London Metropolitan University

Effects of environmental and physiological stress on the functionality of probiotic microorganisms

This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

School of Human Sciences

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly acknowledged.

(Signature)

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Abstract

The aim of this project was to examine whether exposure to environmental and physiological stress conditions could affect some functional properties for the selection of probiotic microorganisms. The study was focused on two commercial strains of *Bifidobacterium animalis* ssp. *lactis* and two non-commercial *Bifidobacterium* strains, namely *B. breve* NCTC 11815 and *B. longum* NCTC 11818. The effects of exposure to acid, bile, osmotic and oxidative stresses on their antimicrobial activity, biofilm formation capacity and antibiotic susceptibility profiles were assessed. The conditions to generate acid stress in the organisms were chosen as pH 3 for one hour, for both *B. animalis* ssp. *lactis* strains, and pH 4 for one hour, for *B. breve* and *B. longum*. Conditions for bile stress were 1% (w/v) bile for one hour, for both *B. animalis* ssp. *lactis* strains and *B. breve*, and 0.5% (w/v) bile for one hour, for *B. longum*. Osmotic stress conditions were 3% (w/v) NaCl for one hour, for both *B. animalis* ssp. *lactis* strains and *B. breve*, and 2% (w/v) NaCl for one hour, for *B. longum*. Oxidative stress was generated for all organisms by shaking at 200 rpm for two hours. The antimicrobial activities of all four bifidobacteria against pathogenic bacteria, namely *Escherichia coli* NCTC 12900, *Salmonella enterica* ser. Typhimurium DT124 and *S. enterica* ser. Enteritidis PT4, were maintained after exposure to each stress, although there appeared to be lower inhibition after exposure to stress. This varied with strain and type of stress. The antibiotic susceptibility profiles of all four bifidobacteria for five antibiotics, namely tetracycline, erythromycin, ampicillin, chloramphenicol and vancomycin, were unchanged after exposure to each stress. The expression of tetracycline resistance gene *tet*(W) in one of the *B.*

animalis ssp. *lactis* strains, designated as strain C, was significantly higher ($P \leq 0.05$) after exposure to acid, bile and osmotic stresses, although this did not translate to higher resistance of *B. animalis* ssp. *lactis* (C) to tetracycline. Effects of each stress on biofilm formation in the four bifidobacteria varied with the strain. In general, more positive effects of exposure to stress were observed in both *B. animalis* ssp. *lactis* strains, while more negative effects of exposure to stress were shown by *B. breve* and *B. longum*. The expression of exopolysaccharide-synthesis gene *gtf*01207 in *B. animalis* ssp. *lactis* (C) was significantly higher after exposure to osmotic stress, although it also appeared to be higher after exposure to acid and bile stresses. Studying the effects of exposure to stress on *in vitro* probiotic selection properties could give a better reflection of what applies *in vivo*, since microorganisms for probiotic use would be inevitably exposed to stresses. This could give a more accurate insight on the potential to provide health benefit. The results of this study may justify the commercial use of the *B. animalis* ssp. *lactis* strains.

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Abbreviations

°C	degrees Centigrade
µg	micrograms
µl	microlitres
µm	micrometres
ATP	adenosine triphosphate
ATR	acid tolerance response
BLIS	bacteriocin-like inhibitory substances
BSH	bile salt hydrolase
cDNA	complementary DNA
cFDA	carboxyfluorescein diacetate
cfu	colony-forming unit
CLA	conjugated linoleic acid
DNA	deoxyribonucleic acid
e.g.	example
EFSA	European Food Safety Authority
EPS	exopolysaccharide
et al.	and others
F6PPK	fructose-6-phosphate phosphoketolase

FAO	Food and Agriculture Organisation of the United Nations
Fig.	figure
g	grams
G+C	guanine+cytosine
GALT	gut-associated lymphoid tissue
gDNA	genomic DNA
GIT	gastrointestinal tract
GRAS	generally recognised as safe
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HMO	human milk oligosaccharide
HPLC	high performance liquid chromatography
i.e.	that is
K^+	potassium ion
KCl	potassium chloride
L	litre
LAB	lactic acid bacteria
M	molar
m^2	square metres

MIC	minimum inhibitory concentration
ml	millilitres
mm	millimetres
mRNA	messenger RNA
Na^+	sodium ion
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide
NCCLS	National Committee for Clinical Laboratory Standards
NCTC	National Collection of Type Cultures
nm	nanometres
OD	optical density
PBS	phosphate buffered saline
p-GTF	priming glycosyltransferase
PI	propidium iodide
PMF	proton motive force
PNDR	pyridine nucleotide-disulfide reductase
QPS	qualified presumption of safety
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RCA	reinforced clostridial agar

RCM	reinforced clostridial medium
RNA	ribonucleic acid
rRNA	ribosomal RNA
ROS	reactive oxygen species
rpm	revolutions per minute
SCFA	short-chain fatty acid
sec	seconds
SEM	standard error of the mean
SOD	superoxide dismutase
spp.	species
ssp.	sub-species
TLR	toll-like receptor
VRBG	Violet Red Bile Glucose
w/v	weight per volume
WHO	World Health Organisation

CHAPTER ONE: INTRODUCTION

1.1 Microbiology of the human gastrointestinal tract

The human gastrointestinal tract (GIT) is the largest tube running through the body. It comprises the oral cavity (mouth), oesophagus, stomach, small intestine and large intestine. The main function of the GIT is for the digestion of food and absorption of nutrients (Shigwedha and Jia 2013). Its estimated surface area of 200 m^2, in addition to being rich in molecules for use as nutrients by microbes, make it a major surface for microbial colonisation (Sekirov *et al.* 2010).

The human GIT contains more than 10^{14} metabolically diverse and active microorganisms (Fig. 1.1), which constitute more than 70% of the total microbes in the human body (Sekirov *et al.* 2010). The population of gut microbiota alone is at least ten times more than the number of cells in the human body. The human “microbiome” plays a major role in the functions of the intestinal epithelium and consequently human health and disease (Bakhtiar *et al.* 2013). Intestinal microbiota play important roles in digestion of food, development of the gut immune system, production of short-chain fatty acids and essential vitamins, and resistance to colonisation by pathogenic microbes (Behnsen *et al.* 2013).

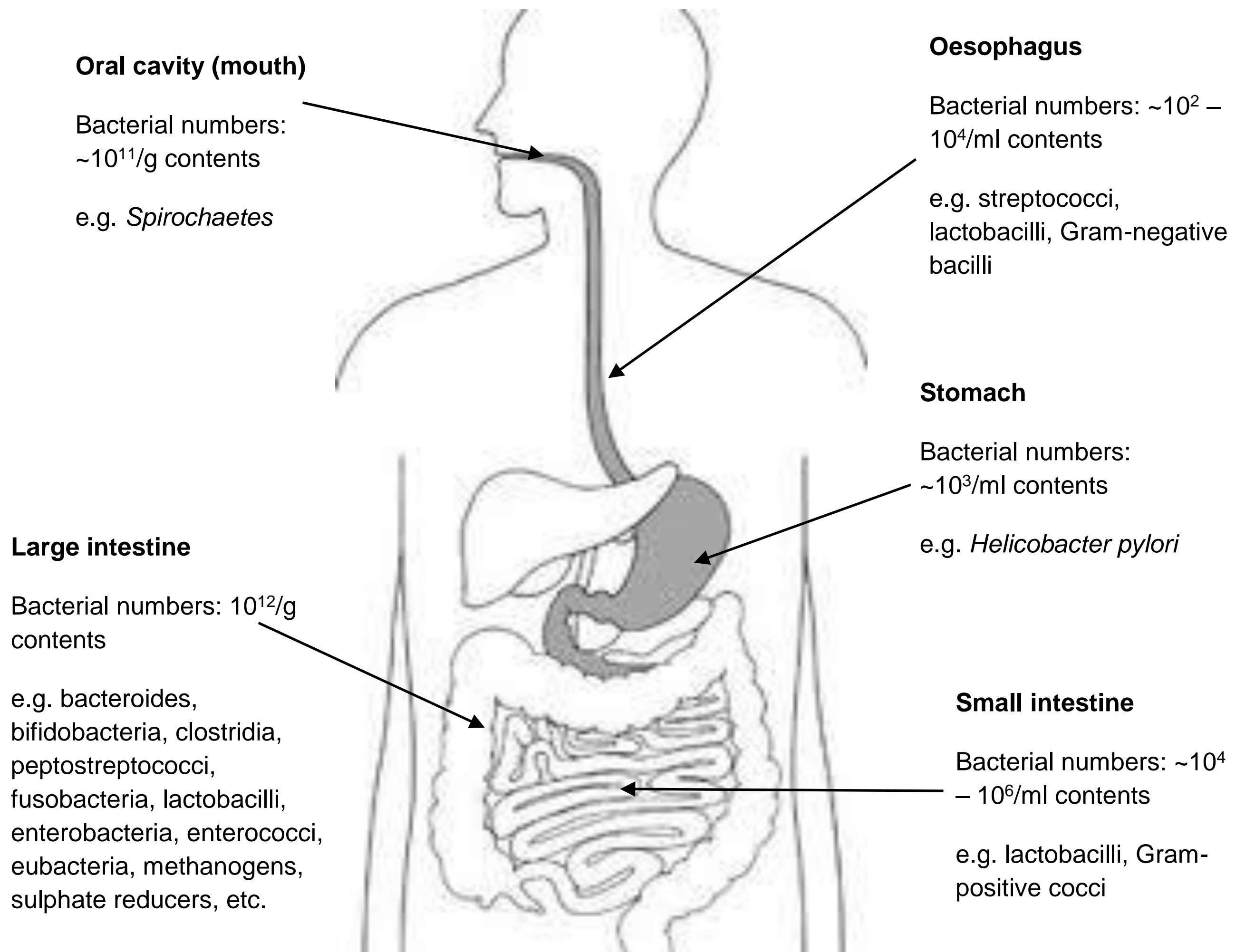

Fig. 1.1 Microbiota of the human gastrointestinal tract (Adapted from Shigwedha and Jia 2013)

The intestine of a foetus is sterile until birth, at which point, microbial colonisation begins via inoculation with the mother's vaginal and faecal flora, as well as the environment. Subsequent development of the intestinal microbiota is largely influenced by diet, i.e. breast feeding or formula feeding, as well as environmental factors. Upon weaning, the microbiota composition normalises and remains stable throughout most of adult life (O'Grady and Gibson 2005; Ray and Bhunia 2008).

Different ecological niches are found in the GIT, with the composition and population varying with location. The oral cavity is made up of the mouth, nose and throat. The mouth is densely populated by microorganisms, including members of *Prevotella*, *Porphyromonas*, *Desulfovibrio*, *Bacteroides*, *Fusobacterium*, *Eubacterium* and *Peptostreptococcus* (O'Grady and Gibson 2005). The stomach is almost sterile, with numbers $<10^3$ cfu/ml of gastric contents. This is due to its low pH, which is bactericidal. Microorganisms found in the stomach are transient rather than resident, and include acid-tolerant lactobacilli, yeasts and *Helicobacter pylori*, which can survive the unfavourable and peristaltic conditions in the stomach (Chadwick *et al.* 2003).

There is a progressive increase in the microbial numbers and species in the small intestine, from the duodenum (upper part) along to the jejunum and ileum (lower parts). The numbers in the duodenum are lower ($\sim10^3$ per gram) because of the secretion of bile, which inhibits bacterial growth, and the short transit time. In the ileum, the bacterial numbers start to grow, up to 10^7 per gram. Species found include enterococci, enterobacteria, lactobacilli, bacteroides and clostridia. There is a rapid increase in population in the colon (large intestine), where microbial numbers can reach 10^{12} per gram of

content. The large intestine is favourable for microbial growth due to favourable pH, availability of nutrients, and slow transit time. The dominant bacteria in the large intestine are non-spore-forming anaerobes, including *Bifidobacterium*, *Bacteroides*, *Eubacterium*, *Lactobacillus* (O'Grady and Gibson 2005; Shigwedha and Jia 2013).

1.2 Probiotics

Probiotics, as defined by FAO/WHO (2002), are "live microorganisms which when administered in adequate amounts, confer a health benefit on the host". Various species of bacteria and yeast have been used as probiotics (Table 1.1). The most commonly used are strains of species of *Lactobacillus* and *Bifidobacterium*. Several health benefits have been associated with probiotic microorganisms and these benefits are strain-specific. Health claims should be supported by scientific evidence which can be based on results from clinical trials. Some of the potential claims are listed in Table 1.2.

Table 1.1 Examples of species which include probiotic strains

Genus	Species	Example strains
Bacillus	*coagulans*, *subtilis*	*B. subtilis* HU58
Bifidobacterium	*adolescentis*, *animalis*, *bifidum*, *breve*, *infantis*, *lactis*, *longum*	*B. animalis* ssp. *lactis* BB-12, *B. animalis* ssp. *lactis* HN019, *B. breve* Yakult, *B. infantis* UCC35624
Enterococcus	*faecium*	*E. faecium* NCIMB10415
Escherichia	*coli*	*E. coli* Nissle
Lactobacillus	*acidophilus*, *casei*, *helveticus*, *johnsonii*, *paracasei*, *plantarum*, *reuteri, rhamnosus*, *salivarus*	*Lb. acidophilus* La-5, *Lb. casei* Shirota, *Lb. johnsonii* La-1, *Lb. plantarum* 299v, *Lb. rhamnosus* ATCC 53013 (LGG)
Lactococcus	*lactis*	*Lc. lactis* ssp. *lactis* HV219
Propionibacterium	*freudenrenchii*, *jensenii*	*P. freudenrenchii* SI 41, *P. jensenii* 702
Saccharomyces	*cerevisiae* var. *boulardii*	*S. cerevisiae* var. *boulardii* CNCM I-3799

(Adapted from Vasiljevic and Shah 2008; Baker *et al.* 2009)

Table 1.2 Probiotics and associated potential health claims

Health claims	**Probiotic organisms**
Diarrhoea treatment, cholesterol reduction, inhibition of colon cancer	*Lactobacillus acidophilus*
Treatment and prevention of diarrhoea, alleviation of atopic dermatitis	*Lactobacillus rhamnosus* GG
Immune system enhancement, modulation of intestinal microflora, vaccine adjuvant, *Helicobacter pylori* treatment	*Lactobacillus johnsonii* La1
Immune system enhancement, alleviation of atopic dermatitis, diarrhoea prevention and treatment (traveller's diarrhoea, rotavirus diarrhoea)	*Bifidobacterium lactis* Bb-12
Treatment of viral diarrhoea, modulation of intestinal bacteria	*Bifidobacterium bifidum*
Treatment of rotavirus diarrhoea, acute diarrhoea	*Lactobacillus reuteri*
Modulation of intestinal microflora, inhibition of superficial bladder cancer	*Lactobacillus casei* Shirota
Modulation of intestinal microflora, increase of short-chain fatty acid production	*Lactobacillus plantarum* 299v
Diarrhoea prevention and treatment (traveller's diarrhoea, *Clostridium difficile*-associated diarrhoea)	*Saccharomyces boulardii*

(Adapted from Saarela *et al.*2000; Dunne *et al.* 2001; Parvez *et al.* 2006)

Dairy products such as yoghurt are the most common vehicles for delivering probiotics. Probiotic microorganisms should be present in these products in large enough numbers to compensate for losses during gastric passage. Daily intake has been suggested at 10^8 CFU/g (Granato *et al.* 2010a), which can be achieved by regular consumption of 400-500 g of product per week (Tamime *et al.* 1995). It has been recommended that minimum counts of around 10^6-10^7 CFU/g of product should be present by the time of consumption/expiry (Talwalkar and Kailasapathy 2004). Fermented and non-fermented dairy products are commonly used because of the healthy image of such dairy products historically, as well as consumer familiarity with the live microbial content of these products (Charteris *et al.* 1998; Heller 2001; Ozer and Kirmaci 2010).

Despite dairy products being the most common means of probiotic administration, there has been development in the area of non-dairy probiotic products, in a bid to increase consumer choice and to satisfy people with lactose intolerance or people who do not like dairy products for their higher fat content. Increasing vegetarianism and allergies to milk proteins are also contributing factors (Granato *et al.* 2010b; Ranadheera *et al*. 2010). Fruit juices, soy-based and cereal-based products have been found to be suitable as carriers of probiotics. *Lactobacillus plantarum* 299v, *Lactobacillus reuteri* and *Lactobacillus rhamnosus* GG have been used to develop probiotic fruit juices/drinks under brand names like ProViva, Gefilus, Biola, Rela (Molin 2001; Prado *et al.* 2008).

1.2.1 Probiotic selection criteria

Microorganisms intended for use as probiotics would be required to meet certain criteria for selection. They would have to meet criteria based on safety characteristics, technological characteristics, functional characteristics and eventually they would be expected to provide the health benefits for which they are used.

Safety criteria: Safety is one of the most important selection criteria for bacterial strains which are to be used in the food industry, including probiotics (Gueimonde *et al.* 2013). Microorganisms for probiotic use would preferably be of human origin and isolated from a healthy human gastrointestinal tract, be non-pathogenic and non-toxic (generally recognised as safe [GRAS]), and possess no transferrable antibiotic resistance genes (Saarela *et al.* 2000). Most bacteria for use in probiotic products have been isolated from humans to increase the likelihood of compatibility with the human gut and its microflora, and improve chances of survival (Rivera-Espinoza and Gallardo-Navarro 2010). Non-pathogenicity implies that there is no risk of infection or other adverse side-effects from the consumption of probiotics (Reid 2006). Antibiotic resistance itself is not a safety issue, since microorganisms can possess inherent resistance. However, it becomes a safety issue where there is a risk of resistance transfer, i.e. transfer of genetic determinants to intestinal pathogens (Aureli *et al.* 2011).

Technological criteria: Desired technological properties include good sensory properties, viability during propagation and processing, stability in the product and during storage, phage resistance and oxygen resistance (Ouwehand *et al.* 1999). Probiotic microorganisms chosen for incorporation

into food products should remain alive during the harsh conditions of food manufacture, and during storage of the food product for the shelf-life period (Sanchez *et al.* 2012). Many probiotic cultures are produced in dried form, which provides a longer shelf life. As such, the probiotic organisms would have to survive the extremes of osmotic and temperature conditions and the exposure to oxygen that occur during the drying process (Jancovic *et al.* 2010). The conditions during the fermentation process, i.e. biomass production, such as composition of the fermentation medium, harvesting time, growth temperature and fermentation pH may also affect the survival of extreme temperature and stability during storage (Makinen *et al.* 2012). The addition of probiotics to food should also not compromise the sensory attributes of the food product (Sanchez *et al.* 2012).

