a review. *International Journal of Food Microbiology,* 44**,** 93-106.

SANDERS, M. E. & HUIS IN'T VELD, J. H. I. 1999. Bringing a probiotic-containing functional food to the market: microbiological, product, regulatory and labeling issues. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology*, 76, 293-315.

SANDERS, M. E. 1993. Summary of conclusions from a consensus panel of experts on health attributes of lactic cultures - significance to fluid milk-products containing cultures. *Journal of Dairy Science*, 76, 1819-1828.

SANTIVARANGKNA, C., KULOZIK, U. & FOERST, P. 2007. Alternative drying processes for the industrial preservation of lactic acid starter cultures. *Biotechnology Progress*, 23, 302-315.

SARKER, S. A. & GYR, K. 1992. Nonimmunological defense-mechanisms of the gut. *Gut,* 33**,** 987-993.

SAULNIER, D. M., SPINLER, J. K., GIBSON, G. R. & VERSALOVIC, J. 2009. Mechanisms of probiosis and prebiosis: considerations for enhanced functional foods. *Current Opinion in Biotechnology*, 20, 135-141.

SAVAGE, D. C. 1977. Microbial ecology of gastrointestinal-tract. *Annual Review of Microbiology,* 31**,** 107-133.

SAVAGE, D. C. 1986. Gastrointestinal microflora in mammalian nutrition. *Annual Review of Nutrition,* 6**,** 155-178.

SAVINI, M., CECCHINI, C., VERDENELLI, M. C., SILVI, S., ORPIANESI, C. & CRESCI, A. 2010. Pilot-scale production and viability analysis of freeze-dried probiotic bacteria using different protective agents. *Nutrients*, 2, 330-339.

SAXELIN, M. 2008. Probiotic formulations and applications, the current probiotics market, and changes in the marketplace: a European perspective. *Clinical Infectious Diseases,* 46 Suppl 2**,** S76-9; discussion S144-S151.

SCHAARSCHMIDT, B. & LAMPRECHT, I. 1978. Micro-calorimetric study of yeast growth, utilization of different carbohydrates. *Thermochimica Acta,* 22**,** 333-338.

SCHAEFFER, A. B. & FULTON, M. D. 1933. A simplified method of staining endospores. Science New York, N.Y., 77, 194-194.

SCHAFFER, B. & LORINCZY, D. 2005. Isoperibol calorimetry as a tool to evaluate the impact of the ratio of exopolysaccharide-producing microbes on the properties of sour cream. *Journal of Thermal Analysis and Calorimetry,* 82**,** 537-541.

SCHAFFER, B., SZAKALY, S. & LORINCZY, D. 2004. Examination of the growth of probiotic culture combinations by the isoperibolic batch calorimetry. *Thermochimica Acta,* 415**,** 123-126.

SCHELINE, R. R. 1973. Metabolism of foreign compounds by gastrointestinal microorganisms. *Pharmacological Reviews,* 25**,** 451-523.

SCHEUTZ, F. & STROCKBINE, A. N. 2005. In Bergey's manual of systemic bacteriology, 2nd ed. Bergey's manual trust, Springer Science Business Media. Inc., 233 Spring Street: New York, 2, 607-625.

SCHLEIFER, K. & BELL, A. J. 2009. In Bergey's manual of systematic bacteriology, 2nd ed. Bergey's manual trust, Springer Dordrecht Heidelberg: London New York 3, 392-400.

SCHLEIFER, K. 2009. In Bergey's manual of systematic bacteriology, 2nd ed. Bergey's manual trust, Springer Dordrecht Heidelberg: London New York 3, 465-491.

SCHMIDT, H. A., FRITZLAR, G., DOLLE, W. & GOEBELL, H. 1970. Histamine and insulin-stimulated acid and pepsin secretion in patients with duodenal ulcers and normal subjects. *Deutsche Medizinische Wochenschrift,* 95**,** 2011-2016.

SCHMIDT, J. K., KONIG, B. & REICHL, U. 2007. Characterization of a three bacteria mixed culture in a chemostat: evaluation and application of a quantitative terminal-restriction fragment length polymorphism (T-RFLP) analysis for absolute and species specific cell enumeration. *Biotechnology and Bioengeneering,* 96**,** 738-756.

SCHMIDT, J. K., RIEDELE, C., REGESTEIN, L., RAUSENBERGER, J. & REICHL, U. 2011. A Novel Concept Combining Experimental and Mathematical Analysis for the Identification of Unknown Interspecies Effects in a Mixed Culture. Biotechnology and Bioengineering, 108, 1900-1911.

SCHOSTER, A., KOKOTOVIC, B., PERMIN, A., PEDERSEN, P. D., DAL BELLO, F. & GUARDABASSI, L. 2013. In vitro inhibition of Clostridium difficile and Clostridium perfringens by commercial probiotic strains. *Anaerobe,* 20**,** 36-41.

SCHWAN, A., SJOLIN, S., TROTTESTAM, U. & ARONSSON, B. 1984. Relapsing Clostridium difficile enterocolitis cured by rectal infusion of normal feces. *Scandinavian Journal of Infectious Diseases,* 16**,** 211-215.

SCHWAN, C., STECHER, B., TZIVELEKIDIS, T., VAN HAM, M., ROHDE, M., HARDT, W.-D., WEHLAND, J. & AKTORIES, K. 2009. Clostridium difficile Toxin CDT Induces Formation of Microtubule-Based Protrusions and Increases Adherence of Bacteria. *Plos Pathogens,* 5.

SCYTHES, K. D., LOUIE, M. & SIMOR, A. E. 1996. Evaluation of nutritive capacities of 10 broth media. *Journal of Clinical Microbiology*, 34, 1804-1807.

SEDLACZE.L, CZERNIAW.E, RADZIEJE.J & ZABLOCKI, B. 1966. Thermogenesis and growth yield of Escherichia coli in media containing various nitrogen sources. *Bulletin De L Academie Polonaise Des Sciences-Serie Des Sciences Biologiques,* 14**,** 613.

SEKIROV, I., RUSSELL, S. L., ANTUNES, L. C. & FINLAY, B. B. 2010. Gut microbiota in health and disease. *Physiological Reviews,* 90**,** 859-904.

SEKSIK, P. 2010. Gut microbiota and IBD. *Gastroentérologie clinique et biologique*, 34 Suppl 1, S44-S51.

SEPOVA, H. K., DUBNICKOVA, M., BILKOVA, A., BUKOVSKY, M. & BEZAKOVA, L. 2011. Identification and biological activity of potential probiotic bacterium isolated from the stomach mucus of breast-fed lamb. *Brazilian Journal of Microbiology,* 42**,** 1187-1195.

SERVIN, A. L. 2004. Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. *Fems Microbiology Reviews,* 28**,** 405-440.

SETLOW, P. 2007. I will survive: DNA protection in bacterial spores. *Trends in Microbiology,* 15**,** 172-180.

SGHIR, A., CHOW, J. M. & MACKIE, R. I. 1998. Continuous culture selection of bifidobacteria and lactobacilli from human faecal samples using fructooligosaccharide as selective substrate. *Journal of Applied Microbiology,* 85**,** 769-777.

SHAH, N. P. 2000. Probiotic bacteria: Selective enumeration and survival in dairy foods. *Journal of Dairy Science,* 83**,** 894-907.

SHAH, N. P. 2007. Functional cultures and health benefits. *International Dairy Journal*, 17, 1262-1277.

SHEARER, C. 1921. On the amount of heat liberated by Bacillu coli when grown in the presence of free amino-acids. *Journal of Physiology-London*, 55, 50-60.

SIMIONE, F. P. & BROWN, E. M. 1991. ATCC preservation methods. Freezing and freeze drying. American Type Culture Collection, Rockville, Maryland.

SIMONCIC, Z., ZUPANCIC, P., ROSKAR, R., GARTNER, A., KOGEJ, K. & KMETEC, V. 2007. Use of microcalorimetry in determination of stability of enalapril maleate and enalapril maleate tablet formulations. *International Journal of Pharmaceutics,* 342**,** 145-151.

SINHA, V. R. & KUMRIA, R. 2003. Microbially triggered drug delivery to the colon. *European Journal of Pharmaceutical Sciences*, 18, 3-18.

SMITH, E. A. & MACFARLANE, G. T. 1996. Enumeration of human colonic bacteria producing phenolic and indolic compounds: Effects of pH, carbohydrate availability and retention time on dissimilatory aromatic amino acid metabolism. *Journal of Applied Bacteriology,* 81**,** 288-302.

SMITH, K. 2013. Therapy: Probiotics do not reduce antibiotic-associated or Clostridium difficile diarrhoea in older hospitalized patients. *Nature Reviews Gastroenterology & Hepatology*, 10, 566.

SOHIER, D., PAVAN, S., RIOU, A., COMBRISSON, J. & POSTOLLEC, F. 2014. Evolution of microbiological analytical methods for dairy industry needs. *Frontiers in Microbiology,* 5**,** 1-10.

SOKOLNICKA, I. 2000. The effect of probiotics on the immune system. *Pneumonologia i alergologia polska*, 68, 471-477.

SOKOLSKI, W. T., STAPERT, E. M. & FERRER, E. B. 1964. Liquid nitrogen freezing in microbiological assay systems. I. Preservation of Lactobacillus leichmannii for direct use in the vitamin B12 assay. *Applied Microbiology*, 12, 327-329.