Functional criteria: Functional requirements, which are initially determined *in vitro*, include resistance to acid/gastric acid, resistance to bile, adhesion to the intestinal epithelium and ability to transiently colonise the gut, stimulation of the immune system, antagonistic activity against pathogens (production of antimicrobial compounds or competitive exclusion), anticarcinogenic and antimutagenic properties (Ouwehand *et al.* 1999; Saarela *et al.* 2000). Since probiotics are usually administered orally, it is crucial that they survive the acidic conditions of the stomach and the bile secreted into the small intestine (Chou and Weimer 1999). Therefore, candidate probiotics are screened for tolerance to acid and bile. Adhesion to intestinal surfaces is important for colonisation of the human gut, as it prevents the elimination of probiotics by peristalsis and provides competitive advantage over pathogens (Rivera-Espinoza and Gallardo-Navarro 2010). Protective effects against pathogens

may also be exerted by direct antagonism, through the production of inhibitory compounds such as organic acids, bacteriocins and hydrogen peroxide, and by competitive exclusion, through the competition for nutrients (Baugher and Klaenhammer 2011). Probiotics may directly or indirectly influence the immune system, as well as bind mutagens to the cell surface and reduce sources of procarcinogens which lead to tumour development (Shah 2007). Functional properties, however, do not necessarily predict health benefits in humans, and are not requirements for a strain to be beneficial to health. Therefore, health benefits of candidate probiotic strains should always be demonstrated by well-designed human trials (Makinen *et al.* 2012).

1.2.2 Mechanisms of probiotic action

Probiotic benefits may occur directly or indirectly by a number of mechanisms. These potential mechanisms revolve around modulation of the intestinal ecosystem, improved colonisation resistance and modulation of immune resistance (Fig. 1.2).

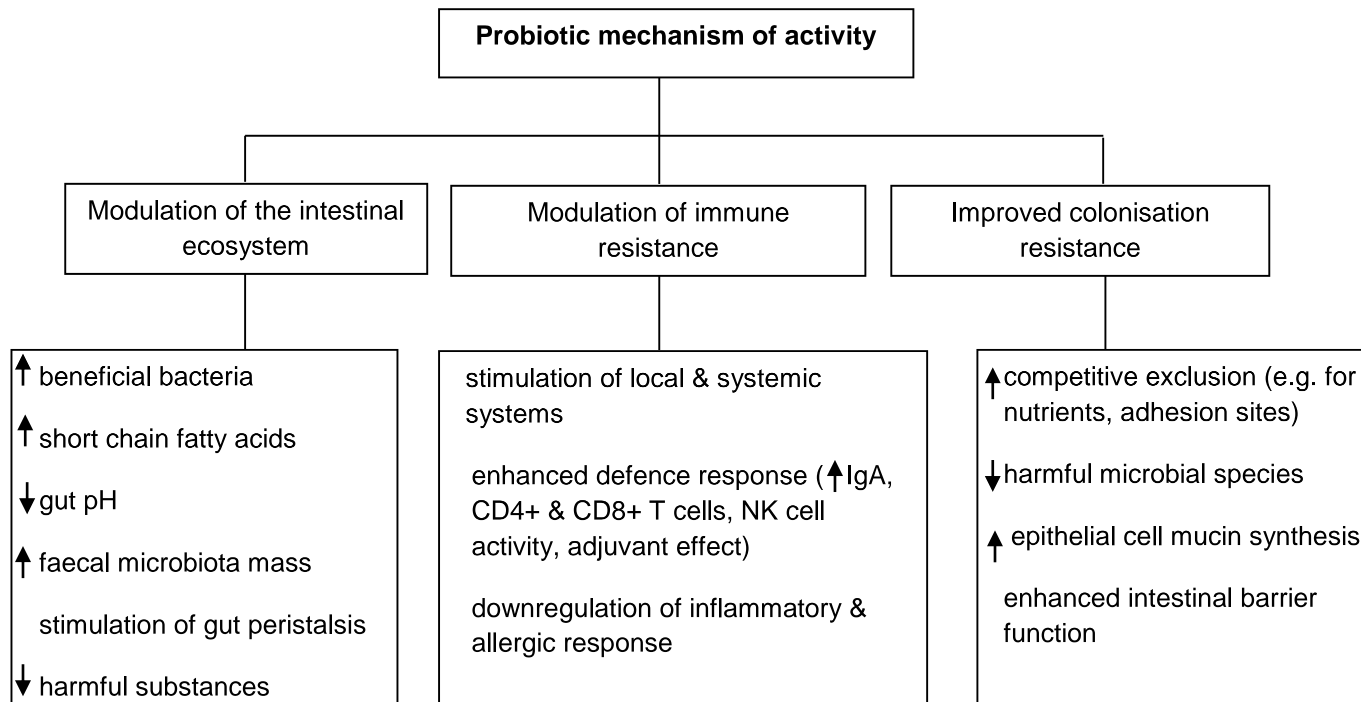

Fig. 1.2 Overview of mechanisms of activity attributed to probiotic microorganisms (adapted from Baker *et al.* 2009)

Modulation of intestinal ecosystem: The consumption of probiotics, and the transient colonisation which occurs, can influence the composition and activity of the natural gut microflora and help to maintain a beneficial balance by increasing the population of beneficial bacteria and decreasing the population of harmful microorganisms. This may be achieved by the production of organic acids or short chain fatty acids, which lower the gut pH, and the production of antimicrobial agents such as bacteriocins, thus making the gut more favourable to beneficial bacteria such as lactobacilli and bifidobacteria and consequently, less favourable to pathogenic microorganisms (Baker *et al.* 2009).

Improved colonisation resistance: The growth and metabolism of probiotic microorganisms can alter the intestinal environment such that colonisation resistance is improved. This may result from adhesion to epithelial cells by probiotics, thus blocking the adhesion of pathogens (i.e. competitive exclusion), as well as the stimulation of mucin production, which enhances intestinal barrier function (Oelschlaeger 2010; Wohlgemuth *et al.* 2010).

Modulation of immune resistance: Probiotics can stimulate mucosal immunity and modulate immune responses by their interaction with the gut-associated lymphoid tissue (GALT). GALT is the largest lymphoid tissue in the human body and contains various cells of the immune system, which interact with intestinal microorganisms. Metabolites, cell wall components and DNA of probiotic microorganisms are recognised by host cells which are sensitive to them, e.g. toll-like receptors (TLRs), and the activation of these receptors lead to modulation of pro- and anti- inflammatory cytokine

expression, i.e. down-regulation of inflammatory and allergic responses (Baker *et al.* 2009; Wohlgemuth *et al.* 2010).

1.3 Genus *Bifidobacterium*

The genus *Bifidobacterium* consists of anaerobic, non-motile, heterofermentative Gram-positive rod-shaped bacteria which normally constitute part of the human and animal intestinal microflora (Sela *et al.* 2010). The genus *Bifidobacterium* belongs to the *Actinomycetaceae* family. *Bifidobacterium* have a distinctly high G + C content (42% - 67%) (Klijn *et al.* 2005) and were first isolated from breast-fed infant faeces by Tissier in 1899, who named it *Bacillus bifidus*, due to the characteristic Y shape. The genus contains 36 recognised species, which are listed in Table 1.3. Bifidobacteria have been isolated from various ecological habitats such as the human oral cavity, human and animal intestine/faeces, human vagina, insect gut and sewage (Sela *et al.* 2010).

The optimum growth temperature is 37 – 41 °C, and no growth occurs below 20 °C and above 46 °C. The optimum pH for growth to occur is 6.5 – 7.0 and no growth generally occurs below 4.5 and above 8.5. Fermentation of carbohydrates by bifidobacteria is via a fructose-6-phosphate phosphoketolase shunt (Fig. 1.3) differentiating bifidobacteria from lactobacilli, which use a glucose-6-phosphate shunt (Tamime *et al.* 1995; Klijn *et al.* 2005).

Table 1.3 Species of *Bifidobacterium* and their ecological origin

Species	Ecological origin						
	Human gut	**Oral cavity**	**Human blood**	**Insect**	**Food**	**Animal gut**	**Sewage**
B. actinocoloniiforme				x			
B. adolescentis	x						
B. angulatum	x						
B. animalis							
ssp. *animalis*						x	
ssp. *lactis*					x		
B. asteroides				x			
B. bifidum	x						
B. bohemicum				x			
B. bombi				x			
B. boum						x	
B. breve	x						
B. catenulatum	x						
B. choerinum						x	
B. coryneforme				x			
B. crudilactis					x		
B. cuniculi						x	
B. dentium		x					
B. gallicum	x						
B. gallinarum						x	
B. indicum				x			
B. longum							
ssp. *infantis*	x						
ssp. *longum*	x						
ssp. *suis*						x	
B. magnum						x	
B. merycicum						x	
B. minimum							x
B. mongoliense					x		
B. pseudocatenulatum	x						
B. pseudolongum							
ssp. *globosum*						x	
ssp. *pseudolongum*						x	
B. psychraerophilum						x	
B. pullorum						x	
B. ruminantium						x	
B. saeculare						x	
B. scardovii			x				
B. stercoris	x						
B. subtile							x
B. thermacidophilum							
ssp. *porcinum*						x	
ssp. *thermacidophilum*							x
B. thermophilum							x
B. tsurumiense		x					

(Adapted from Lee and O’Sullivan 2010; Russell *et al*. 2011)

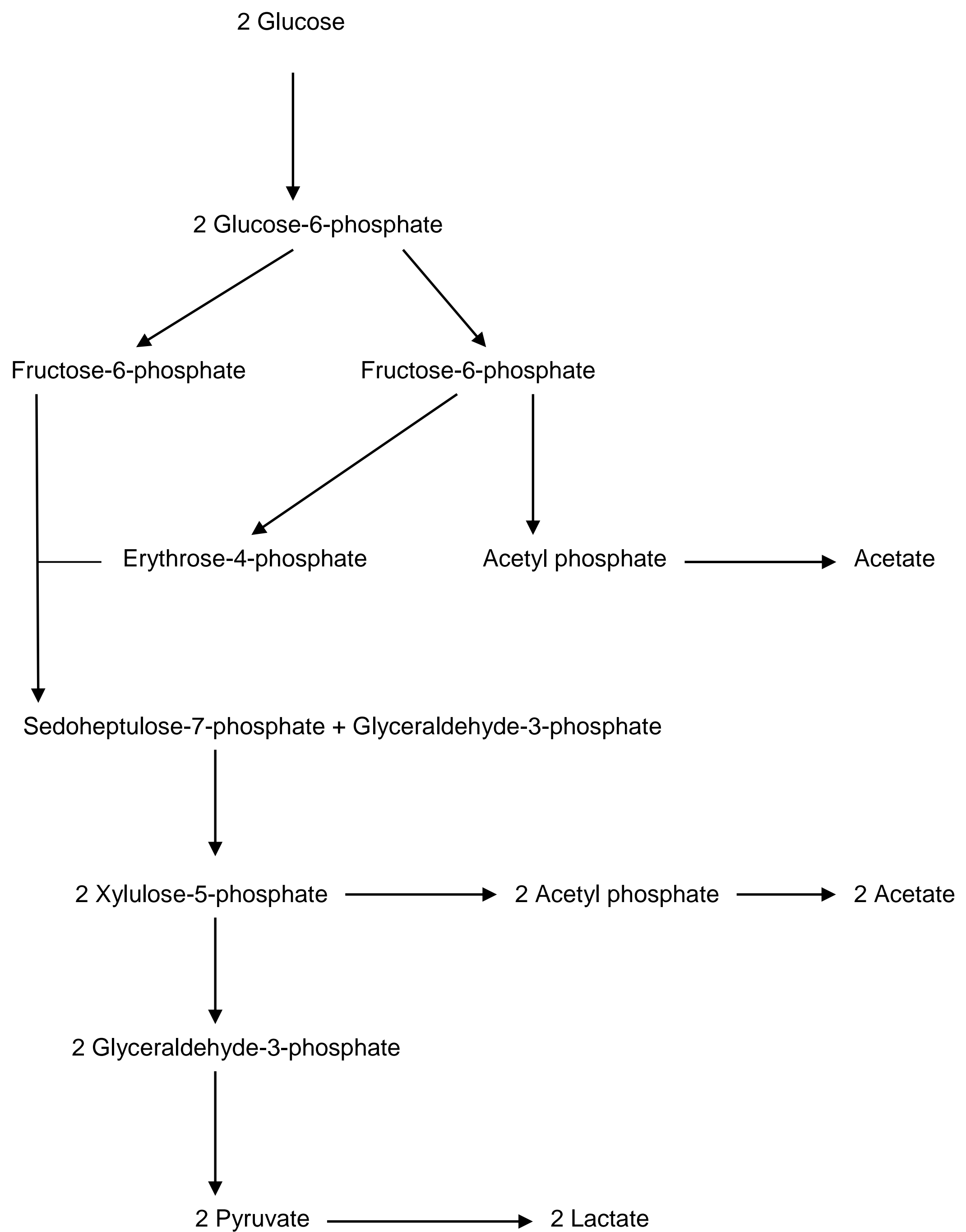

Fig. 1.3 Carbohydrate metabolism in bifidobacteria via fructose-6-phosphoketolase pathway (Adapted from Tamime *et al.* 1995)

Bifidobacteria are normal inhabitants of the human GIT, and they are reported to play certain metabolically important roles. These include production of conjugated linoleic acid (CLA), immunostimulation, production of short chain fatty acids (SCFAs), prebiotic utilisation and exopolysaccharide (EPS) production (Russell *et al.* 2011).

Bifidobacteria predominate the microflora found in breast-fed infants, before weaning, in comparison to formula-fed infants. This has been associated with better health in breast-fed babies compared with formula-fed babies (Roger *et al.* 2010). Human breastmilk has been shown to contain *Bifidobacterium*-stimulating or 'bifidogenic' factors, such as lactulose, *N*-acetyl-glucosamine-containing saccharides and other human milk oligosaccharides (HMOs), which consist of short-chain trisaccharides, e.g. sialyllactose, and complex, high-molecular-weight glycans, e.g. *N*-acetyllactosamine polymers. These oligosaccharides are only partially digested in the small intestine, and are therefore able to reach the colon to selectively stimulate the development of bifidobacteria in the gut microbiota (Gonzalez *et al.* 2008; Lee and O'Sullivan 2010). Genomic analyses of bifidobacteria found in the infant gut have revealed the possession of specialised genes that encode enzymes dedicated to the utilisation of HMOs (Ventura *et al.* 2012).

The population of bifidobacteria decreases with age, from 90-95% of the total gut microbial population at infancy to 3-6% in adulthood, and this remains stable until elderly age, where further decline has been observed (Leahy *et al.* 2005; Russell *et al.* 2011). Age-related changes in gut microflora have been linked to increased susceptibility to gastrointestinal disorders in the elderly (Baker *et al.* 2009).

1.3.1 Bifidobacteria as probiotics

There has been a lot of interest in bifidobacteria because of their association with maintenance of gut health. This has led to their incorporation in probiotic dairy products and other food products (Jayamanne and Adams 2006). Probiotic properties have been found in strains of *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve* and *B. longum* (Russell *et al.* 2011).

Various potential health benefits have been associated to strains of bifidobacteria, based on animal and human studies. Some of these studies have been criticised for poor design and weak conclusions (Lee and O'Sullivan 2010). Potential probiotic health benefits associated to bifidobacteria include prevention of diarrhoea (Saavedra *et al.* 1994; Chouraqui *et al.* 2004), alleviation of lactose intolerance (Jiang *et al.* 1996; He *et al.* 2008), cancer prevention (Rowland *et al.* 1998; Le Leu *et al.* 2010), cholesterol reduction (Xiao *et al.* 2003; Ataie-Jafari *et al.* 2009), modulation of the immune system (Arunachalam *et al.* 2000; Lee *et al.* 2004) and establishment of healthy microflora in premature infants (Li *et al.* 2004; Wang *et al.* 2007).

Bifidobacteria have a long history of safe use as probiotics, with low risk of causing infection (Russell *et al.* 2011). The five species of *Bifidobacterium* with Qualified Presumption of Safety (QPS) status, i.e. *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve* and *B. longum* (EFSA 2012), which contain probiotic strains, have not been linked to infections in healthy individuals (Gueimonde *et al.* 2013).

Some probiotic strains of bifidobacteria have been demonstrated to show no mucin-degrading activity, as well as no translocation ability in a normal host (Abe *et al.* 2010). Mucin degradation and translocation are important for safety because bacterial translocation from probiotic consumption could result in infections such as bacteraemia, sepsis and endocarditis (bloodstream infections). Mucin on the intestinal wall is important to prevent bacterial translocation. Translocation starts with the invasion of bacteria through the intestinal wall, therefore mucin degradation activity is used as an index of safety (Ishibashi and Yamazaki 2001; Abe *et al.* 2010). Furthermore, the transferability of antibiotic resistance genes from bifidobacteria to other enteric bacteria has not been demonstrated (Gueimonde *et al.* 2013).

Although the bifidobacteria used as probiotics are known to be safe, correct identification and labelling is crucial to ensure safety, because some rare cases of bifidobacteria causing infection have been reported, and it is not uncommon to have the species of *Bifidobacterium* used not indicated on the label, or the organism simply referred to as 'bifidus' (O'Brien *et al.* 1999). *Bifidobacterium dentium* has been implicated as an opportunistic pathogen associated with the development of dental caries (Ventura *et al.* 2009). Similarly, *B. scardovii* has been associated with infection in an immunocompromised elderly patient (Barberis *et al.* 2012). However, the administration of probiotic bifidobacteria has also been linked to sepsis, but involving an immunocompromised patient as well (Ohishi *et al.* 2010). Therefore, care may have to be taken when administering probiotics to vulnerable patients.

Generally, bifidobacteria grow slowly in milk and also show poor survival in fermented milk products (Goderska and Stanton 2010). They are therefore usually not used for fermentation on their own, but rather added as adjunct cultures to traditional yoghurt starter cultures, i.e. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, either at the start of the fermentation process, or after fermentation has finished (Maus and Ingham 2003). Furthermore, the growth of bifidobacteria in milk may affect the sensory properties of the final product, due to the production of acetic acid (Margolles and Sanchez 2012).

Among the probiotic bifidobacteria known, the most commonly used bifidobacteria commercially appear to be strains of *B. animalis* ssp. *lactis*. This may be due to better technological properties in comparison to the other bifidobacteria, which are of human intestinal origin (Raeisi *et al.* 2013). *B. animalis* ssp. *lactis* has been shown to have higher acid tolerance, as well as better tolerance to oxygen and heat and survival during storage (Simpson *et al.* 2005; Jayamanne and Adams 2006; Jayamanne and Adams 2009; Ruiz *et al.* 2012).

1.4 Microbial stress

For bacteria, there are conditions which can be considered as optimum, where growth and multiplication is facilitated. Conditions outside the optimum range, with the potential to decrease bacterial growth, can be considered as stressful. Therefore, environmental stress can be defined as external factors which have adverse impacts on the physiological welfare of bacterial cells,

resulting in reduced growth rate or, in extremes, resulting in inhibition (i.e. bacteriostatic) and/or death of individual cells or populations of cells (i.e. bacteriocidal) (McMahon *et al.* 2007b).

Examples of stresses include nutrient depletion (starvation), presence of toxic or inhibitory compounds, extremes of temperature, pH, osmotic pressure. Such conditions can induce the expression of specific genes in response and may also result in sublethally injured microorganisms (Wesche *et al.* 2009).

Stress can be classified on the basis of severity into suboptimal, sublethal and lethal (Ray and Bhunia 2008). Suboptimal conditions are those still within the growth range, but where growth is reduced. Bacterial cells show stress adaptation under suboptimal conditions. Under sublethal conditions, growth is less likely to occur and bacterial cells can suffer sublethal injury, which is reversible. Lethal conditions are those severe enough to cause cell death or irreversible injury.