SONOIKE, K., MADA, M. & MUTAI, M. 1986. Selective agar medium for counting viable cells of bifidobacteria in fermented milk. *Journal of the Food Hygienic Society of Japan,* 27**,** 238-244.

SORG, J. A. & SONENSHEIN, A. L. 2008. Bile salts and glycine as cogerminants for Clostridium difficile spores. *Journal of Bacteriology,* 190**,** 2505-2512.

SOURABH, A., KANWAR, S. S. & SHARMA, O. P. 2011. Antagonistic potential of indigenous bacterial probiotics of Western Himalayas against antibiotic-resistant bacterial pathogens. *Current Science,* 101**,** 1351-1356.

SOUSA, T., PATERSON, R., MOORE, V., CARLSSON, A., ABRAHAMSSON, B. & BASIT, A. W. 2008. The gastrointestinal microbiota as a site for the biotransformation of drugs. *International Journal of Pharmaceutics,* 363**,** 1-25.

SPIEGELMAN, D., WHISSELL, G. & GREER, C. W. 2005. A survey of the methods for the characterization of microbial consortia and communities. *Canadian Journal of Microbiology,* 51**,** 355-386.

STAPERT, E. M., SOKOLSKI, W. T., KANESHIRO, W. M. & COLE, R. J. 1964. Liquid nitrogen freezing in microbiological assay systems. II. Preservation of Sarcina Lutea for antibiotic assays. *Journal of Bacteriology*, 88, 532-533.

STAPLES, B. R., PROSEN, E. J. & GOLDBERG, R. N. 1973. National Bureau of Standards NBSIR Washington, D. C. 73-181.

STECHER, B. & HARDT, W.-D. 2008. The role of microbiota in infectious disease. *Trends in Microbiology,* 16**,** 107-114.

STECHER, B., ROBBIANI, R., WALKER, A. W., WESTENDORF, A. M., BARTHEL, M., KREMER, M., CHAFFRON, S., MACPHERSON, A. J., BUER, J., PARKHILL, J., DOUGAN, G., VON MERING, C. & HARDT, W.-D. 2007. Salmonella enterica serovar typhimurium exploits inflammation to compete with the intestinal microbiota. *Plos Biology*, 5, 2177-2189.

STEER, T., CARPENTER, H., TUOHY, K. & GIBSON, G. R. 2000. Perspectives on the role of the human gut microbiota and its modulation by pro- and prebiotics. *Nutrition Research Reviews*, 13, 229-254.

STEINGOETTER, A., FOX, M., TREIER, R., WEISHAUPT, D., MARINCEK, B., BOESIGER, P., FRIED, M.& SCHWIZER, W. 2006. Effects of posture on the physiology of gastric emptying: a magnetic resonance imaging study. *Scandinavian Journal of Gastroenterology*, 41,1155–1164.

STRINGER, S. C., WEBB, M. D. & PECK, M. W. 2011. Lag time variability in individual spores of Clostridium botulinum. *Food Microbiology*, 28, 228-235.

SUN, W. R. & GRIFFITHS, M. W. 2000. Survival of bifidobacteria in yogurt and simulated gastric juice following immobilization in gellan-xanthan beads. *International Journal of Food Microbiology*, 61, 17-25.

SWIDSINSKI, A., LOENING-BAUCKE, V., VERSTRAELEN, H., OSOWSKA, S. & DOERFFEL, Y. 2008. Biostructure of fecal microbiota in healthy subjects and patients with chronic idiopathic diarrhea. *Gastroenterology*, 135, 568-579.

TABASCO, R., GARCIA-CAYUELA, T., PELAEZ, C. & REQUENA, T. 2009. Lactobacillus acidophilus La-5 increases lactacin B production when it senses live target bacteria. *International Journal of Food Microbiology*, 132, 109-116.

TABASCO, R., PAARUP, T., JANER, C., PELAEZ, C. & REQUENA, T. 2007. Selective enumeration and identification of mixed cultures of Streptococcus thermophilus, Lactobacillus delbrueckii subsp bulgaricus, L-acidophilus, L-paracasei subsp paracasei and Bifidobacterium lactis in fermented milk. *International Dairy Journal*, 17, 1107-1114.

TAN, A. M. & LU, J. H. 1999. Microcalorimetric study of antiviral effect of drug. *Journal of Biochemical and Biophysical Methods*, 38, 225-228.

TANG, X. L. & PIKAL, M. J. 2004. Design of freeze-drying processes for pharmaceuticals: Practical advice. *Pharmaceutical Research*, 21, 191-200.

TANGL, F. 1903. Articles on the energetic of ontogenesis. Second announcement - On the consumption of chemical energy during the development of bacteria cultures. *Archiv Fur Die Gesamte Physiologie Des Menschen Und Der Tiere*, 98, 475-489.

TANNOCK, G. W. 2008. Molecular analysis of the intestinal microflora in IBD. *Mucosal Immunology*, 1, S15-S18.

TEDESCHI, R. & DE PAOLI, P. 2011. Collection and preservation of frozen microorganisms. *Methods in Molecular Biology*, 675, 313-326.

TEDESCO, F. J. 1981. Antibiotic-associated colitis--an abating enigma. *Journal of clinical gastroenterology*, 3, 221-224.

TEIXEIRA, P., CASTRO, H. & KIRBY, R. 1995. Spray-drying as a method for preparing concentrated cultures of Lactobacillus-bulgaricus. *Journal of Applied Bacteriology*, 78, 456-462.

TEJERO-SARINENA, S., BARLOW, J., COSTABILE, A., GIBSON, G. R. & ROWLAND, I. 2013. Antipathogenic activity of probiotics against Salmonella Typhimurium and Clostridium difficile in anaerobic batch culture systems: is it due to synergies in probiotic mixtures or the specificity of single strains? *Anaerobe*, 24, 60-65.

TEJERO-SARINENA, S., BARLOW, J., COSTABILE, A., GIBSON, G. R. & ROWLAND, I. 2012. In vitro evaluation of the antimicrobial activity of a range of probiotics against pathogens: Evidence for the effects of organic acids. *Anaerobe*, 18, 530-538.

TEMMERMAN, R., POT, B., HUYS, G. & SWINGS, J. 2001. A quality analysis of commercial probiotic products. *Meded Rijksuniv Gent Fak Landbouwkd Toegep Biol Wet*, 66, 535, 537-542.

TEMMERMAN, R., SCHEIRLINCK, I., HUYS, G. & SWINGS, J. 2003. Culture-independent analysis of probiotic products by denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology*, 69, 220-226.

TENNYSON, C. A. & FRIEDMAN, G. 2008. Microecology, obesity, and probiotics. *Current Opinion in Endocrinology Diabetes and Obesity*, 15, 422-427.

TERROINE, E. F. & WURMSER, R. 1922. Energy of growth. I. Development of Aspergillus niger. *Bulletin de la Société de Chimie Biologique*, 4, 519.

THADEPALLI, H., LOU, M. A., BACH, V. T., MATSUI, T. K. & MANDAL, A. K. 1979. Microflora of the human small-intestine. *American Journal of Surgery,* 138**,** 845-850.

THAM, C. S.-C., PEH, K.-K., BHAT, R. & LIONG, M.-T. 2012. Probiotic properties of bifidobacteria and lactobacilli isolated from local dairy products. *Annals of Microbiology*, 62, 1079-1087.

THARMARAJ, N. & SHAH, N. P. 2003. Selective enumeration of Lactobacillus delbrueckii ssp bulgaricus, Streptococcus thermophilus, Lactobacillus acidophilus, bifidobacteria, Lactobacillus casei, Lactobacillus rhamnosus, and propionibacteria. *Journal of Dairy Science,* 86**,** 2288-2296.

THERMOMETRIC AB. 2002. Thermal Activity Monitor Technical Specifications.

THOMPSON-CHAGOYAN, O. C., MALDONADO, J. & GIL, A. 2007. Colonization and impact of disease and other factors on intestinal microbiota. *Digestive Diseases and Sciences*, 52, 2069-2077.

TIMMERMAN, H. M., KONING, C. J. M., MULDER, L., ROMBOUTS, F. M. & BEYNEN, A. C. 2004. Monostrain, multistrain and multispecies problotics - A comparison of functionality and efficacy. *International Journal of Food Microbiology*, 96, 219-233.

TIRLONI, E., CATTANEO, P., RIPAMONTI, B., AGAZZI, A., BERSANI, C. & STELLA, S. 2014. In vitro evaluation of Lactobacillus animalis SB310, Lactobacillus paracasei subsp paracasei SB137 and their mixtures as potential bioprotective agents for raw meat. *Food Control*, 41, 63-68.

TISSIER, H. 1906. Treatment of intestinal infection by way of transforming the bacterial flora of the intestine. *Comptes Rendus Des Seances De La Societe De Biologie Et De Ses Filiales*, 58, 359-361.

TOBA, T., YOSHIOKA, E. & ITOH, T. 1991. Acidophilucin-a, a new heat-labile bacteriocin produced by lactobacillus-acidophilus lapt 1060. *Letters in Applied Microbiology,* 12**,** 106-108.

TOBA, T., YOSHIOKA, E. & ITOH, T. 1991. Potential of Lactobacillus-gasseri isolated from infant feces to produce bacteriocin. *Letters in Applied Microbiology*, 12, 228-231.

TONNA, I. & WELSBY, P. D. 2005. Pathogenesis and treatment of Clostridium difficile infection. *Postgraduate Medical Journal*, 81, 367-369.