Various types of damage can be caused by stressful conditions. When cells are exposed to stress, they are either unaffected, sublethally injured, or lose viability completely. Sublethal injury may cause a change in physiology. Stress may cause damage to the cell wall and cytoplasmic membrane, damage to ribosomes and RNA or damage to DNA. This injury may be manifested as loss of virulence, sensitivity to selective agents (in media), sensitivity to secondary stress, sensitivity to oxidative stress (reactive oxygen species), or extended lag periods, which allow for repair of damage (Stephens and Mackey 2003). These are summarized in Table 1.4.

Table 1.4 Sublethal injury in bacterial cells

Manifestation of injury	Possible cause of injury
Altered metabolism	Enzyme inactivation Membrane damage, leading to changes in enzyme/substrate accessibility
Extended lag	Need to resynthesise damaged membranes, nucleic acids, ribosomes, etc.
Sensitivity to oxidative stress	Inactivation of catalase, superoxide dismutase Increased metabolic production of H_2O_2 or O_2^- Loss of cofactors for DNA repair enzymes Loss of intracellular reductants
Sensitivity to acid, alkali, osmotic stress	Cytoplasmic membrane damage
Sensitivity to bile salts, hydrophobic antibiotics, lytic enzymes	Damage to Gram-negative outer membrane or Gram-positive surface protein layer
Increased frequency of mutation	Damage to DNA

(Adapted from Mackey 2000)

Understanding how microorganisms are affected by stress is important, especially in food processing, and prevention and control of food-borne pathogens. It is particularly necessary to understand the various strategies employed by these microorganisms to survive stress.

1.5 Stress and probiotic microorganisms

Microorganisms for probiotic use are faced with stressful conditions at various stages from processing to storage and gut transit, which could affect viability (Lacroix and Yildirim 2007). Stresses could affect the physiological activity of the probiotic microorganisms, and as a consequence, affect their functionality (Kheadr *et al.* 2007). Stresses faced by probiotic microorganisms may be classified as technological, which occur during preparation of probiotic formulations in large-scale and during storage in the products, or as gastrointestinal, which occur during transit through the

human GIT (Ruiz *et al.* 2011). Stress conditions probiotic microorganisms may be exposed to are summarised in Table 1.5.

Table 1.5 Stress conditions faced by probiotic microorganisms

Type	**Stage**	**Stress vector**
Technological	Fermentation and processing	• Composition of growth medium • Presence of organic acids during cultivation • High osmotic pressure and low water activity during cell dehydration • Extremes of temperature during spray drying and freeze drying • Oxygen exposure during fermentation, drying and storage
	Storage in probiotic-containing product (carrier)	• Increased acidity (pH < 5) • Presence of oxygen • Strain antagonism • Nutrient depletion • Presence of antimicrobial compounds • Storage temperature
Gastrointestinal	Mouth	Oxygen, enzymes
	Stomach	Acid, enzymes
	Small intestine	Bile, enzymes
	Colon	Nutrient starvation, microbiota

(Adapted from Lacroix and Yildirim 2007; Vasiljevic and Shah 2008; Mills *et al.* 2011; Ruiz *et al.* 2011)

Probiotic microorganisms should be able to survive these stress conditions. Lactic acid bacteria (LAB) and bifidobacteria have been shown to possess defence mechanisms to aid their survival during exposure to various stress conditions (Corcoran *et al.* 2008). Numerous studies have been carried out to understand these mechanisms in various LAB and bifidobacteria. Such mechanisms include proteases to degrade damaged proteins, chaperone proteins, which facilitate the folding of misfolded proteins, proton pumps, transporters and decarboxylases to combat decreases in intracellular pH,

catalases and superoxide dismutase to combat reactive oxygen species, and transport systems to maintain correct osmolarity (Mills *et al.* 2011). Some of these mechanisms are summarised in Table 1.6 and further illustrated in Figure 1.4.

1.5.1 Acid stress response

Acid stress occurs during passage through the stomach (De Dea Lindner *et al.* 2007). The stomach has a low pH due to the presence of hydrochloric acid in gastric juice, and is thus almost sterile (Chadwick *et al.* 2003). In addition, the presence or production of lactic and other organic acids in fermented dairy products used for probiotic delivery, owing to fermentation of lactose by lactic acid bacteria, reduces the pH of the milk, thereby creating acid stress (Sanchez *et al.* 2007a).

Low environmental pH has negative implications for bacterial cells, and could cause severe damage. Acid exposure causes a reduction in intracellular pH due to intracellular accumulation of protons, and also affects transmembrane pH. This alters the proton motive force (PMF) which is necessary for transport processes across the membrane. Acid stress could cause damage to the cell membrane, DNA and proteins, and is one of the most crucial stresses, as well as accumulation of oxidative intermediates. Thus, acid resistance is one of the criteria for probiotic selection (Corcoran *et al.* 2008).

Table 1.6 Strategies of stress response in some probiotic *Bifidobacterium* spp.

Stress source	Strategy of response	Molecular mechanisms/players
Heat	Proper protein folding	Molecular chaperones: GroEL, GroES, GrpE, DnaJ, DnaK, ClpB, Hsp20
	Degradation of misfolded proteins	Proteases: ClpC, ClpP
	Regulatory network	Transcriptional regulators: HrcA, HspR, CglR
Acid	Proton extrusion	F_1F_0-ATPase
	Cytoplasm buffering/ammonia production	Branched-chain amino acid production Glutamine synthetase
	Unknown	CysD, MetE
Bile	Bile salt/acid detoxification	Multidrug transporters and bile efflux pumps (BetA, Ctr)
	Bile salt deconjugation	Bile salt hydrolase
	Alteration of cell surface	Production of extracellular exopolysaccharides Changes in fatty acid composition Changes in surface-associated proteins: enolase, oligopeptide binding proteins
	Changes in energetic metabolism	Increase in ATP synthesis Changes in the ratios of final glycolytic products
	Modification of redox status	Methionine synthetase, Peroxidase
	Proper protein folding	Molecular chaperones: ClpB, HtrA, GrpE, GroES,
	Degradation of misfolded proteins	Proteases: ClpC
Oxygen	ROS-scavenging enzymes	NADH oxidase, NADH peroxidase, Mn-superoxide dismutase
	DNA/RNA-protective proteins	Dpr (DNA-binding ferritin-like protein) NrdA (ribonucleotide reductase) MutT1 (NTP phosphohydrolases)
	Oxygen stress-protective proteins	AhpC (alkyl hydroperoxide reductase C22) PNDR (Pyridine nucleotide-disulfide reductase)
Osmotic	Proper protein folding	Molecular chaperones: GroEL, GroES, Hsp20, DnaK, GrpE, DnaJ1, DnaJ2
	Transport systems	ATP-binding cassettes (ABC): OpuA, BusA Ion motive-force driven transporters: BetP, ProP
Cold	Small heat shock proteins	Hsp18.5, Hsp19.3, Hsp18.55
	Cold shock proteins	CspA, CspL,
	Cold induced proteins	ClpP (Clp ATPase family, members act as chaperones and regulators of proteolysis) Pyruvate kinase Glycoprotein endopeptidase

(Adapted from Corcoran *et al.* 2008; Mills *et al.* 2011; Ruiz *et al.* 2011)

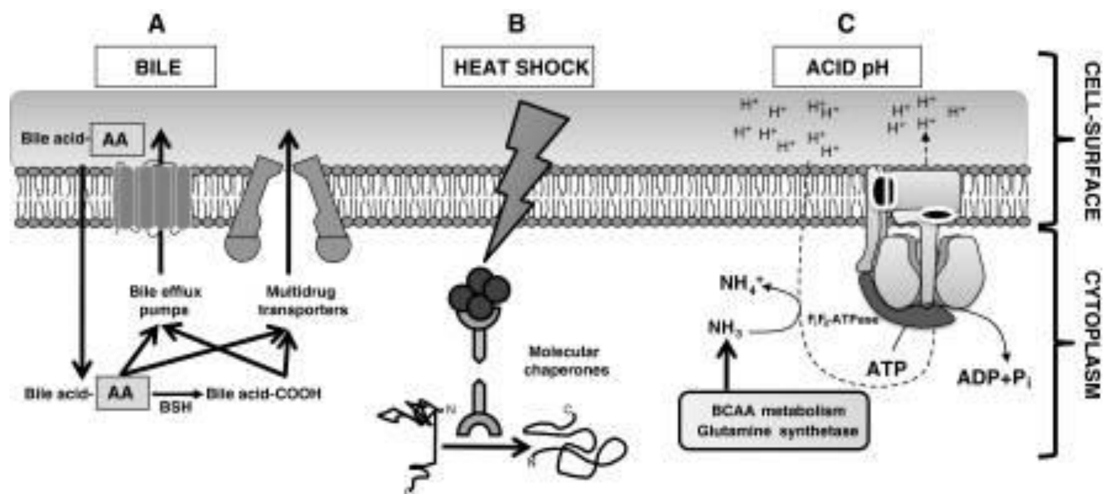

Fig. 1.4 Molecular mechanisms involved in the response of bifodobacteria to different stresses. **A)** Bile is detoxified from the cytoplasm by the activity of bile efflux pumps and/or multidrug transporters. Conjugated bile acids are deconjugated by the bile salt hydrolase (BSH), although the relationship of this enzyme with the resistance to bile is unclear. **B)** Both bile and heat shock induce protein aggregation and misfolding, which is counteracted by the action of chaperones and proteases. **C)** The F_1F_0-ATPase is used by bifidobacteria for counteracting the cytoplasm acidification that occurs in acidic environments. In addition, production of branched-chain amino acids is coupled with glutamine deamination, rendering ammonia that acts as a cytoplasmic buffer (Ruiz *et al.* 2011).

Lactobacilli and bifidobacteria have been demonstrated to possess an acid tolerance response (ATR), which enables their survival under acidic conditions, by maintaining their cytoplasmic pH near neutral (De Dea Lindner *et al.* 2007). The main mechanisms are via proton translocation (extrusion) by the F_1F_0-ATPase, which is a multi-subunit enzyme whose activity in anaerobic bacteria is enhanced at low pH; and via alkalinization of the cytoplasm by ammonia formation, which occurs from glutamine deamination. The ammonia formed captures protons, thus acting as a cytoplasmic buffer at low pH (Sanchez *et al.* 2010b; Ruiz *et al.* 2011).

1.5.2 Bile stress response

In the small intestine, the presence of bile salts is inhibitory to microbial growth, and thus, low numbers of microorganisms are found (Chadwick *et al.* 2003). Bile salts are biological detergents and are found in bile, which is secreted into the small intestine for the emulsification and absorption of fats. The antimicrobial action of bile is displayed by inducing membrane damage, protein misfolding, and causing DNA injury by oxidative shock and low intracellular pH (Sanchez *et al.* 2007b; Mills *et al.* 2011). Resistance to the lethal action of bile salts is thus crucial for probiotic microorganisms.

There are various bile tolerance mechanisms in response to the various effects of bile on cells. Unconjugated bile acids such as cholic acid can passively accumulate in cells by freely crossing the lipid bilayer. This leads to reduction in intracellular pH, causing leakage of ions and cellular components, thus leading to cell death. To combat this, bile tolerant cells

have efflux pumps for extrusion of bile acids. In response to the higher energy requirements under stress, bile tolerant cells are able to modify their sugar metabolism (in order to produce more energy) and their redox status in the presence of bile (Sanchez *et al.* 2010b).

Oxidative damage caused by bile exposure (resulting from production of oxygen free radicals) can be dealt with by the induction of enzymes involved in the SOS response, i.e. a stress response to DNA damage, such as a thioredoxin-dependent thiol peroxidase and a Dps protein (DNA-binding protein from starved cells). Another mechanism to deal with oxidative damage is by reduced production of enzymes involved in methionine biosynthesis (the methionine sulphur group is susceptible to oxidation). The effect of bile on protein conformation can be combated by the overproduction of chaperones and proteases to conduct proper folding of proteins and promote quicker recycling of misfolded proteins (Ruiz *et al.* 2011)

In response to the effects of bile on the cell surface, which is the first target of bile action, increased production of exopolysaccharide (EPS) can confer protection against bile. Also, changes in the lipid composition of the cell membrane are induced in the presence of bile, which can reduce the bile salt diffusion rate into the cytoplasm, thus enhancing bile tolerance. Also, a relationship has been observed between bile adaptation and increase in production of bile salt hydrolase (BSH) enzyme, which is responsible for bile salt deconjugation, though its role is not clear (Sanchez *et al.* 2010b; Ruiz *et al.* 2011).

1.5.3 Heat stress response

Probiotics are commonly grown to high numbers before undergoing drying processes to produce powders which can be added to probiotic products. The bacteria are exposed to high temperatures during spray-drying, up to 200 °C, for a short time, which can disrupt the integrity of viable bacterial cells (De Dea Lindner *et al.* 2007; Mills *et al.* 2011). Lactobacilli and bifidobacteria are sensitive to temperatures above 50 °C. Heat stress affects microbial activity and growth by affecting the bacterial membrane, which consists of fatty acids susceptible to heat damage. Subsequent aggregation of proteins and damage to ribosomes and RNA also occurs, as a result of protein denaturation from the destabilisation of non-covalent interactions at high temperatures (Corcoran *et al.* 2008; Champomier-Verges *et al.* 2010).

Heat shock has been widely studied in lactobacilli and bifidobacteria. They can utilise several heat shock proteins, including chaperones such as GroES, GroEL, DnaK and proteases such as HtrA (high temperature requirement), ClpC, ClpP (caseinolytic protease) to combat heat stress. These are induced by increasing temperatures. Small heat shock proteins (sHsps), which are ATP-independent chaperones, are also associated with enhanced bacterial survival during exposure to heat stress. They are necessary for growth, stability of DNA and RNA and preventing the formation of inclusion bodies, but not involved in protein re-folding (Mills *et al.* 2011; Ruiz *et al.* 2011).

1.5.4 Oxidative stress response

Lactobacilli are microaerophilic and bifidobacteria are obligate anaerobes, though some species exhibit tolerance to microaerophilic levels, e.g. *Bifidobacterium psychraerophilum*. Probiotic bacteria are exposed to oxidative stress at different stages in their production, as well as in the GIT (Corcoran *et al.* 2008). Probiotic microorganisms in yoghurts are exposed to dissolved oxygen as a result of the mixing processes in manufacture which incorporate oxygen in the product, as well as diffusion of oxygen through the packaging materials (Talwalkar *et al.* 2004). Exposure to oxygen has been suggested as one of the reasons for loss in viability of probiotics. This is due to the formation of reactive oxygen species (ROS) such as superoxide, hydroxyl and hydrogen peroxide (H_2O_2) from incomplete reduction of oxygen, which can cause damage by reacting with proteins, lipids and DNA (Corcoran *et al.* 2008; Li *et al.* 2010). Tolerance to oxygen is therefore a desirable characteristic for probiotic microorganisms.

Anaerobes lack the enzymes catalase and superoxide dismutase (SOD) which can decompose and detoxify ROS (Li *et al.* 2010). Enzymes such as NADH oxidase and NADH peroxidase are required by anaerobic lactobacilli and bifidobacteria to scavenge environmental oxygen and H_2O_2 respectively. Higher levels of these enzymes are found in the more aerotolerant organisms, thus protecting against oxygen toxicity (Talwalkar and Kailasapathy 2003). Damage to proteins is combated by the induction of protective proteins like AhpC and PNDR, and DNA and RNA are protected from damage by induction of proteins like Dpr, NrdA, MutT1 and enolase, in the presence of oxygen (Xiao *et al.* 2011). Dpr (dps-like peroxide resistance)

induction leads to production of a feritin-like iron scavenger upon exposure to H_2O_2, which prevents the overabundance of free iron that leads to oxidative damage (Wesche *et al.* 2009, Yamamoto *et al.* 2011).

1.5.5 Cold stress response

Probiotics in dairy products are exposed to low temperature prior to consumption, during production and storage (Corcoran *et al.* 2008). It is known that numbers of probiotic microorganisms decline with time during cold storage, and low temperatures are considered as a reason for loss in viability (Maus and Ingham 2003). Low (freezing) temperatures also cause membrane damage and affect replication, transcription and translation (Champomier-Verges *et al.* 2010). Cryotolerance (i.e. cold tolerance) is thus a desirable feature in probiotic bacteria.

The induction of chaperones and proteases such as ATP-dependent ClpP is important for enhancing cold tolerance. Also, cold shock proteins e.g. CspL are over-expressed after cold shock and appear to stabilize mRNA. Some small heat shock proteins are also expressed following cold shock. The protease functions by proteolysis of misfolded and damaged proteins generated by cold shock (Champomier-Verges *et al.* 2010). The induction of ATP-dependent ClpP is the main adaptive response to cold in lactobacilli. Also, alterations (increase) in the unsaturated fatty acid composition of membranes occur during cold adaptation, which maintains membrane fluidity during cold stress, thus enhancing cryotolerance (Wang *et al.* 2005; Corcoran *et al.* 2008).

1.5.6 Osmotic stress response

Osmotic stress may occur during spray drying and freeze drying, and may also occur due to the presence of salt (NaCl or KCl). In the gut, osmotic stress in bacteria results from fluctuations in diet (De Dea Lindner *et al.* 2007). Osmotic stress can occur from shifts in external osmolarity, which leads to movement of water out of the cell, excessive movement of which, can lead to cell damage or death (Wesche *et al.* 2009). Cells need to adjust their intracellular osmolyte concentration, in order to retain turgour during osmotic upshift (De Angelis and Gobbetti 2004; Corcoran *et al.* 2008).

Lactobacilli and bifidobacteria are unable to accumulate sufficiently high concentrations of Na^+ or K^+ to maintain turgour, and they synthesise very low levels of compatible solutes which serve as osmotic balancers. As such, transport systems are necessary to take up solutes and enhance osmotic tolerance (Corcoran *et al.* 2008; Mills *et al.* 2011). Molecular chaperones are also produced under osmotic shock to promote proper protein folding. GroEL and DnaK, which are heat shock proteins, are induced under osmotic shock, thus indicating some overlaps between heat and osmotic stress responses (Prasad *et al.* 2003).

1.6 Effects of stress on probiotic functionality

There have been several studies about the behaviour and responses of probiotic microorganisms to the various stresses they can be exposed to. The studies on lactic acid bacteria and bifidobacteria in relation to stress have been mainly in the context of monitoring their survival and viability, and

understanding the genes and proteins involved in their stress response, and how these aid their survival of sublethal stress, and their viability (Schmidt and Zink 2000; De Angelis and Gobbetti 2004). Some of these have been highlighted in the previous section.

Similar studies have been done on other organisms, especially pathogens, e.g. *Salmonella* (Alvarez-Odonez *et al.* 2011), *Bacillus cereus* (Mols and Abee 2011), *Listeria* (Moorhead and Dykes 2004), *Campylobacter* (Birk *et al.* 2012), on how they respond to environmental and physiological stress, with focus on growth/survival and pathogenicity. These have been aimed at developing strategies for preventing and controlling such pathogens, such as in food processing and preservation, to ensure food safety.