TRAMPUZ, A., SALZMANN, S., ANTHEAUME, J. & DANIELS, A. U. 2007. Microcalorimetry: a novel method for detection of microbial contamination in platelet products. *Transfusion*, 47, 1643-1650.

TRANSPARENCY MARKET RESEARCH. August 2013. Probiotics Market – Global Industry Analysis, Market Size, Share, Trends, Analysis, Growth and Forecast, 2012 – 2018. [online]. Available from: http://www.prweb.com/releases/2013/8/prweb11077944.htm [Accessed 4 January 2014]

TREJO, F. M., MINNAARD, J., PEREZ, P. F. & DE ANTONI, G. L. 2006. Inhibition of Clostridium difficile growth and adhesion to enterocytes by Bifidobacterium supernatants. *Anaerobe*, 12, 186-193.

TREJO, F. M., PEREZ, P. F. & DE ANTONI, G. L. 2010. Co-culture with potentially probiotic microorganisms antagonises virulence factors of Clostridium difficile in vitro. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology*, 98, 19-29.

TSUTSAEVA, A. A. & VYSEKANTSEV, I. P. 1983. The infectivity of t-4 phage frozen to minus 196 celsius in the presence of cryoprotectors. *Mikrobiologiya*, 52, 519-521.

TURNBAUGH, P. J., HAMADY, M., YATSUNENKO, T., CANTAREL, B. L., DUNCAN, A., LEY, R. E., SOGIN, M. L., JONES, W. J., ROE, B. A., AFFOURTIT, J. P., EGHOLM, M., HENRISSAT, B., HEATH, A. C., KNIGHT, R. & GORDON, J. I. 2009. A core gut microbiome in obese and lean twins. *Nature*, 457, 480-484.

TURSI, A., BRANDIMARTE, G., GIORGETTI, G. M., ELISEI, W. & AIELLO, F. 2007. Balsalazide and/or high-potency probiotic mixture (VSL#3) in maintaining remission after attack of acute, uncomplicated diverticulitis of the colon. *International Journal of Colorectal Disease*, 22, 1103-1108.

UKAI, M., TOMURA, A. & ITO, M. 1976. Cholesterol synthesis in germfree and conventional rats. *The Journal of Nutrition*, 106, 1175-1183.

USP, 2008. The United States Pharmacopeia USP 31, the National Formulary NF 26. The United States Pharmacopeial Convention, Inc. Rockville.

VAN DE WIELE, T., BOON, N., POSSEMIERS, S., JACOBS, H. & VERSTRAETE, W. 2004. Prebiotic effects of chicory inulin in the simulator of the human intestinal microbial ecosystem. *Fems Microbiology Ecology*, 51, 143-153.

VAN DEN ABBEELE, P., VENEMA, K., VAN DE WIELE, T., VERSTRAETE, W. & POSSEMIERS, S. 2013. Different human gut models reveal the distinct fermentation patterns of arabinoxylan versus inulin. *Journal of Agricultural and Food Chemistry*, 61, 9819-9827.

VAN REENEN, C. A., DICKS, L. M. T. & CHIKINDAS, M. L. 1998. Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by Lactobacillus plantarum. *Journal of Applied Microbiology*, 84, 1131-1137.

VANHOUTTE, T., HUYS, G., BRANDT, E. & SWINGS, J. 2004. Temporal stability analysis of the microbiota in human feces by denaturing gradient gel electrophoresis using universal and group-specific 16S rRNA gene primers. *Fems Microbiology Ecology*, 48, 437-446.

VAUGHAN, E. E., CAPLICE, E., LOONEY, R., OROURKE, N., COVENEY, H., DALY, C. & FITZGERALD, G. F. 1994. Isolation from food sources, of lactic-acid bacteria that produced antimicrobials. *Journal of Applied Bacteriology*, 76, 118-123.

VAZQUEZ, C., LAGO, N., LEGIDO, J. L., ARIAS, I., CASAS, L. M. & MATO, M. M. 2013. Microcalorimetric study of the growth of Enterococcus faecalis, Klebsiella pneumoniae and their mixtures in an enriched culture medium. *Journal of Thermal Analysis and Calorimetry*, 113, 1415-1420.

VAZQUEZ, C., LAGO, N., MATO, M. M., CASAS, L. M., ESARTE, L., LEGIDO, J. L. & ARIAS, I. 2014. Microcalorimetric performance of the growth in culture of Escherichia coli, Proteus mirabilis and their mixtures in different proportions. *Journal of Thermal Analysis and Calorimetry*, 116, 107-112.

VEDANTAM, G., CLARK, A., CHU, M., MCQUADE, R., MALLOZZI, M. & VISWANATHAN, V. K. 2012. Clostridium difficile infection: toxins and non-toxin virulence factors, and their contributions to disease establishment and host response. *Gut Microbes*, 3, 121-134.

VEKEMAN, B., HOEFMAN, S., DE VOS, P., SPIECK, E. & HEYLEN, K. 2013. A generally applicable cryopreservation method for nitrite-oxidizing bacteria. *Systematic and Applied Microbiology*, 36, 579-584.

VELDKAMP, H. & JANNASCH, H. W. 1972. Mixed culture studies with chemostat. *Journal of Applied Chemistry and Biotechnology*, 22, 105-123.

VENEMA, K., VAN NUENEN, M. H. M. C., VAN DEN HEUVEL, E. G., POOL, W. & VAN DER VOSSEN, J. M. B. M. 2003. The effect of lactulose on the composition of the intestinal microbiota and short-chain fatty acid production in human volunteers and a computer-controlled model of the proximal large intestine. *Microbial Ecology in Health and Disease*, 15, 94-105.

VERTZONI, M., DRESSMAN, J., BUTLER, J., HEMPENSTALL, J. & REPPAS, C. 2005. Simulation of fasting gastric conditions and its importance for the in vivo dissolution of lipophilic compounds. *European Journal of Pharmaceutics and Biopharmaceutics*, 60, 413-417.

VERTZONI, M., PASTELLI, E., PSACHOULIAS, D., KALANTZI, L. & REPPAS, C. 2007. Estimation of intragastric solubility of drugs: In what medium? *Pharmaceutical Research*, 24, 909-917.

VILELA, E. G., FERRARI, M. D. D., TORRES, H. O. D., PINTO, A. G., AGUIRRE, A. C. C., MARTINS, F. P., GOULART, E. M. A. & DA CUNHA, A. S. 2008. Influence of Saccharomyces boulardii on the intestinal permeability of patients with Crohn's disease in remission. *Scandinavian Journal of Gastroenterology*, 43, 842-848.

VINE, G. J. & BISHOP, A. H. 2005. The analysis of microorganisms by microcalorimetry in the pharmaceutical industry. *Current Pharmaceutical Biotechnology*, 6, 223-238.

VISCIDI, R., WILLEY, S. & BARTLETT, J. G. 1981. Isolation rates and toxigenic potential of Clostridium difficile isolates from various patient populations. *Gastroenterology*, 81, 5-9.

VON AH, U., WIRZ, D. & DANIELS, A. 2009. Isothermal microcalorimetry - a new method for MIC determinations: results for 12 antibiotics and reference strains of E. coli and S. aureus. *Bmc Microbiology*, 9, 1-14.

VON AH, U., WIRZ, D. & DANIELS, A. U. 2008. Rapid differentiation of methicillin-susceptible Staphylococcus aureus from methicillin-resistant S. aureus and MIC determinations by isothermal microcalorimetry. *Journal of Clinical Microbiology*, 46, 2083-2087.

VON STOCKAR, U. & BIROU, B. 1989. The heat generated by yeast cultures with a mixed metabolism in the transition between respiration and fermentation. *Biotechnology and Bioengineering*, 34, 86-101.

VONBERG, R. P., REICHARDT, C., BEHNKE, M., SCHWAB, F., ZINDLER, S. & GASTMEIER, P. 2008. Costs of nosocomial Clostridium difficile-associated diarrhoea. *Journal of Hospital Infection*, 70, 15-20.

VRIEZE, A., HOLLEMAN, F., ZOETENDAL, E. G., DE VOS, W. M., HOEKSTRA, J. B. & NIEUWDORP, M. 2010. The environment within: how gut microbiota may influence metabolism and body composition. *Diabetologia*, 53, 606-613.

WADSO, I. & GOLDBERG, R. N. 2001. Standards in isothermal microcalorimetry (IUPAC technical report). *Pure and Applied Chemistry*, 73, 1625-1639.

WADSO, I. 2009. Characterization of microbial activity in soil by use of isothermal microcalorimetry. *Journal of Thermal Analysis and Calorimetry*, 95, 843-850.

WADSO, L. & GALINDO, F. G. 2009. Isothermal calorimetry for biological applications in food science and technology. *Food Control*, 20, 956-961.

WADSO, L. 2010. Operational issues in isothermal calorimetry. *Cement and Concrete Research*, 40, 1129-1137.

WADSO, L., LI, Y. J. & BJURMAN, J. 2004. Measurements on two mould fungi with a calorespirometric method. *Thermochimica Acta*, 422, 63-68.

WALKER, A. W. & LAWLEY, T. D. 2013. Therapeutic modulation of intestinal dysbiosis. *Pharmacological Research*, 69, 75-86.