There have been no known studies which have examined if and/or how exposure to stress can affect the functionality of viable bacteria for potential probiotic use. Research has been done on pathogenic microorganisms, which suggest that exposure to stressful conditions might aid their virulence. For instance, a study by Begley *et al.* (2009) showed that exposure to bile influences biofilm formation in *Listeria monocytogenes*, which may contribute to its survival and virulence in the human gastrointestinal tract. Links have been found between the induction of stress-tolerance responses and increased virulence in pathogenic *Escherichia coli*, *Listeria monocytogenes* and *Salmonella typhimurium*, in which the synthesis of virulence genes is regulated by the exposure to hostile conditions (Archer 1996; Gahan and Hill 1999).

To draw a parallel with probiotic microorganisms, it could be hypothesised that exposure to stress may aid probiotic microorganisms in their functionality, or otherwise impede them. Studies have been conducted on the influences on manufacturing conditions on some *in vitro* properties of probiotic lactobacilli. Grzeskowiak *et al.* (2011) reported differences in the *in vitro* properties of *Lactobacillus rhamnosus* GG isolates from different sources (probiotic products). It was suggested that different manufacturing processes may have an impact on strain properties. Similar suggestions were made by Nivoliez *et al.* (2012) in their comparative study of *Lactobacillus rhamnosus* Lcr35 and four of its commercial formulations. These variations between isolates may be due to genetic rearrangements within the genome of the strain as a result of differing manufacturing conditions, thus impacting on functionality (Sybesma *et al.* 2013). However, none of these studies have explicitly linked any of these differences with specific stress conditions.

Resistance of probiotic *Lactobacillus* strains to low pH and bile has been demonstrated to be influenced by the type carbohydrate used as carbon source in the medium the lactobacilli were grown on (Hernandez-Hernandez *et al.* 2012; Nazzaro *et al.* 2012). In addition, Fayol-Messaoudi *et al.* (2005) showed that the temperature at which *Lb. rhamnosus* GG was grown had an influence on its anti-pathogen activity. A significant reduction in the killing activity against *Salmonella enterica* ser. Typhimurium was observed when *Lb. rhamnosus* GG was grown at 32 °C, compared to when grown at 37 °C. This report may indicate an effect of stress on functional properties of probiotics.

It is important to study the functionality of probiotics exposed to stress conditions because they are usually available within food products,

consequently implying that they are inevitably faced with stressful conditions, not to mention the stress they go through in the human gastrointestinal tract, upon consumption. Any significant effects of stress conditions on the functional properties could have implications on how probiotics are produced and delivered. Therefore, research is needed to provide knowledge on the specific effects of individual stresses on different aspects of probiotic functionality.

1.7 Aims and objectives

Potential probiotic microorganisms are usually screened *in vitro* for acid and bile tolerance, in order to predict *in vivo* survival capacity when encountering acid and bile stress. However, the need to consider other stresses, including osmotic and oxidative stress, when selecting probiotic strains, has been highlighted (Lebeer *et al.* 2008). Acid stress occurs in the stomach, but also during fermentation, due to the production and accumulation of organic acids such as lactic acid. Bile stress occurs in the small intestine, due to the secretion of bile salts. Oxidative stress occurs in the mouth, but also during production and storage, because of the presence of oxygen. Osmotic stress occurs due to changes in the bacterial environment, and also due to the presence of salt ions, which result in movement of water in or out of the cell.

It is expected that probiotics are able to confer health benefits despite exposure to stressful conditions. Whilst reduction in numbers may occur during gut transit, sufficient numbers should still be able to survive. Injury may occur among the surviving cells, but they should be able to recover and

retain their health-promoting properties. Probiotic microorganisms should be able to form biofilms in the gut and produce antagonistic (antimicrobial) compounds in order to out-compete undesirable bacteria and colonise the gut. They should also be able to tolerate antibiotics in order to aid re-colonisation of the gut, during antibiotic therapy.

Therefore, the aim of this project was to examine possible effects of environmental and physiological stresses on various functional properties of probiotic microorganisms, with specific interest in *Bifidobacterium* spp., using *in vitro* studies. Specific objectives of this project were:

- to identify conditions for inducing acid, bile, oxidative and osmotic stresses in the bifidobacterial strains studied;
- to assess the effect of exposure to these stresses on the functional properties, namely antimicrobial activity, biofilm formation and antibiotic susceptibility profile, in comparison with controls (unstressed cells); and
- to provide insight into how the functional properties of probiotic microorganisms may be affected due to exposure to stressful conditions and discuss possible implications.

CHAPTER TWO: ESTABLISHMENT OF STRESS TREATMENT CONDITIONS

2.1 Introduction

Microorganisms intended for probiotic use are preferably of human intestinal origin, an environment in which their existence is relatively stress-free, as it is nutrient-rich, anaerobic and of neutral pH (Corcoran *et al.* 2008). When selected for probiotic use, however, these microorganisms become exposed to various stressful conditions. Conditions which are outside the optimum can be considered as stressful. These may be classed as suboptimal, sublethal and lethal (Ray and Bhunia 2008).

Optimum conditions for bifidobacteria are typically anaerobic, with a pH between 6.5 – 7, and temperature between 37 – 41 °C (Tamime *et al.* 1995). However they can survive suboptimal conditions, as stress tolerance/response mechanisms are elicited upon exposure. Under such suboptimal conditions, growth can still occur due to adaptation, though at a slower rate. Sublethal conditions may usually result in sublethal injury, and it is less likely that growth would occur, until the cells are under optimum conditions and repair has occurred. Lethal conditions would usually cause irreparable damage and cell death.

Yoghurt, which is a common vehicle for delivery of probiotics, usually has a pH of 4.5. As such, bifidobacteria are usually added after fermentation has ended, in the required numbers since no growth will occur during storage/refrigeration. Furthermore, the stomach pH ranges between 1 – 3, and this is potentially lethal to bifidobacteria during passage in the gastrointestinal tract. Transit time in the gut can range between 1 – 4 hours (Li *et al.* 2010).

The estimated physiological bile concentration in the human GIT is between 0.3 – 2% (w/v) (Noriega *et al.* 2004). Bifidobacteria can tolerate bile concentrations of up to 2% (w/v), but this varies with the type of strain (Margolles *et al.* 2003; Jia *et al.* 2010).

The osmolarity of the upper small intestine is equivalent to 0.3M NaCl, which suggests high salt concentration and low water activity (a_w) (Sheehan *et al.* 2007; Alvarez-Ordonez *et al.* 2011). Similar osmolarity can be achieved by supplementing the medium with 1.5% (w/v) NaCl or 6% (w/v) sucrose (Sheehan *et al.* 2007). Concentrations of NaCl in physiological gastrointestinal conditions are not less than 0.5% (w/v), and in food fermentations range up to 6-8% (w/v) (Collado and Sanz 2007).

The processses of manufacture and storage of probiotic-containing food products expose probiotic strains to aerobic conditions. The toxic effect of oxygen is considered one of the main factors influencing the survival of bifidobacteria in probiotic yoghurts (Talwalkar *et al.* 2004; Ruiz *et al.* 2012). Steep oxygen gradients may also be present in the gastrointestinal tract (Corcoran *et al.* 2008). Tolerance to oxygen is therefore desirable in probiotics, to ensure stability and viability in end products (Ruiz *et al.* 2012).

The aim of this chapter was to examine the survival of different bifidobacteria under varying levels of acid, bile, osmotic and oxidative conditions in order to select sublethal stress conditions which could elicit stress in the bifidobacteria without being lethal. The expectation was that no growth would occur under such sublethal conditions, thus forming the basis of their selection, as no growth would be indicative of stress and/or sublethal injury in

the cells. These selected conditions would then be used to induce stress in the bifidobacteria prior to subsequent experiments in the further chapters.

2.2 Materials and methods

2.2.1 Bacterial cultures

Bifidobacterial cultures were made available from the culture collection of the Microbiology Research Unit of London Metropolitan University, namely *Bifidobacterium breve* NCTC 11815, *B. longum* NCTC 11818 (National Collection of Type Cultures, Collindale, UK), and two strains of *B. animalis* ssp. *lactis*, designated C and D. *B. breve* NCTC 11815 was of human origin, isolated from the intestine of an infant. *B. longum* NCTC 11818 was also of human origin, isolated from the intestine of an adult. *B. animalis* ssp. *lactis* (C) was isolated from a commercial culture, *Bifidobacterium* BB-12, kindly provided by Chr. Hansen (Berkshire, UK). *B. animalis* ssp. *lactis* (D) was of dairy origin, isolated from a fermented dairy product of mixed microbial culture. Both were confirmed as *B. animalis* ssp. *lactis* by 16S rRNA gene sequencing. All cultures were stored on beads in cryovials (Microbank, Pro-Lab Diagnostics UK) and kept at -20 ^{o}C. Cultures were streaked on Reinforced Clostridial Agar (RCA) (CM 0151; Oxoid, Hampshire, UK) and incubated under anaerobic conditions (10% CO_2, 10% H_2, 80% N_2) in an anaerobic cabinet (Don Whitley Scientific, UK) for 48 hours at 37 ^{o}C. The resulting colonies were used for subsequent experiments.

2.2.2 Inoculum preparation

Colonies from a 48-hour culture were suspended in normal saline (8.5 g/L NaCl solution) to achieve an optical density equivalent to 0.5 McFarland using a Sensititre nephelometer (Trek Diagnostics, UK).

2.2.3 Survival under acid conditions

A 1 ml volume of the suspension was inoculated into 19 ml of MRSc broth (de Man, Rogosa, Sharpe broth, Oxoid CM0359 + 0.05% w/v L-cysteine hydrochloride) in 25 ml screw-capped Universal bottles, adjusted to pH 2.0, 3.0 and 4.0 using 2M hydrochloric acid (HCl). The pH of the MRSc broths were adjusted after autoclaving. The control set up was unadjusted MRSc broth (pH 6.2). Enumeration was done on RCA at the point of inoculation (Time 0), and after 1, 2, 3 and 24 hours of incubation. The cultures were incubated under anaerobic conditions at 37 °C. Experiments were carried out in duplicate.

2.2.4 Survival in the presence of bile

A 1 ml volume of the suspension was inoculated into 19 ml of MRSc broth in 25 ml screw-capped Universal bottles, supplemented with ox-bile (B-3883; Sigma, St. Louis, USA) to reach ox-bile concentrations of 0.5% (w/v) and 1% (w/v). A stock solution of 10% (w/v) ox-bile was prepared in sterile distilled water and filter sterilised using a Nalgene 0.2 µm pore-size syringe filter (Thermo Scientific, UK). The MRSc broths were supplemented with appropriate quantities of bile solution after autoclaving, to achieve the final concentrations of 0.5% (w/v) and 1% (w/v). The control set up was non-supplemented MRSc broth. Enumeration was done on RCA at the point of inoculation (Time 0), and after 1, 2, 3 and 24 hours of incubation. The cultures were incubated under anaerobic conditions at 37 °C. Experiments were carried out in duplicate.

2.2.5 Survival under osmotic conditions

A 1 ml volume of the suspension was inoculated into 19 ml of MRSc broth in 25 ml screw-capped Universal bottles, supplemented with sodium chloride (NaCl) (Sigma) to achieve concentrations of 1%, 2%, 3%, 4% and 5% (w/v). The media were supplemented with NaCl before autoclaving. The control set up was non-supplemented MRSc broth. Enumeration was done on RCA at the point of inoculation (Time 0), and after 2, 4 and 24 hours of incubation. The cultures were incubated under anaerobic conditions at 37 ^{0}C. Experiments were carried out in duplicate.

2.2.6 Survival under oxidative conditions

A 1 ml volume of the suspension was inoculated into 19 ml of MRSc broth in 25 ml screw-capped Universal bottles and incubated under anaerobic conditions for 24 hours. Cells were harvested by centrifugation (26,700 x *g* for 15 minutes) and resuspended in 100 ml MRS broth in 250 ml conical flasks. Flasks were incubated in a shaking incubator with shaking speed of 200 rpm at 37 ^{0}C and enumeration was done at point of inoculation (Time 0), and after 2, 4 and 24 hours. The control set up was in MRSc broth incubated under anaerobic conditions in 100 ml Duran bottles, with no shaking. Experiments were carried out in duplicate.

2.3 Results

2.3.1 Survival under acid conditions

Cultures were inoculated in MRSc broth of pH 2, 3 or 4 and enumerated at 0, 1, 2, 3 and 24 hours to monitor survival in acidic conditions. For all cultures, pH 2 proved to be very inhibitory, with no colonies recovered after one hour from the undiluted sample (Fig. 2.1a). At pH 3, the *B. animalis* ssp. *lactis* strains showed good survival over the 24 hour period, while no *B. longum* and *B. breve* colonies were recoverable after one hour from the undiluted sample (Fig. 2.1b). At pH 4, all cultures showed good survival over the 24 hour period (Fig. 2.1c).

2.3.2 Survival in the presence of bile

Cultures were inoculated in MRSc broth of 0.5% or 1% (w/v) bile and enumerated at 0, 1, 2, 3 and 24 hours to monitor survival in the presence of bile. Generally colonies were recoverable after 24 hours under both conditions, except *B. longum*, where colonies were not recovered from the undiluted sample at 3 and 24 hours. All showed no large reduction in numbers after one hour of exposure to both 0.5% (Fig. 2.2a) and 1% (w/v) bile (Fig. 2.2b), except *B. longum,* which fared better after one hour of exposure to 0.5% than 1% (w/v) bile.

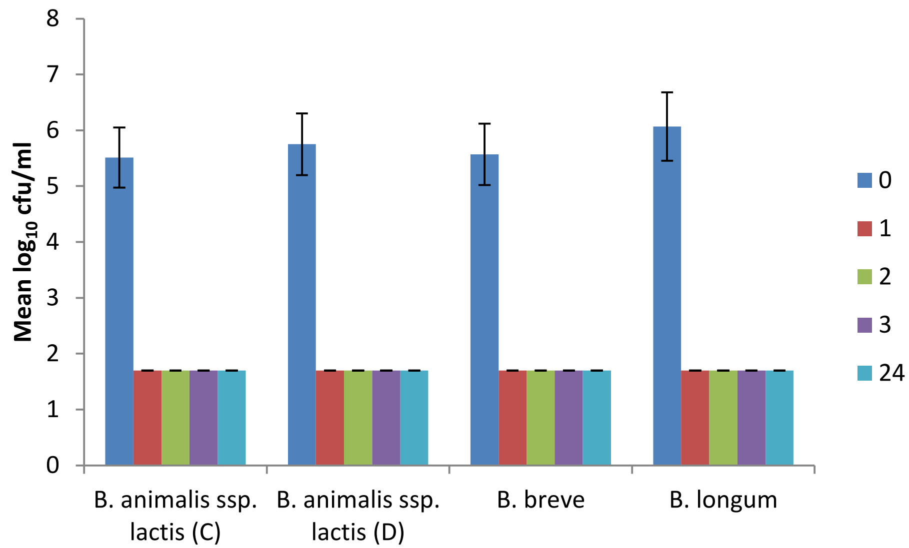

Fig. 2.1a Enumeration of bifidobacteria in MRSc broth with pH adjusted to pH 2, at 0, 1, 2, 3 and 24 hours after incubation. Error bars represent standard error of the mean (SEM)

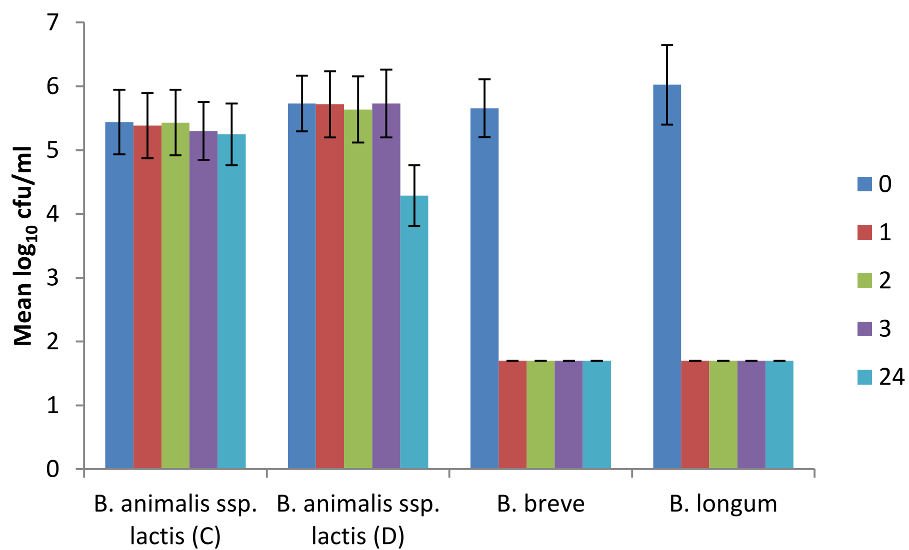

Fig. 2.1b Enumeration of bifidobacteria in MRSc broth with pH adjusted to pH 3, at 0, 1, 2, 3 and 24 hours after incubation. Error bars represent standard error of the mean (SEM)

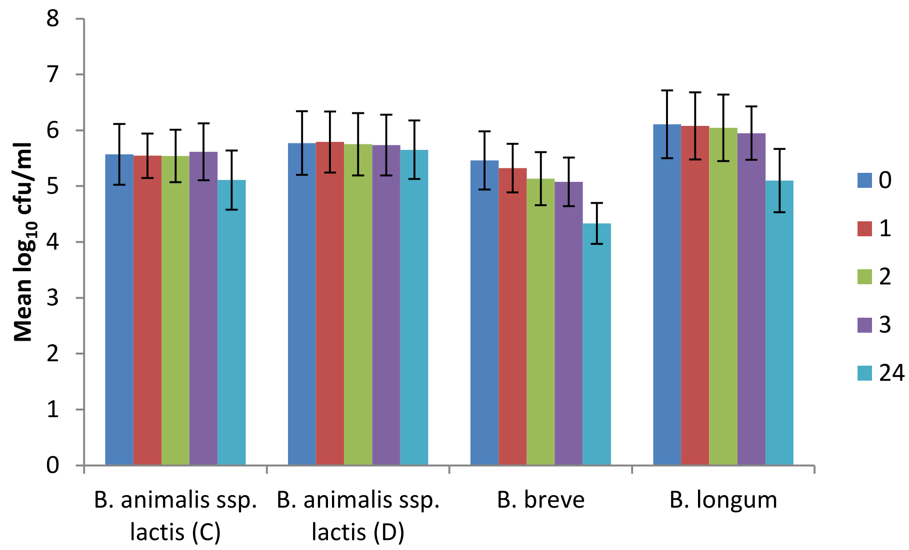

Fig. 2.1c Enumeration of bifidobacteria in MRSc broth with pH adjusted to pH 4, at 0, 1, 2, 3 and 24 hours after incubation. Error bars represent standard error of the mean (SEM)

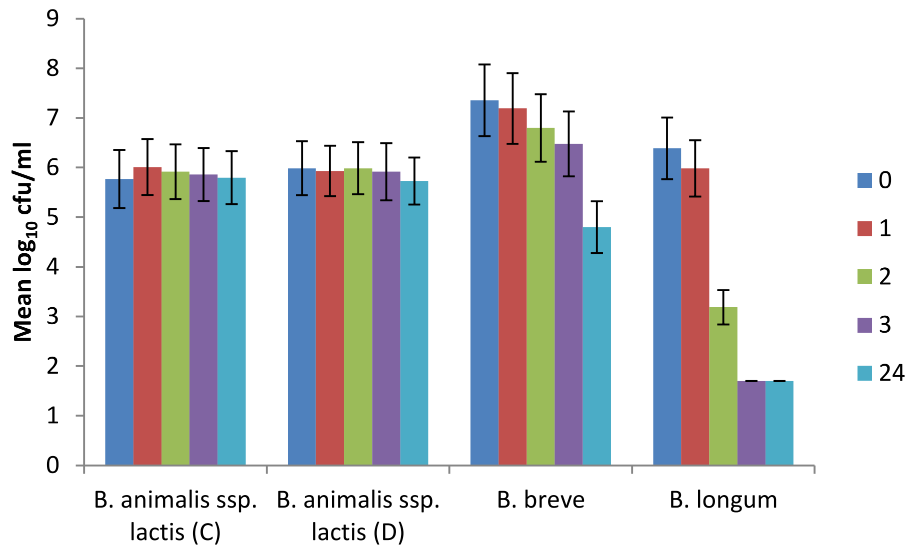

Fig. 2.2a Enumeration of bifidobacteria in MRSc broth containing 0.5% (w/v) bile, at 0, 1, 2, 3 and 24 hours after incubation. Error bars represent standard error of the mean (SEM)

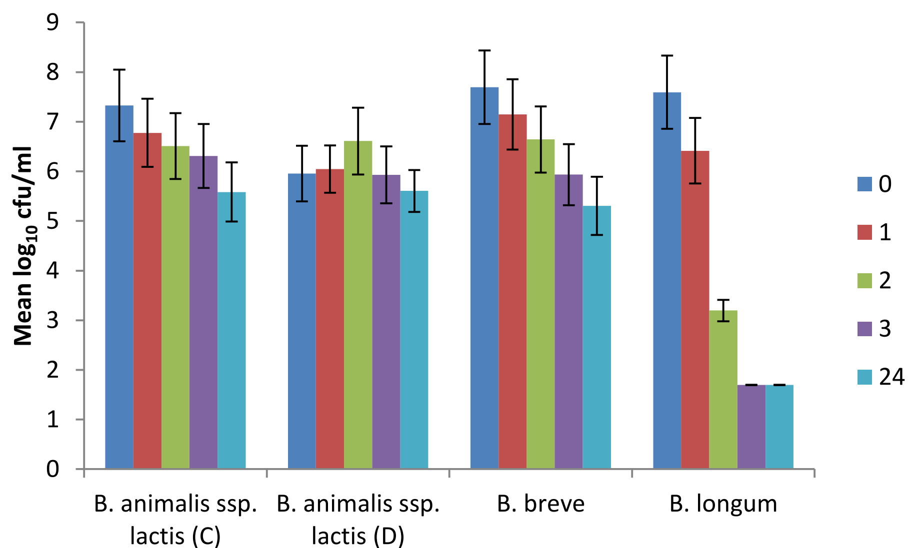

Fig. 2.2b Enumeration of bifidobacteria in MRSc broth containing 1% (w/v) bile, at 0, 1, 2, 3 and 24 hours after incubation. Error bars represent standard error of the mean (SEM)

2.3.3 Survival under varying osmotic conditions

Cultures were inoculated in MRSc broth of 1%, 2%, 3%, 4% or 5% (w/v) NaCl and enumerated at 0, 2, 4 and 24 hours to monitor survival in the presence of NaCl. Fig. 2.3 shows survival of the bifidobacteria under osmotic conditions. At NaCl concentrations of 1% and 2% (w/v) growth was observed after 24 hour incubation for *B. animalis* ssp. *lactis* strains (Fig. 2.3a and 2.3b) and *B. breve* (Fig. 2.3c). However, at concentrations from 3% (w/v) and above, no growth was observed, and no marked reduction was observed in the first 2 hours. For *B. longum* (Fig. 2.3d), no growth was observed under osmotic conditions, but survival after 24 hours was observed up to 2% (w/v) NaCl. No colonies were recovered from the undiluted sample after 24 hours at NaCl concentrations above 2% (w/v) for *B. longum*.

2.3.4 Survival under oxidative conditions

Cultures were inoculated in MRS broth and incubated in a shaking incubator at 200 rpm to aerate the medium. Fig. 2.4 shows survival under aerobic conditions. In general, under aerobic conditions, no colonies were recovered from the undiluted samples after 24 hours. However, there was no large reduction in numbers within the first two hours of aerobic incubation.

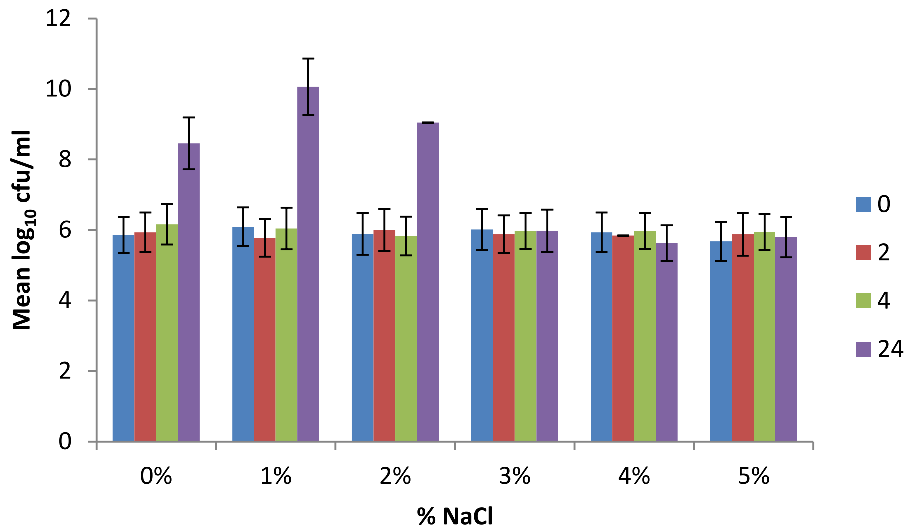

Fig. 2.3a Enumeration of *B. animalis* ssp. *lactis* (strain D) in MRSc broth adjusted to different NaCl concentrations (0%, 1%, 2%, 3%, 4%, 5%) at 0, 2, 4 and 24 hours after incubation. Error bars represent standard error of the mean (SEM)

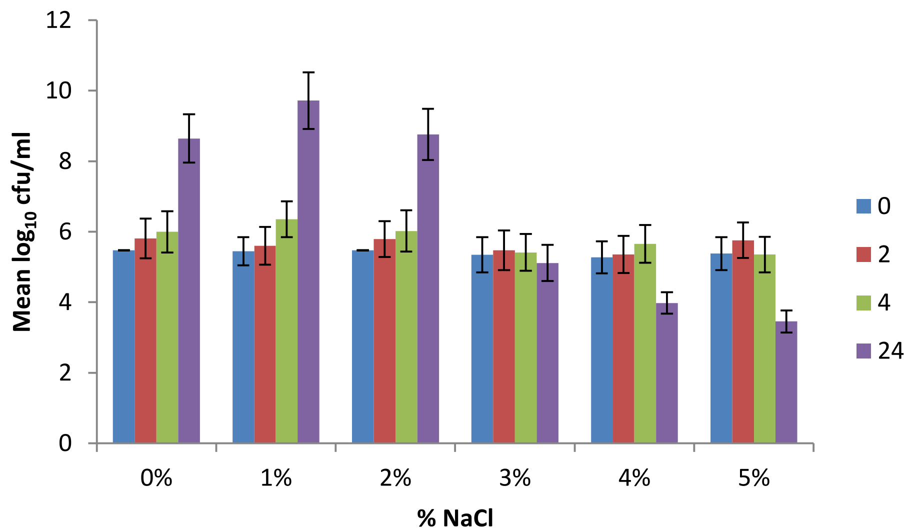

Fig. 2.3b Enumeration of *B. animalis* ssp. *lactis* (strain C) in MRSc broth adjusted to different NaCl concentrations (0%, 1%, 2%, 3%, 4%, 5%) at 0, 2, 4 and 24 hours after incubation. Error bars represent standard error of the mean (SEM)

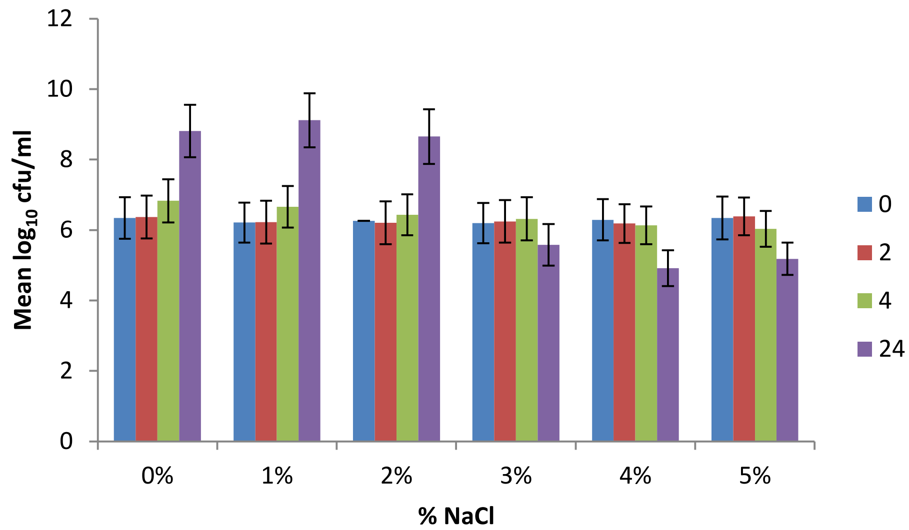

Fig. 2.3c Enumeration of *B. breve* in MRSc broth adjusted to different NaCl concentrations (0%, 1%, 2%, 3%, 4%, 5%) at 0, 2, 4 and 24 hours after incubation. Error bars represent standard error of the mean (SEM)

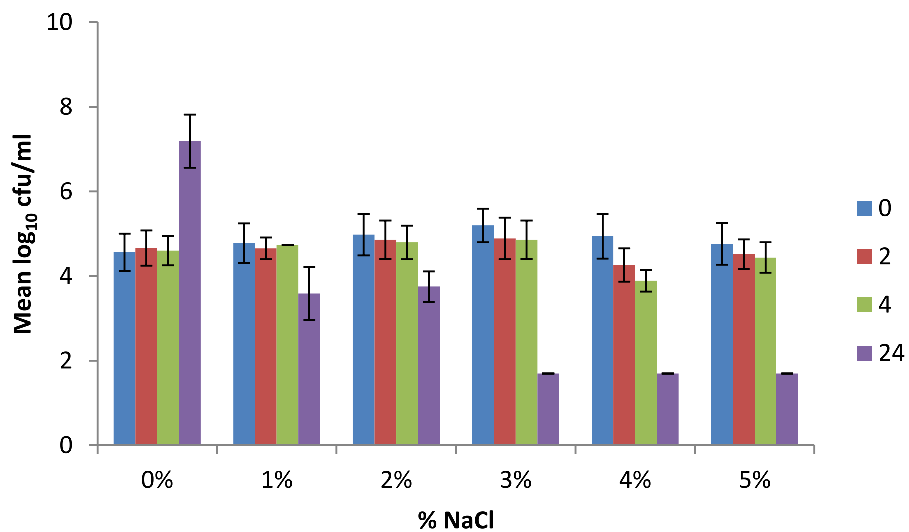

Fig. 2.3d Enumeration of *B. longum* in MRSc broth adjusted to different NaCl concentrations (0%, 1%, 2%, 3%, 4%, 5%) at 0, 2, 4 and 24 hours after incubation. Error bars represent standard error of the mean (SEM)

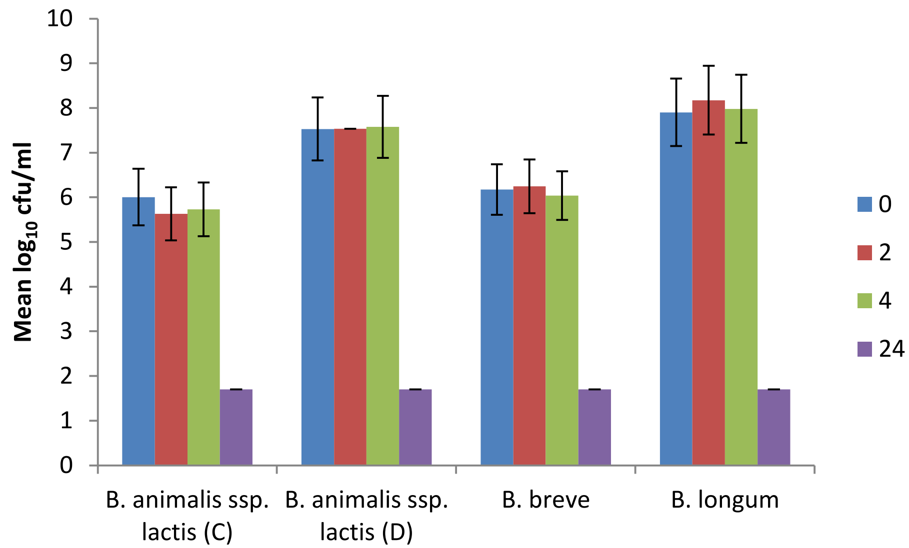

Fig. 2.4 Enumeration of bifidobacteria in MRS broth at 0, 2, 4 and 24 hours after incubation, under aerobic incubation (shaking at 200 rpm). Error bars represent standard error of the mean (SEM)

2.4 Discussion

Commercial probiotic microorganisms are exposed to several environmental stresses, during large-scale production and product storage, and during passage through the oral cavity, stomach and small intestine. These stresses include oxygen and oxygen-derived radicals, acids, bile, osmotic, heat and cold stress, which could negatively affect viability and functionality (Zomer *et al.* 2009).

Stress may be manifested in microorganisms in different ways, and these manifestations can be identified by various methods. Stress can result in changes in cell morphology, which can be identified by microscopic examination (Wesche *et al.* 2009). For instance, bifidobacteria exposed to oxidative stress showed intracellular granule formation and changes in shape when stained with simple stains like safranin or Loeffler's methylene blue and examined under a microscope (Qian *et al.* 2011). Stress can also result in membrane damage, and this can be identified by flow cytometry. Ben Amor *et al.* (2002) were able to distinguish between live, dead and injured bifidobacteria after bile stress, using multiparameter flow cytometer, involving three dyes – propidium iodide (PI) stains dead cells, carboxyfluorescein diacetate (cFDA) stains intact cells, and bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$] stains injured cells.

Another method of confirming injury in microorganisms is by following repair of injury in their cells in a repair medium. This can be done by differential plating on restrictive medium, as described by Mackey (2000). The method is based on the theory that injured bacteria will not be able to grow on restrictive media, such as selective media, on which they would otherwise

grow if intact. The selective media would be inhibitory to injured bacteria, possibly due to membrane damage, for instance. Enumeration is done on selective and non-selective or optimum media. The optimum medium estimates the entire population while the selective medium estimates the healthy portion of cells. As enumeration is performed at intervals, and repair occurs, colonies are formed on the selective medium, and the counts increase with time. This has mainly been demonstrated in bacteria such as *Campylobacter jejuni*, *Escherichia coli*, *Salmonella* and *Listeria monocytogenes* (Wesche *et al.* 2009); no reports have been found for assessment of repair in lactic acid bacteria and bifidobacteria.

Different stress conditions can affect cells in different ways, and the effects would usually be identified by certain methods. For the purpose of this study where different stress conditions were being studied, it was considered more practical to use an effect which was more universal irrespective of the type of stress factor, and which could be assessed using minimal resources. Therefore, the effect of stress on growth was selected as the criterion for determining stress in cells. A similar technique was carried out by McMahon *et al.* (2007b) to determine sublethal stress conditions of NaCl, pH and temperature stresses for *Salmonella*, *Staphylococcus aureus* and *E. coli*. Different NaCl concentrations, pH values and temperatures were assessed and growth was monitored for 24 hours by optical density readings at 600 nm (OD_{600}). The sublethal stress levels of each stressor were determined as that at which a 75% reduction in OD_{600} of stressed cultures was observed, compared to that of the unstressed (control) cultures.

In this present study, survival under low pH was assessed at pH values of 2, 3, and 4 for up to 3 hours, and then 24 hours. Similar conditions have been used in various other studies to assess acid tolerance of lactobacilli and bifidobacteria, such as Prasad *et al.* (1998), Collado and Sanz (2007), Bao *et al.* (2010), Jia *et al.* (2010) and Faye *et al.* (2012). Assessing survival up to 3 hours is reflective of the time spent by food in the stomach (Maragkoudakis *et al.* 2006). Food transit time through the stomach is at least 1.5 hours (Chou and Weimer 1999). Studying higher pH (i.e. pH 4) is reflective of the increase in pH that could occur in the stomach due to ingesting a meal, or the probiotic delivery medium (e.g. yoghurt) (Vernazza *et al.* 2006a). Furthermore, fermented food products have a pH of about 4.5, in which probiotic microorganisms have to survive for long periods during refrigerated storage (Jia *et al.* 2010).

At pH 2, there were no recoverable cells after 24 hours, and hourly enumeration showed no recoverable cells even after 1 hour, indicating that it was an extreme condition (Fig. 2.1a). At pH 3, *B. animalis* ssp. *lactis* strains C and D showed better survival than *B. breve* and *B. longum* (Fig. 2.1b) and all the strains showed better survival at pH 4 (Fig. 2.1c). Sanchez *et al.* (2010a) also observed drastic loss in viability of *B. animalis* ssp. *lactis* at pH 2, and no loss in viability at pH 3. Vernazza *et al.* (2006a) reported that the *Bifidobacterium* spp. studied showed better survival at pH 3 and pH 4, while pH 2 was lethal. The *B. lactis* strain (Bb12) they studied showed good survival at all three pH values (2, 3 and 4). B. *animalis* ssp. *lactis*, which is more commercially used, has been known to show greater acid tolerance in

comparison to other non-commercial bifidobacteria (Maus and Ingham 2003), and this is consistent with the results of this study.

The acid tolerance of bifidobacteria of human origin, such as *B. breve*, *B. longum*, *B. bifidum*, *B. infantis* and *B. adolescentis* have been shown to be generally weaker in comparison to those of animal origin (Collado and Sanz 2007; Russell *et al.* 2011). The poorer acid tolerance of *B. breve* and *B. longum* in comparison to *B. animalis* ssp. *lactis* observed in this present study may be due to the lack of an inducible acid tolerance response (ATR) (Waddington *et al.* 2010).