WANG, F., YAO, J., CHEN, H., ZHOU, Y., CHEN, Y., CHEN, H., GAI, N., ZHUANG, R., TIAN, L., MASKOW, T., CECCANTI, B., TREBSE, P. & ZARAY, G. 2009. Microcalorimetric measurements of the microbial activities of single- and mixed-species with trivalent iron in soil. *Ecotoxicology and Environmental Safety*, 72, 128-135.

WANG, X. & GIBSON, G. R. 1993. Effects of the in-vitro fermentation of oligofructose and inulin by bacteria growing in the human large-intestine. *Journal of Applied Bacteriology*, 75, 373-380.

WARD, L. J. H. & TIMMINS, M. J. 1999. Differentiation of Lactobacillus casei, Lactobacillus paracasei and Lactobacillus rhamnosus by polymerase chain reaction. *Letters in Applied Microbiology*, 29, 90-92.

WARNY, M., PEPIN, J., FANG, A., KILLGORE, G., THOMPSON, A., BRAZIER, J., FROST, E. & MCDONALD, L. C. 2005. Toxin production by an emerging strain of Clostridium difficile associated with outbreaks of severe disease in North America and Europe. *Lancet*, 366, 1079-84.

WEESE, J. S. & MARTIN, H. 2011. Assessment of commercial probiotic bacterial contents and label accuracy. *Canadian Veterinary Journal*, 52, 43-46.

WEESE, J. S. 2002. Microbiologic evaluation of commercial probiotics. *Journal of the American Veterinary Medical Association*, 220, 794-797.

WEST, P. A., HEWITT, J. H. & MURPHY, O. M. 1979. Influence of methods of collection and storage on the bacteriology of human-milk. *Journal of Applied Bacteriology*, 46, 269-277.

WHEELDON, L. J., WORTHINGTON, T., HILTON, A. C., ELLIOTT, T. S. J. & LAMBERT, P. A. 2008. Physical and chemical factors influencing the germination of Clostridium difficile spores. *Journal of Applied Microbiology*, 105, 2223-2230.

WIEGEL, J. 2009. In Bergey's manual of systematic bacteriology, 2nd ed. Bergey's manual trust, Springer Dordrecht Heidelberg: London New York, 3, 736-772.

WIJSMAN, M. R., HEREIJGERS, J. & DEGROOTE, J. 1989. Selective enumeration of bifidobacteria in fermented dairy-products. *Netherlands Milk and Dairy Journal,* 43**,** 395-405.

WILLS R. 2012. In vitro assessment of the survivability of Lactobacillus casei DN-114 001 and Lactobacillus casei Shirota, within commercialised food matrices, in the upper gastrointestinal tract. *The Plymouth Student Scientist*, 5, 80-89.

WILSON, K. H. 1983. Efficiency of various bile salt preparations for stimulation of Clostridium difficile spore germination. *Journal of Clinical Microbiology*, 18, 1017-1019.

WILSON, K. H., SHEAGREN, J. N. & FRETER, R. 1985. Population-dynamics of ingested clostridium-difficile in the gastrointestinal-tract of the syrian-hamster. *Journal of Infectious Diseases*, 151, 355-361.

WINKELMANN, M., HUTTL, R. & WOLF, G. 2004. Application of batch-calorimetry for the investigation of microbial activity. *Thermochimica Acta*, 415, 75-82.

WISPLINGHOFF, H., BISCHOFF, T., TALLENT, S. M., SEIFERT, H., WENZEL, R. P. & EDMOND, M. B. 2004. Nosocomial bloodstream infections in US hospitals: Analysis of 24,179 cases from a prospective nationwide surveillance study. *Clinical Infectious Diseases*, 39, 309-317.

WLODARSKA, M., WILLING, B., KEENEY, K. M., MENENDEZ, A., BERGSTROM, K. S., GILL, N., RUSSELL, S. L., VALLANCE, B. A. & FINLAY, B. B. 2011. Antibiotic treatment alters the colonic mucus layer and predisposes the host to exacerbated citrobacter rodentium-induced colitis. *Infection and Immunity*, 79, 1536-1545.

WONG, J. M. W., DE SOUZA, R., KENDALL, C. W. C., EMAM, A. & JENKINS, D. J. A. 2006. Colonic health: fermentation and short chain fatty acids. *Journal of Clinical Gastroenterology*, 40, 235-243.

XIE, C. L., TANG, H. K., SONG, Z. H., QU, S. S., LIAO, Y. T. & LIU, H. S. 1988. Microcalorimetric study of bacterial-growth. *Thermochimica Acta*, 123, 33-41.

XING, X., ZHAO, Y., KONG, W., ZHONG, Y., YAN, D., ZHANG, P., HAN, Y., JIA, L., JIN, C. & XIAO, X. 2011. Application of microcalorimetry and chemometric analysis. *Journal of Thermal Analysis and Calorimetry*, 109, 381-385.

XU, J. & GORDON, J. I. 2003. Honor thy symbionts. *Proceedings of the National Academy of Sciences of the United States of America,* 100**,** 10452-10459.

YADAV, V., GAISFORD, S., MERCHANT, H. A. & BASIT, A. W. 2013. Colonic bacterial metabolism of corticosteroids. *International Journal of Pharmaceutics*, 457, 268-274.

YANG, L. N., XU, F., SUN, L. X., ZHAO, Z. B. & SONG, C. G. 2008. Microcalorimetric studies on the antimicrobial actions of different cephalosporins. *Journal of Thermal Analysis and Calorimetry*, 93, 417-421.

YEUNG, P. S. M., SANDERS, M. E., KITTS, C. L., CANO, R. & TONG, P. S. 2002. Species-specific identification of commercial probiotic strains. *Journal of Dairy Science*, 85, 1039-1051.

YILDIRIM, Z. & JOHNSON, M. G. 1998. Characterization and antimicrobial spectrum of bifidocin B, a bacteriocin produced by Bifidobacterium bifidum NCFB 1454. *Journal of Food Protection*, 61, 47-51.

ZAHARIA, D. C., MUNTEAN, A. A., POPA, M. G., STERIADE, A. T., BALINT, O., MICUT, R., IFTENE, C., TOFOLEAN, I., POPA, V. T., BAICUS, C., BOGDAN, M. A. & POPA, M. I. 2013. Comparative analysis of Staphylococcus aureus and Escherichia coli microcalorimetric growth. *Bmc Microbiology*, 13.

ZHANG, G., FAN, M., LI, Y., WANG, P. & LV, Q. 2012. Effect of growth phase, protective agents, rehydration media and stress pretreatments on viability of Oenococcus oeni subjected to freeze-drying. *African Journal of Microbiology Research,* 6**,** 1478-1484.

ZHANG, H., DIBAISE, J. K., ZUCCOLO, A., KUDRNA, D., BRAIDOTTI, M., YU, Y., PARAMESWARAN, P., CROWELL, M. D., WING, R., RITTMANN, B. E. & KRAJMALNIK-BROWN, R. 2009. Human gut microbiota in obesity and after gastric bypass. *Proceedings of the National Academy of Sciences U S A*, 106, 2365-2370.

ZHANG, J., ZHANG, X., YONG, Q., XU, Y. & YU, S. 2008. In vitro fermentation characterization of low concentration of xylooligosaccharides prepared from corncob xylan by Bifidobacterium adolescentis. *Proceedings of International Conference on Pulping, Papermaking and Biotechnology* 2008: Icppb '08, I, 707-711.

ZHAO, G. & ZHANG, G. 2005. Effect of protective agents, freezing temperature, rehydration media on viability of malolactic bacteria subjected to freeze-drying. *Journal of Applied Microbiology*, 99, 333-338.

ZHAO, R. M., LIU, Y., XIE, Z. X., SHEN, P. & QU, S. S. 2000. A microcalorimetric method for studying the biological effects of La3+ on Escherichia coli. *Journal of Biochemical and Biophysical Methods*, 46, 1-9.

ZHOU, J., TUN, T. N., HONG, S. H., MERCER-CHALMERS, J. D., LAABEI, M., YOUNG, A. E. & JENKINS, A. T. 2011. Development of a prototype wound dressing technology which can detect and report colonization by pathogenic bacteria. *Biosens Bioelectron*, 30, 67-72.

ZHU, H., HART, C. A., SALES, D. & ROBERTS, N. B. 2006. Bacterial killing in gastric juice--effect of pH and pepsin on Escherichia coli and Helicobacter pylori. *Journal of Medical Microbiology*, 55, 1265-70.

ZHU, W. M., LIU, W. & WU, D. Q. 2000. Isolation and characterization of a new bacteriocin from Lactobacillus gasseri KT7. *Journal of Applied Microbiology*, 88, 877-886.

ZHU, Y., XIAO, L., SHEN, D. & HAO, Y. 2010. Competition between yogurt probiotics and periodontal pathogens in vitro. *Acta Odontologica Scandinavica*, 68, 261-268.

ZOETENDAL, E. G., AKKERMANS, A. D. & DE VOS, W. M. 1998. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. *Applied and Environmental Microbiology*, 64, 3854-3859.

ZOETENDAL, E. G., AKKERMANS, A. D. L., AKKERMANS-VAN VLIET, W. M., DE VISSER, J. A. G. M. & DE VOS, W. M. 2001. The host genotype affects the bacterial community in the human gastrointestinal tract. *Microbial Ecology in Health and Disease,* 13**,** 129-134.

ZOETENDAL, E. G., VAUGHAN, E. E. & DE VOS, W. M. 2006. A microbial world within us. *Molecular Microbiology*, 59, 1639-1650.