In this study, survival in the presence of bile was assessed at bile concentrations of 0.5% and 1% (w/v). Some studies have assessed bile tolerance at 0.3% (w/v), while others have assessed it at higher concentrations, from 0.5 % (w/v) up to 1-2% (w/v) (Margolles *et al.* 2003; Maragkoudakis *et al.* 2006; Vernazza *et al.* 2006a; Pan *et al.* 2009; Bao *et al.* 2010). The average physiological concentration of bile in humans ranges between 0.3 to 0.5% (w/v) (Wu *et al.* 2010). However, this fluctuates with time, being about 1.5% to 2% (w/v) within the first hour of digestion, and decreasing to around 0.3% (w/v) (Noriega *et al.* 2004). No growth occurred in the presence of either 0.5% or 1% (w/v) bile, in this study. Growth may have occurred at lower concentrations, as shown in Margolles *et al.* (2003), but this may depend on the strain or species. *B. longum* appeared to be more sensitive to bile than *B. animalis* ssp. *lactis* and *B. breve*, as survival was observed after 24 hours in all except *B. longum* (Fig. 2.2). A similar pattern was observed in the study of Vernazza *et al.* (2006a), where the *B. longum*

strains showed undetectable counts in the presence of 0.5% (w/v) bile, in contrast to the *B. animalis* ssp. *lactis* strain, which showed higher counts.

Growth of the bifidobacteria in this present study appeared to be inhibited in the presence of higher NaCl content i.e. 3% (w/v) and above. This may imply that there was excessive movement of water from the cells into the environment (Corcoran *et al.* 2008). In this study, *B. longum* appeared to be more sensitive to osmotic conditions than *B. animalis* ssp. *lactis* and *B. breve*, since no growth after 24 hours was observed in the presence of NaCl (1% and above) (Fig. 2.3).

The lack of growth in the bifidobacteria in this study under aerobic conditions (Fig. 2.4) may confirm that they are indeed obligate anaerobes. Shaking at the speed of 200 rpm provided fully aerobic conditions, as reported by Li *et al.* (2010). However, the fact that there was no immediate reduction in numbers in the initial hours sampled may suggest that the bifidobacteria were able to tolerate oxygen to some extent. Growth of different species and strains of bifidobacteria in the presence of varying amounts of oxygen has been reported (Simpson *et al.* 2005; Xiao *et al.* 2011). *B. animalis* ssp. *lactis* has been reported to be more resistant to oxidative stress than bifidobacteria of human origin (Jayamanne and Adams 2006; Ruiz *et al.* 2012).

2.5 Conclusion

Whilst bifidobacteria may be exposed to extreme conditions in the gut, the food they are ingested with may act as a buffer against extreme pH in the stomach. Similarly, the concentration of bile reduces with time. In reality, the amount of individual stresses that probiotics are exposed to would vary with circumstances. The conditions in this study were decidedly more extreme in order to ensure a state of stress in the organisms under study.

Based on the above results, using the criteria of conditions which did not result in growth after 24 hours, and did not result in loss of viability within 1-2 hours of exposure, the selected stress treatment parameters for the subsequent experiments were as follows:

- Acid stress: pH 3 for 1 hour for *B. animalis* ssp. *lactis* strains, and pH 4 for 1 hour for *B. breve* and *B. longum*.
- Bile stress: 1% (w/v) bile for 1 hour for *B. animalis* ssp. *lactis* strains and *B. breve*, and 0.5% (w/v) bile for 1 hour for *B. longum*.
- Osmotic stress: 3% (w/v) NaCl for 1 hour for *B. animalis* ssp. *lactis* strains and *B. breve*, and 2% (w/v) NaCl for 1 hour for *B. longum*.
- Oxidative stress: Shaking at 200 rpm for 2 hours for all organisms.

CHAPTER THREE: EFFECTS OF STRESS ON ANTIMICROBIAL ACTIVITY OF BIFIDOBACTERIUM SPP.

3.1 Introduction

Antagonistic activity against pathogens is one of the desirable properties of microorganisms selected for probiotic use. Reduction of intestinal pH by production of short chain fatty acids (e.g. lactic acid, acetic acid), production of bacteriocins and other substances which are inhibitory to several intestinal pathogens, stimulation of the immune system, and competition against pathogens for nutrients and for intestinal adhesion sites, are possible mechanisms through which probiotic bifidobacteria and lactobacilli could elicit potential health benefits, such as treatment of gastrointestinal infections (Toure *et al.* 2003; Makras and De Vuyst 2006; Wohlgemuth *et al.* 2010).

Production of short chain fatty acids, i.e lactic acid and acetic acid, is suggested as the main mechanism by which bifidobacteria may inhibit enteric pathogens (Fliss *et al.* 2010). Bifidobacteria metabolise sugars via the unique fructose-6-phosphate phosphoketolase (F6PPK) pathway to produce 2 molecules of lactate and 3 molecules of acetate (Sela *et al.* 2010) (see Fig. 1.3). Inhibition of enteric bacteria may result from the lowering of pH by the organic acids, but also from the effects of the undissociated organic acid molecules. In this regard, acetic acid is considered to be a more potent antimicrobial than lactic acid, as its higher pKa value allows it to diffuse across the cell membrane at higher pH. However, the ratio of lactic to acetic acid production in bifidobacteria varies with strain/species as well as culture conditions (Fliss *et al.* 2010).

Other antimicrobial substances ascribed to bifidobacteria include bacteriocins and bacteriocin-like inhibitory substances (BLIS). Bacteriocins are low-molecular-mass proteins synthesised in the ribosomes and released

extracellualrly, which exert bacteriostatic or bactericidal effects on other bacteria (Cheikhyoussef *et al.* 2009). Bacteriocin production in lactobacilli and other lactic acid bacteria (LAB) has been well reported, but fewer reports are available for bifidobacteria (Cheikhyoussef *et al.* 2008).

Only one bacteriocin, Bifidocin B, produced by a *B. bifidum* strain, has been purified and sequenced and characterised (Yildirim *et al.* 1999). Bifidocin B has been shown to exert antimicrobial activity against food-borne pathogens and food spoilage bacteria like *Listeria*, *Enterococcus*, *Bacillus*, *Pediococcus*, *Leuconostoc* (Cheikhyoussef *et al.* 2009). Other bacteriocins from bifidobacteria which have been purified or partially purified include Bifidin and Bifilong (Cheikhyoussef *et al.* 2008; Fliss *et al.* 2010). The term BLIS mainly refers to proteinaceous inhibitory compounds obtained from bifidobacteria which have not been confirmed as bacteriocins (Fliss *et al.* 2010). Various studies have reported the presence of BLIS (Lee *et al.* 2003; Toure *et al.* 2003; Collado *et al.* 2005)

Antimicrobial activity is initially assessed *in vitro* on agar by the presence and size of inhibition zones. Assays include the agar overlay (spot test) and agar well diffusion (Tejero-Sarinena *et al.* 2012). Inhibition zones are clear zones around a bacterial colony or cell-free metabolites, where no growth of another type of bacteria is observed. Inhibition zones indicate a suppression of one type of bacteria by another (Vinderola *et al.* 2008). Potential probiotic bacteria would be desired to show inhibition zones on agar against different enteric pathogens, as this indicates a potential for health benefit. Antimicrobial activity may also be assessed by co-culture of two types of bacteria in liquid medium. A decline in the numbers of one type of bacteria in

the presence of another, may be indicative of antimicrobial/antagonistic activity of one against the other (Drago *et al.* 1997).

Bifidobacteria have been demonstrated to exhibit antimicrobial activity against various Gram negative bacteria, such as *Salmonella enterica* ser. Typhimurium, *Shigella sonnei* and *Escherichia coli* (Hutt *et al.* 2006; Makras and De Vuyst 2006) and Gram positives such as *Clostridium difficile* (Lee *et al.* 2003), *Listeria monocytogenes* (Toure *et al.* 2003), and *Staphylococcus aureus*, to a lesser extent (Lahtinen *et al.* 2007).

Whilst there is knowledge about *in vitro* antimicrobial activity of probiotic bacteria against pathogens under normal conditions, as well as the influence of culture conditions on the amounts of organic acids produced (Talwalkar and Kailasapathy 2003; Jalili *et al.* 2009; Marianelli *et al.* 2010), there are no known recorded studies about the antimicrobial activity of probiotic bacteria after exposure to sub-lethal stress conditions, particularly those encountered during gut transit. Thus this chapter examined the possible effects of the exposure of bifidobacteria to individual stress conditions on their potential for antimicrobial activity against pathogenic bacteria.

3.2 Materials and Methods

3.2.1 Bacterial cultures

Bifidobacterial cultures were namely *Bifidobacterium breve* NCTC 11815, *B. longum* NCTC 11818, *B. animalis* ssp. *lactis* strain C and *B. animalis* ssp. *lactis* strain D, as described in 2.2.1. Indicator bacteria cultures (pathogens) were made available from the Microbiology Research Unit culture collection, namely *Escherichia coli* NCTC 12900, *Salmonella enterica* ser. Typhimurium DT124 and *S. enterica* ser. Enteritidis PT4. All cultures were stored on beads in cryovials (Microbank, Pro-Lab Diagnostics UK) and kept at -20 ^{o}C. Cultures were streaked on nutrient agar and incubated aerobically for 18-24 hours at 37 ^{o}C. The resulting colonies were used for subsequent agar spot and well diffusion experiments.

3.2.2 Stress treatment

3.2.2.1 Acid stress

Colonies from 48-hour cultures on Reinforced Clostridial Agar (RCA) were suspended in 5 ml MRSc broth (MRS + 0.05% w/v L-cysteine) adjusted to pH 3 or pH 4 (using hydrochloric acid) till a turbid suspension was achieved. Suspensions were incubated under anaerobic conditions at 37 ^{o}C for 1 hour. Cells were harvested by centrifuging at 16,100 x *g* for 10 minutes and supernatant discarded. Pellets were resuspended in normal saline and adjusted to turbidity equivalent to 0.5 McFarland.

3.2.2.2 Bile stress

Colonies from 48-hour cultures on RCA were suspended in 5 ml MRSc broth containing 0.5 or 1% w/v ox-bile (B-3883; Sigma, St. Louis, USA) till a turbid suspension was achieved. Suspensions were incubated under anaerobic conditions at 37 ^{o}C for 1 hour. Cells were harvested by centrifuging at 16,100 x *g* for 10 minutes and supernatant discarded. Pellets were resuspended in normal saline and adjusted to turbidity equivalent to 0.5 McFarland.

3.2.2.3 Oxidative stress

Colonies from 48-hour cultures on RCA were suspended in 20 ml MRS broth and incubated under anaerobic conditions for 24 hours. Cells were harvested by centrifuging at 26,700 x *g* for 15 minutes and supernatant discarded. Pellets were suspended in 50 ml MRS broth contained in 250 ml conical flasks and incubated aerobically in a shaking incubator at 37 ^{o}C for 2 hours with shaking at 200 rpm. Cells were harvested by centrifuging at 16,100 x *g* for 10 minutes and supernatant discarded. Pellets were resuspended in normal saline and adjusted to turbidity equivalent to 0.5 McFarland.

3.2.2.4 Osmotic stress

Colonies from 48-hour cultures on RCA were suspended in 5 ml MRSc broth containing 2% or 3% (w/v) sodium chloride till a turbid suspension was achieved. Suspensions were incubated under anaerobic conditions at 37 ^{o}C for 1 hour. Cells were harvested by centrifuging at 16,100 x *g* for 10 minutes

and supernatant discarded. Pellets were resuspended in normal saline and adjusted to turbidity equivalent to 0.5 McFarland.

3.2.2.5 Unstressed cells

Colonies from 48-hour cultures on RCA were suspended in 5 ml unadjusted MRSc broth till a turbid suspension was achieved. Suspensions were incubated under anaerobic conditions at 37 ºC for 1 hour. Cells were harvested by centrifuging at 16,100 x *g* for 10 minutes and supernatant discarded. Pellets were resuspended in normal saline and adjusted to turbidity equivalent to 0.5 McFarland.

3.2.3 Agar overlay (spot test)

A 2 μl aliquot of each suspension of bifidobacteria (stressed or unstressed) was spotted on RCA (up to four spots per plate, in duplicate). Plates were left to dry at room temperature for 30 minutes and afterwards incubated at 37 ˚C for 18-24 h under anaerobic conditions. Resulting colonies were overlaid with 10 ml soft agar (tryptone soya broth (CM 0129, Oxoid) + 0.8% w/v agar (LP 0013, Oxoid) at 45 ˚C which were seeded with 100 μl of suspensions (adjusted to 0.5 McFarland turbidity) of overnight cultures of indicator bacteria. Overlaid plates were incubated aerobically at 37 ˚C for 18-24 hours. Inhibition zones were measured and recorded in mm by subtracting the diameter of the colony from the diameter of the entire halo (Fig 3.1). Eight measurements from two experiments were taken and the means calculated.

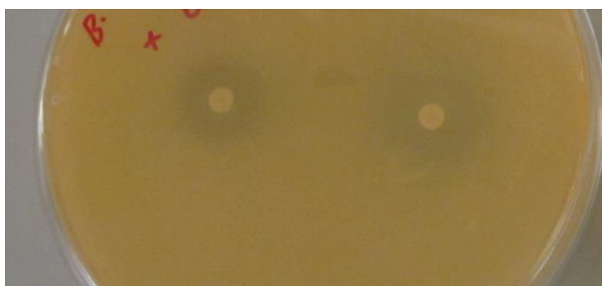

Fig. 3.1 A typical agar overlay (spot test) showing inhibition zones around bifidobacterial colonies

3.2.4 Agar well diffusion assay

A 200 µl volume of each stressed or unstressed bifidobacterial suspension was inoculated into 20 ml of Reinforced Clostridial Medium (RCM) (CM 0149; Oxoid, Hampshire, UK) and incubated under anaerobic conditions for 24 hours. The overnight cultures were centrifuged at 26,700 x *g* for 15 minutes and the supernatants were separated from the pellets and sterilised through a Nalgene 0.2 µm pore-size syringe filter (Thermo Scientific, UK). The sterile supernatants were concentrated to approximately one-twentieth of the original volume, using a vacuum evaporator system (Buchi UK Ltd), and used for the agar well diffusion assay. The supernatants were concentrated because Toure *et al.* (2003) reported a failure of unconcentrated supernatants of bifidobacteria to produce inhibition, which was recovered by concentration of the supernatants. Twenty ml volumes of soft TSA (containing 0.8% w/v agar) were seeded with 200 µl of suspensions (adjusted to 0.5 McFarland turbidity) of overnight cultures of indicator bacteria, poured into sterile Petri dishes and allowed to solidify at 5 ˚C for 2 hours. Wells of 7 mm diameter were punched into the solidified agar and

filled with 100 µl of the concentrated sterile supernatants. The plates were kept at 5 ˚C for 2 hours to allow diffusion of the tested supernatants and then incubated aerobically at 37 ˚C for 18-24 hours. Inhibition zones were measured and recorded in mm by subtracting the diameter of the well from the diameter of the entire halo (Fig. 3.2). Six measurements from two experiments were taken and the means calculated.

Fig. 3.2 A typical agar well diffusion assay showing an inhibition zone around a well

3.2.5 Study of acidification rate

Two ml volumes of each 0.5 McFarland turbidity adjusted bifidobacterial suspension (unstressed, acid, bile and osmotically stressed) were inoculated into 200 ml of RCM in sterile magnetically-stirred 300 ml water jacketed batch fermentation vessels (Soham Scientific, UK) and incubated under anaerobic conditions at 37 ˚C. The temperature was controlled by means of a circulating water bath set at 37 ˚C. The pH of each vessel was monitored by pH electrodes inserted into the vessels (FerMac 260 pH Control, Electrolab, UK), which were connected to a data logger (eLogger, Electrolab, UK). Acidification was monitored over a 48 hour period, with data logged at

regular intervals. These experiments were carried out in three replicates and mean pH at each interval was calculated.

3.2.6 Enumeration of pathogens in co-culture with bifidobacteria

Twenty ml volumes of RCM were inoculated with 200 µl of suspensions (0.5 McFarland turbidity) of overnight cultures of *E. coli* NCTC 12900 or *Salmonella enterica* ser. Typhimurium DT124, either on their own, or with 200 µl of suspensions (adjusted to 0.5 McFarland turbidity) of each of the bifidobacteria of different stress exposure treatments (unstressed, acid, bile, osmotic), and incubated under anaerobic conditions at 37 ˚C. These experiments were carried out in duplicate. Enumeration of the pathogens was done on Violet Red Bile Glucose (VRBG) agar (Oxoid CM1082). VRBG plates were incubated at 37 ˚C aerobically for 18-24 hours.

3.2.7 Statistical analysis

Data from the agar overlay and agar well diffusion assay were analysed by Student's t-test to compare unstressed and stressed data groups, using Microsoft Excel 2007. Statistical significance was set at $P \leq 0.05$.

3.3 Results

3.3.1 Antimicrobial activities by agar overlay

Fig. 3.3 shows comparisons of antimicrobial activities against the indicator organisms by the agar overlay, for the tested bifidobacteria after prior exposure to stress conditions. Antimicrobial activity was quantified by the diameters of the inhibition zones (mm). Overall, unstressed *B. breve* and *B. longum* showed larger inhibition zones than both *B. animalis* ssp. *lactis* strains. Also, inhibitory activities of all four bifidobacteria were maintained after exposure to stress. *B. breve* and *B. longum* exposed to acid stress showed significantly smaller inhibition zones for all three indicator bacteria.

Acid-stressed *B. animalis* ssp. *lactis* (C) showed no significant difference in inhibition zone sizes, while acid-stressed *B. animalis* ssp. *lactis* (D) showed significantly smaller inhibition zones for *E. coli* and *S.* Enteritidis (Fig. 3.3a).

Bile-stressed *B. breve* showed smaller inhibition zones for all three indicator bacteria, while bile-stressed *B. longum* showed significantly smaller inhibition zones for *E. coli* and *S.* Typhimurium. Bile-stressed *B. animalis* ssp. *lactis* (C) showed larger inhibition zones, but only significantly for *S.* Typhimurium, while bile-stressed *B. animalis* ssp. *lactis* (D) showed significantly smaller inhibition zones for both salmonellae, and no significant difference for *E. coli* (Fig. 3.3b).

Osmotically stressed *B. animalis* ssp. *lactis* C and D showed significantly smaller inhibition zones for *S.* Typhimurium and *E. coli* respectively. Significantly smaller inhibition zones for both salmonellae were observed by osmotically stressed *B. breve* and *B. longum* (Fig. 3.3c).

Oxidatively stressed *B. animalis* ssp. *lactis* (C) and *B. longum* showed significantly smaller inhibition zones for all three indicator bacteria. *B. breve* exposed to oxidative stress showed significantly smaller inhibition zones for both salmonellae, and *B. animalis* ssp. *lactis* exposed to oxidative stress showed significantly larger inhibition for *S.* Typhimurium, with no significant difference for the other two indicator bacteria (Fig. 3.3d).

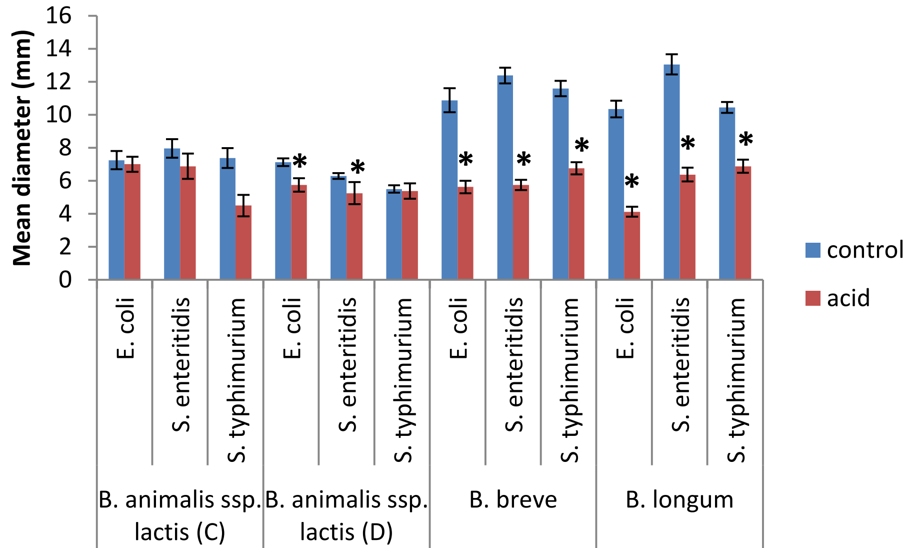

Fig. 3.3a Inhibition zones (mm) of bifidobacteria against indicator organisms by agar overlay after exposure to acid stress. Error bars represent standard error of the mean (SEM). Asterisks represent significant differences ($P \leq 0.05$)

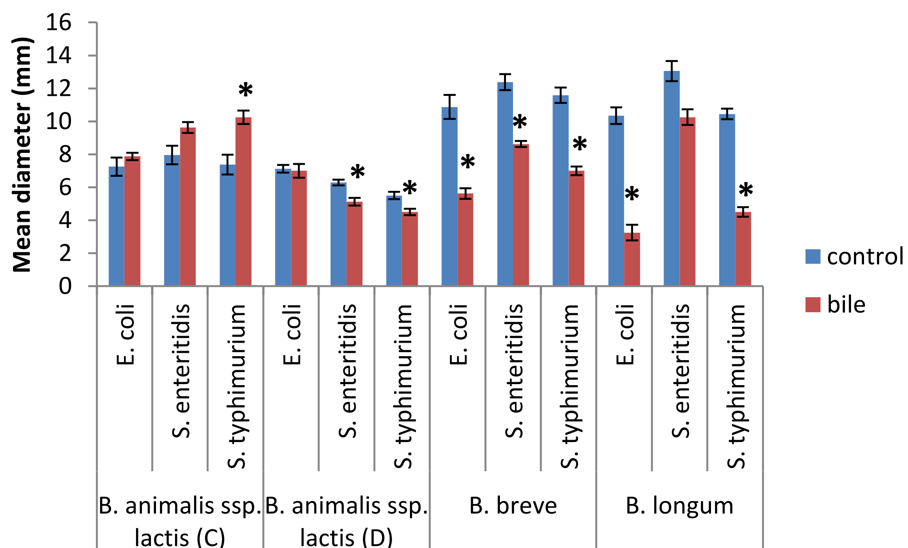

Fig. 3.3b Inhibition zones (mm) of bifidobacteria against indicator organisms by agar overlay after exposure to bile stress. Error bars represent standard error of the mean (SEM). Asterisks represent significant differences ($P \leq 0.05$)

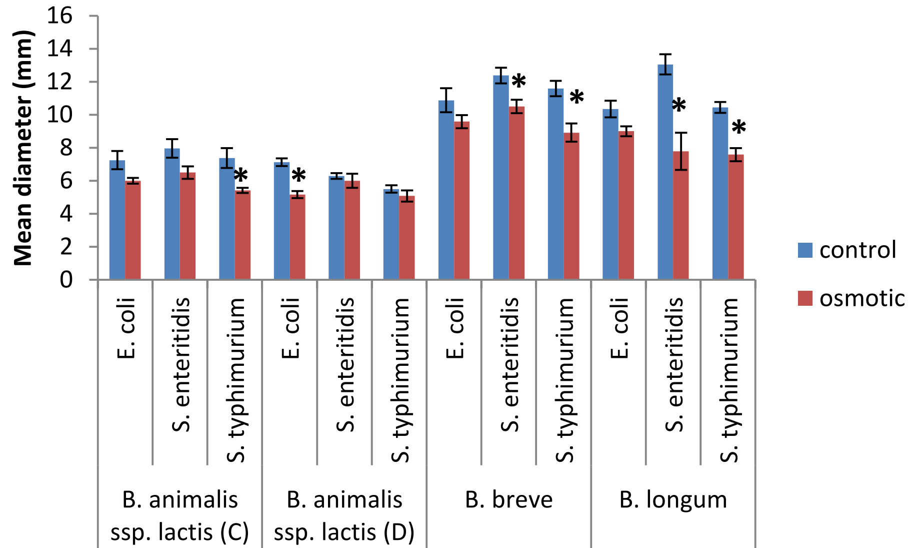

Fig. 3.3c Inhibition zones (mm) of bifidobacteria against indicator organisms by agar overlay after exposure to osmotic stress. Error bars represent standard error of the mean (SEM). Asterisks represent significant differences (*P* ≤ 0.05)

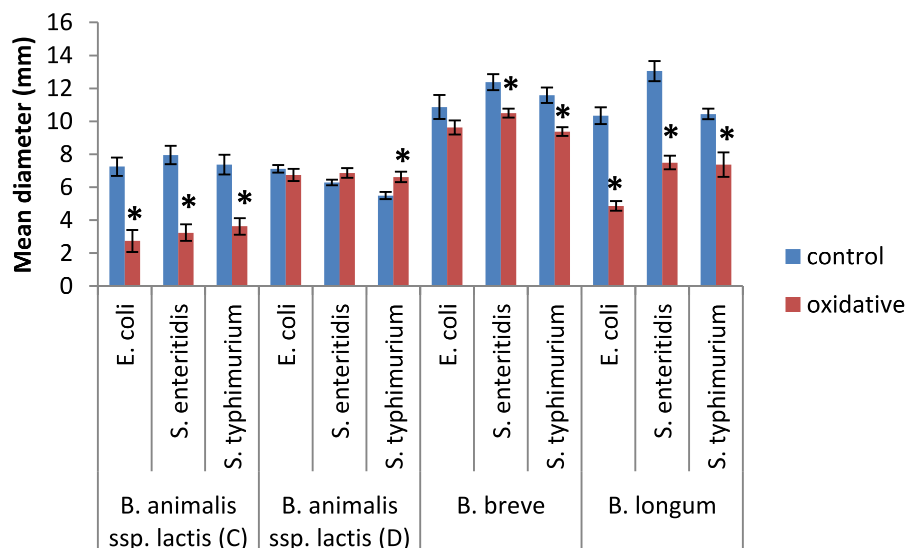

Fig. 3.3d Inhibition zones (mm) of bifidobacteria against indicator organisms by agar overlay after exposure to oxidative stress. Error bars represent standard error of the mean (SEM). Asterisks represent significant differences (*P* ≤ 0.05)

3.3.2 Antimicrobial activities assessed by agar well diffusion

To further examine whether exposure to stress had an effect on the inhibitory substances released into the medium, antimicrobial activities of cell-free supernatants were assessed by agar well diffusion. Fig. 3.4 shows comparisons of antimicrobial activities against the indicator organisms between the stressed and unstressed bifidobacteria, by the agar well diffusion assay. Antimicrobial activity was quantified by the diameters of the inhibition zones (mm). As observed in the agar spot test, supernatants of unstressed *B. breve* and *B. longum* showed larger inhibition zones than both *B. animalis* ssp. *lactis* strains. Also, inhibitory activities of all four bifidobacteria were maintained after exposure to stress.

The supernatants of acid-stressed *B. animalis* ssp. *lactis* (C), *B. breve* and *B. longum* showed significantly smaller inhibition zones than supernatants from unstressed cells, for all three indicator bacteria, while that for acid-stressed *B. animalis* ssp. *lactis* (D) showed significantly smaller inhibition for only *S. enteritidis* (Fig. 3.4a).

Bile-stressed *B. breve* and *B. longum* supernatants showed significantly smaller inhibition for all indicator bacteria, while that for *B. animalis* ssp. *lactis* (D) showed significantly smaller inhibition for *S.* Enteritidis only. No significant differences in inhibition were observed for by supernatants of bile-stressed *B. animalis* ssp. *lactis* (C) (Fig. 3.4b).

No significant differences in inhibition by supernatants of osmotically stressed bifidobacteria were observed except in those of *B. longum* supernatants, where significantly smaller inhibition occurred (Fig. 3.4c).

Supernatants of *B. animalis* ssp. *lactis* (C), *B. breve* and *B. longum* exposed to oxidative stress showed significantly smaller inhibition zones for all indicator bacteria, while those for oxidatively stressed *B. animalis* ssp. *lactis* (D) showed significantly smaller inhibition for only *S.* Enteritidis (Fig 3.4d).

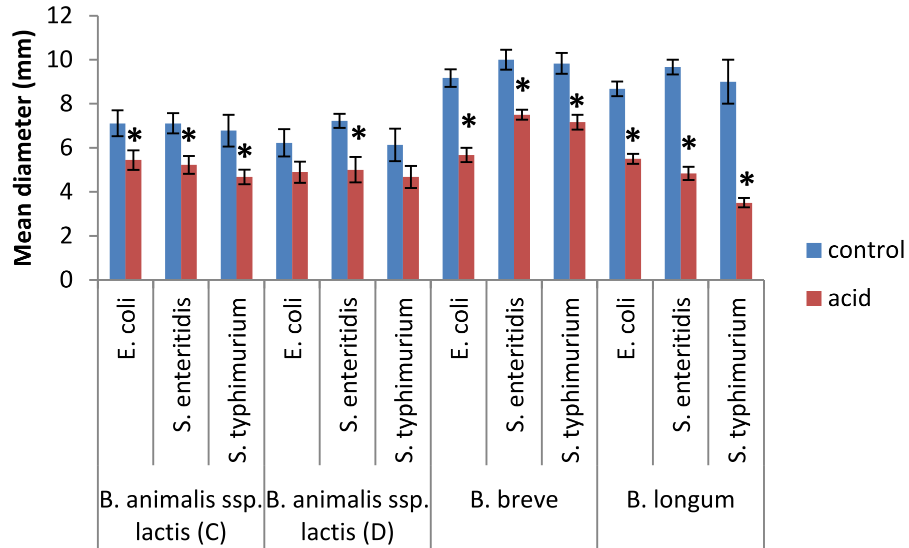

Fig. 3.4a Inhibition zones (mm) of bifidobacteria against indicator organisms by agar well diffusion after exposure to acid stress. Error bars represent standard error of the mean (SEM). Asterisks represent significant differences ($P \leq 0.05$)

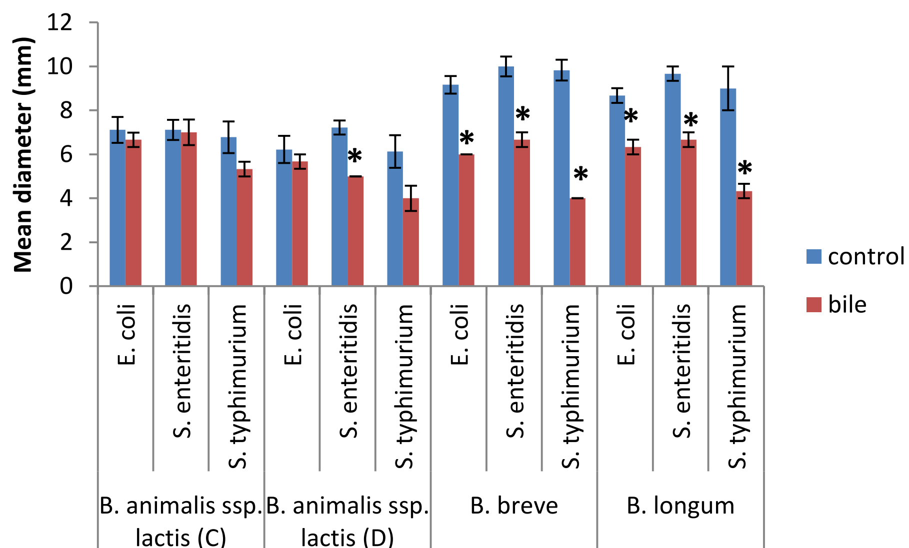

Fig. 3.4b Inhibition zones (mm) of bifidobacteria against indicator organisms by agar well diffusion after exposure to bile stress. Error bars represent standard error of the mean (SEM). Asterisks represent significant differences ($P \leq 0.05$)

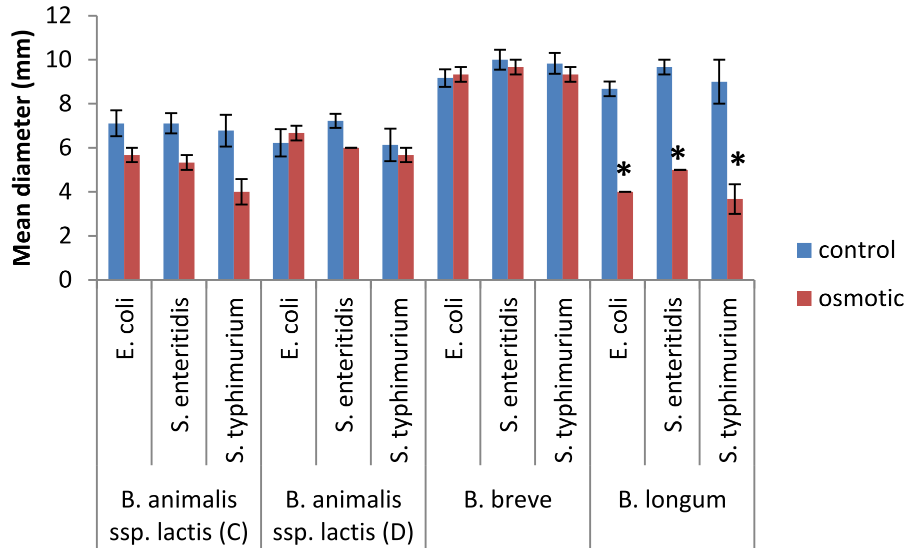

Fig. 3.4c Inhibition zones (mm) of bifidobacteria against indicator organisms by agar well diffusion after exposure to osmotic stress. Error bars represent standard error of the mean (SEM). Asterisks represent significant differences ($P \leq 0.05$)

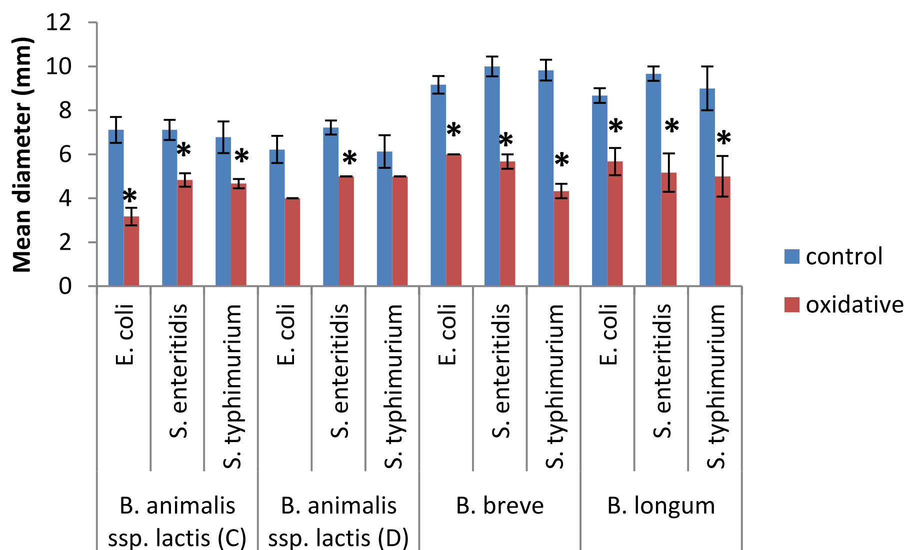

Fig. 3.4d Inhibition zones (mm) of bifidobacteria against indicator organisms by agar well diffusion after exposure to oxidative stress. Error bars represent standard error of the mean (SEM). Asterisks represent significant differences ($P \leq 0.05$)

3.3.3 Acidification rates

To examine whether reductions in inhibition zones after exposure to stress were due to impact of stress-exposure on pH reduction, acidification rates, i.e. pH reduction, by *B. animalis* (strain C), *B. breve* and *B. longum* exposed to acid, bile and osmotic stress, were monitored by a data logger. Oxidative stress was not included due to time constraint. Fig. 3.5 shows graphs for acidification of bifidobacteria of different stress treatments.

The acidification patterns of *B. animalis* ssp. *lactis* (C) (Fig. 3.5a) exposed to acid, bile and osmotic stresses appeared to be similar to the pattern of the unstressed. Acidification patterns of acid and osmotically stressed *B. breve* were also similar to the unstressed. Acidification in *B. breve* exposed to bile appeared to be considerably slower than the unstressed in the first 30 hours, after which it sped up considerably, such that the final pH at the 48 hour point was similar to the other treatments (Fig. 3.5b).

Acidification by *B. longum* exposed to acid, bile and osmotic stress appeared to be faster than the unstressed, and the final pH values at 48 hours were all considerably lower than the unstressed. Acidification by *B. longum* exposed to bile was particularly faster than other treatments (Fig. 3.5c).

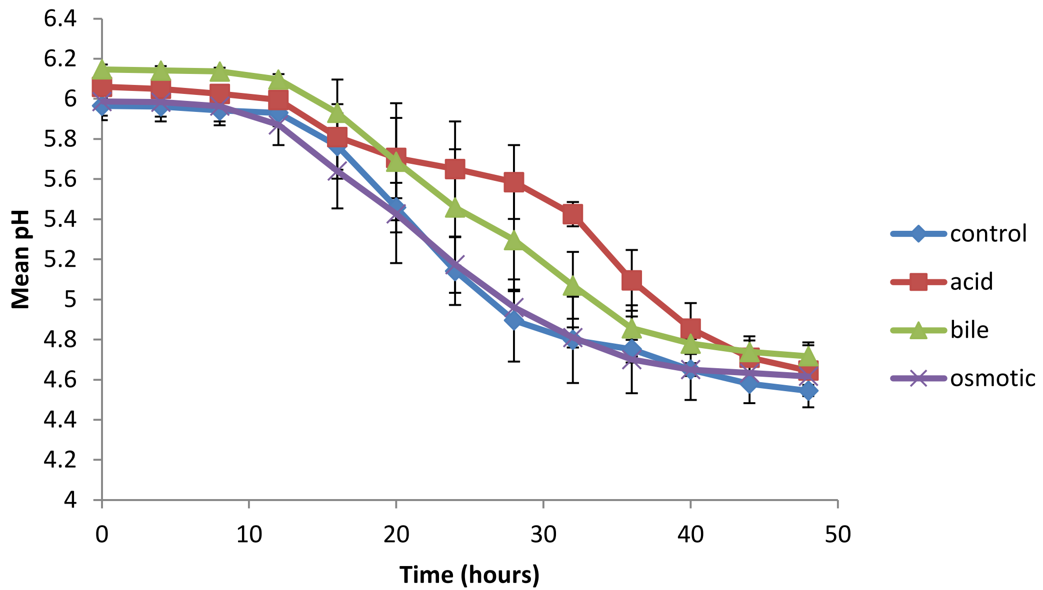

Fig. 3.5a Acidification in RCM by unstressed and stress-treated (acid, bile, osmotic) *B. animalis* subsp. *lactis* (strain C). Error bars represent standard error of the mean (SEM)

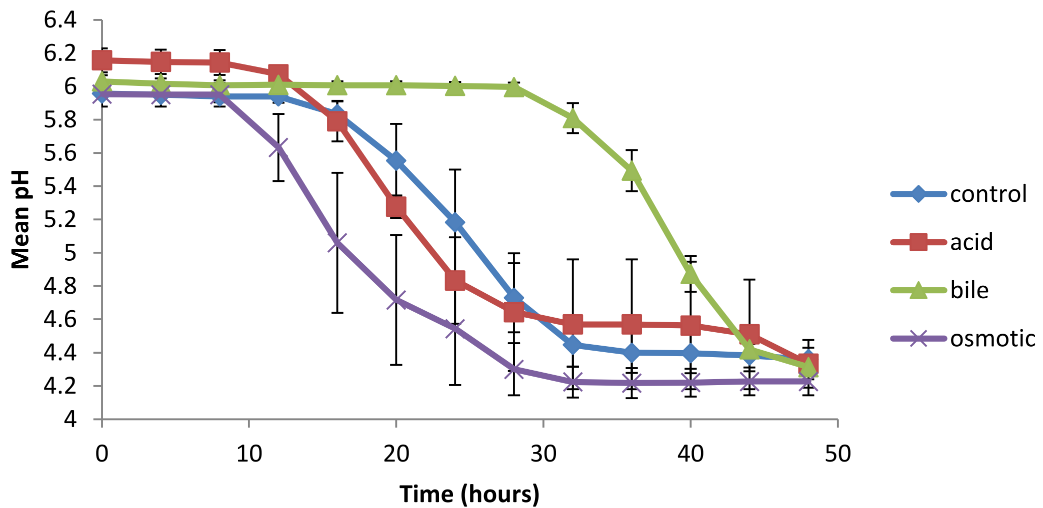

Fig. 3.5b Acidification in RCM by unstressed and stress-treated (acid, bile, osmotic) *B. breve*. Error bars represent standard error of the mean (SEM)

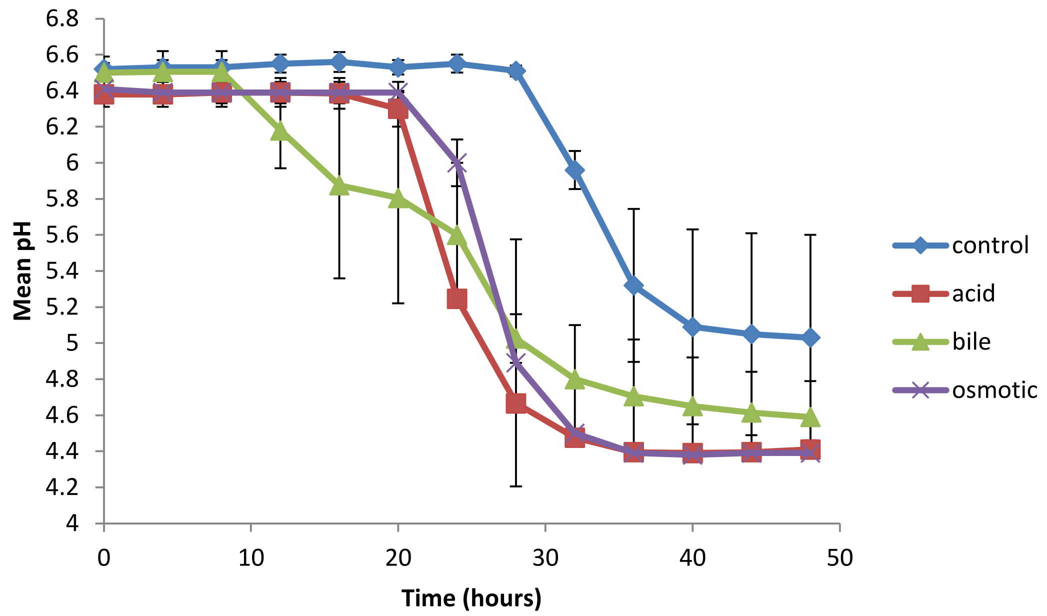

Fig. 3.5c Acidification in RCM by unstressed and stress-treated (acid, bile, osmotic) *B. longum*. Error bars represent standard error of the mean (SEM)

3.3.4 Co-culture

To further study the effect of stress on the antimicrobial activity of bifidobacteria, enumeration of two indicator organisms used in the agar spot and well diffusion assays, namely *E. coli* NCTC 12900 and *S.* Typhimurium DT124, was carried out on VRBG agar, which is selective for *Enterobacteriaceae* and does not support the growth of bifidobacteria. Both bacteria were cultured in RCM with each of the four bifidobacteria, which had been unstressed or exposed to acid, bile or osmotic stress.

Figs. 3.6 – 3.9 show the counts of *E. coli* and *S.* Typhimurium on their own and in co-culture with bifidobacteria of different stress treatments, at 0, 24 and 48 hours. There was growth of *E. coli* and *S.* Typhimurium cultured alone after 24 and 48 hours. In general, growth of *E. coli* and *S.* Typhimurium was better sustained up to 48 hours when cultured on their own than in co-culture, with few exceptions.

Growth was observed in *E. coli* co-cultured with *B. animalis* ssp. *lactis* (D) of different stress treatments after 24 hours, though less than *E. coli* cultured on its own. Counts of *E. coli* co-cultured with unstressed *B. animalis* ssp. *lactis* (D) at 24 hours were slightly lower than with stressed *B. animalis* ssp. *lactis* (D). After 48 hours, counts of *E. coli* in co-culture with *B. animalis* ssp. *lactis* (D) were lower than at the 24 hour point. Counts of *E. coli* co-cultured with acid-stressed *B. animalis* ssp. *lactis* (D) appeared to be lower in comparison to others, at the 48 hour enumeration (Fig. 3.6a).

Growth was observed in *S.* Typhimurium in co-culture with *B. animalis* ssp. *lactis* (D) of different stress treatments after 24 hours. Counts were lower

than *S.* Typhimurium cultured alone, except for counts of *S.* Typhimurium co-cultured with bile-stressed *B. animalis* ssp. *lactis* (D), which were better than even *S.* Typhimurium cultured on its own. After 48 hours, decline was observed in *S.* Typhimurium in co-culture, except for *S.* Typhimurium in co-culture with bile-stressed *B. animalis* ssp. *lactis* (D), where counts were still similar to the 24 hour enumeration. There also appeared to be greater decline in counts of *S.* Typhimurium co-cultured with acid-stressed *B. animalis* ssp. *lactis* (D), in comparison to others, at 48 hours (Fig. 3.6b).

Little growth was observed after 24 hours in *S.* Typhimurium co-cultured with *B. animalis* ssp. *lactis* (C) of different stress treatments, except for that co-cultured with osmotically-stressed *B. animalis* ssp. *lactis* (C), where better growth was observed than *S.* Typhimurium cultured on its own. After 48 hours, no further growth was observed in *S.* Typhimurium in co-culture with *B. animalis* ssp. *lactis* (C). Decline was observed in the counts of *S.* Typhimurium co-cultured with acid-stressed and bile-stressed *B. animalis* ssp. *lactis* (C), and counts of *S.* Typhimurium co-cultured with osmotically stressed *B. animalis* ssp. *lactis* (C) remained similar to the 24 hour counts, showing only slight decline (Fig. 3.7a).

Growth was observed in *E. coli* co-cultured with *B. animalis* ssp. *lactis* (C) of different stress treatments after 24 hours, but less than *E. coli* cultured on its own, with the exception of *E. coli* co-cultured with osmotically-stressed *B. animalis* ssp. *lactis* (C), which had growth similar to *E. coli* cultured alone. After 48 hours, decline was observed in counts of *E. coli* co-cultured with acid-stressed and bile-stressed *B. animalis* ssp. *lactis* (C), while little change was observed in 48-hour counts of *E. coli* co-cultured with unstressed and

osmotically-stressed *B. animalis* ssp. *lactis* (C), in comparison to 24-hour counts (Fig. 3.7b).

Decline was observed in *S.* Typhimurium co-cultured with unstressed and osmotically-stressed *B. breve* after 24 hours, while growth was observed in *S.* Typhimurium co-cultured with acid-stressed and bile-stressed *B. breve*, with better growth in the *S.* Typhimurium co-cultured with bile-stressed *B. breve*, in comparison to *S.* Typhimurium cultured on its own. After 48 hours, decline was also observed in the *S.* Typhimurium co-cultured with acid-stressed and bile-stressed *B. breve*, though counts were better for *S.* Typhimurium co-cultured with bile-stressed *B. breve* (Fig. 3.8a).

Growth was observed in *E. coli* co-cultured with *B. breve* of different stress treatments after 24 hours. Counts at 24 hours were better in co-culture with unstressed and bile-stressed *B. breve*, with counts in co-culture with bile-stressed *B. breve* being similar to *E. coli* cultured on its own. After 48 hours, decline was generally observed in *E. coli* counts, with larger decline observed in *E. coli* co-cultured with unstressed and acid-stressed *B. breve*, in comparison to *E. coli* co-cultured with osmotically-stressed *B. breve*. Slight decline was observed in *E. coli* co-cultured with bile-stressed *B. breve* (Fig. 3.8b).

Growth was observed in *S.* Typhimurium co-cultured with *B. longum* of different stress treatments after 24 hours, with the least growth observed in co-culture with acid-stressed *B. longum*. After 48 hours, large decline was observed in *S.* Typhimurium counts in co-culture with *B. longum*, with the

least decline shown by *S.* Typhimurium co-cultured with bile-stressed *B. longum* (Fig. 3.9a).

Growth was observed in *E. coli* co-cultured with *B. longum* of different stress treatments after 24 hours. Counts were similar to that of *E. coli* cultured on its own. After 48 hours, decline was observed generally, with the least and most decline observed in *E. coli* co-cultured with bile-stressed and osmotically-stressed *B. longum* respectively (Fig. 3.9b)

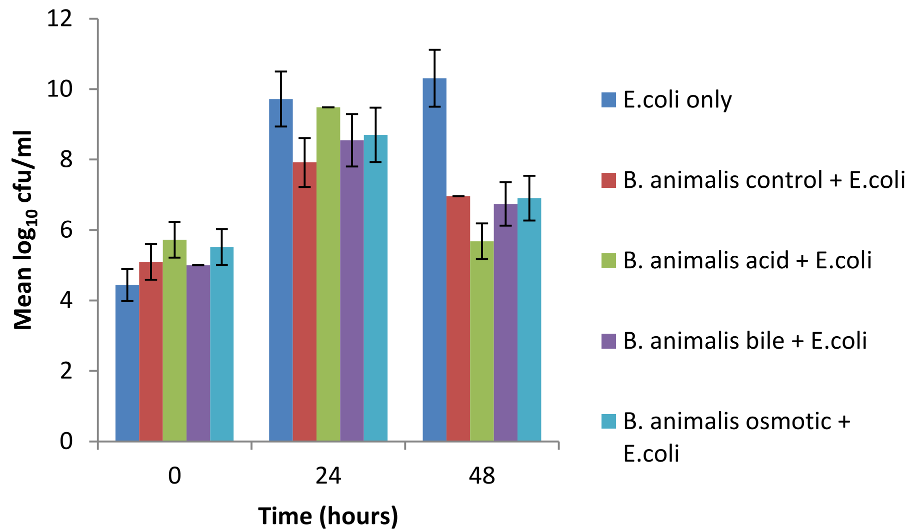

Fig. 3.6a Enumeration on VRBG agar of *E. coli* in co-culture with *B. animalis* ssp. *lactis* (strain D) treatments at 0, 24 and 48 hours. Error bars represent standard error of the mean (SEM)

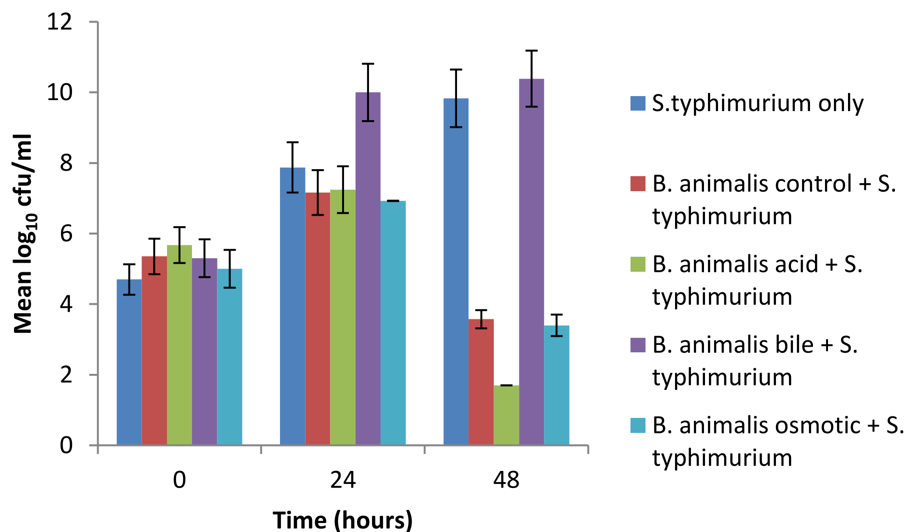

Fig. 3.6b Enumeration on VRBG agar of *S.* Typhimurium in co-culture with *B. animalis* ssp. *lactis* (strain D) treatments at 0, 24 and 48 hours. Error bars represent standard error of the mean (SEM)

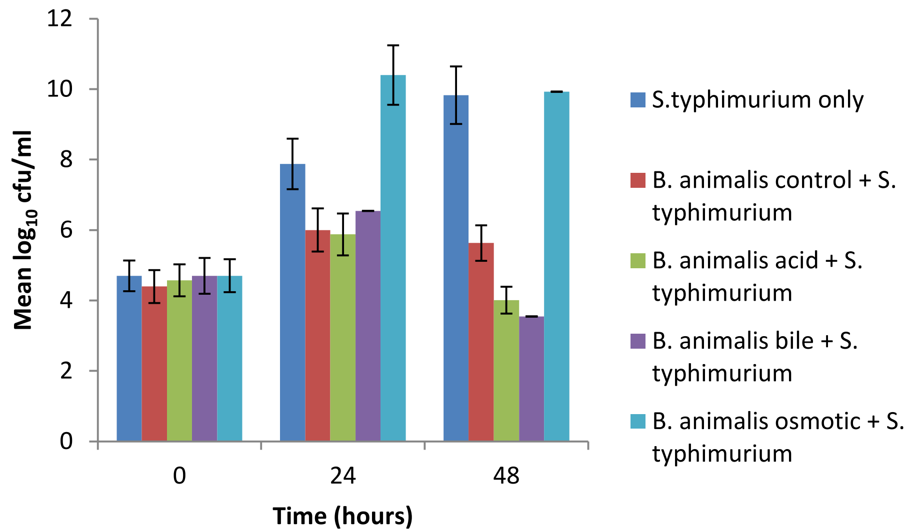

Fig. 3.7a Enumeration on VRBG agar of *S.* Typhimurium in co-culture with *B. animalis* ssp. *lactis* (strain C) treatments at 0, 24 and 48 hours. Error bars represent standard error of the mean (SEM)

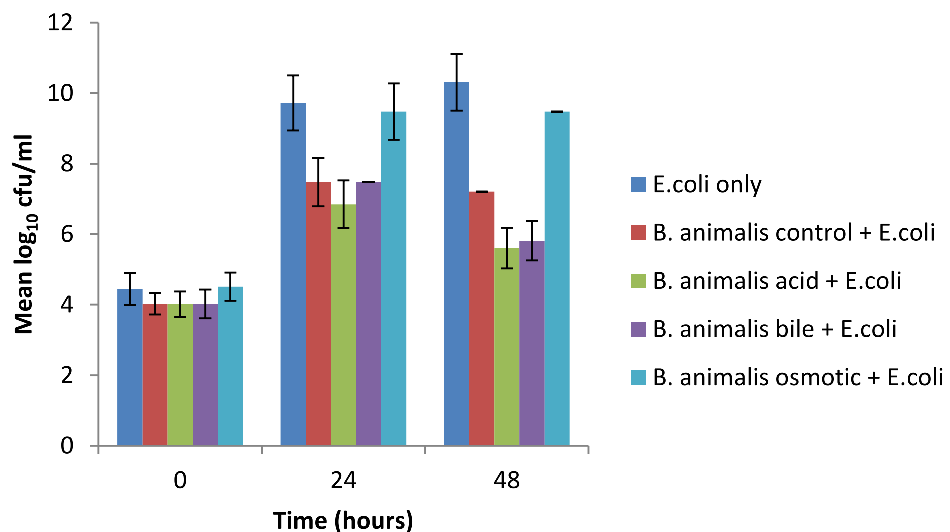

Fig. 3.7b Enumeration on VRBG agar of *E. coli* in co-culture with *B. animalis* ssp. *lactis* (strain C) treatments at 0, 24 and 48 hours. Error bars represent standard error of the mean (SEM)

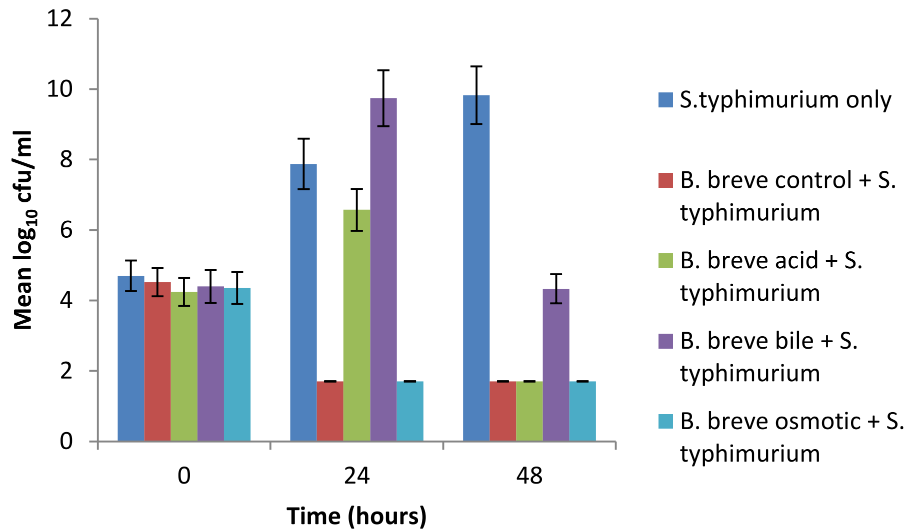

Fig. 3.8a Enumeration on VRBG agar of *S.* Typhimurium in co-culture with *B. breve* treatments at 0, 24 and 48 hours. Error bars represent standard error of the mean (SEM)

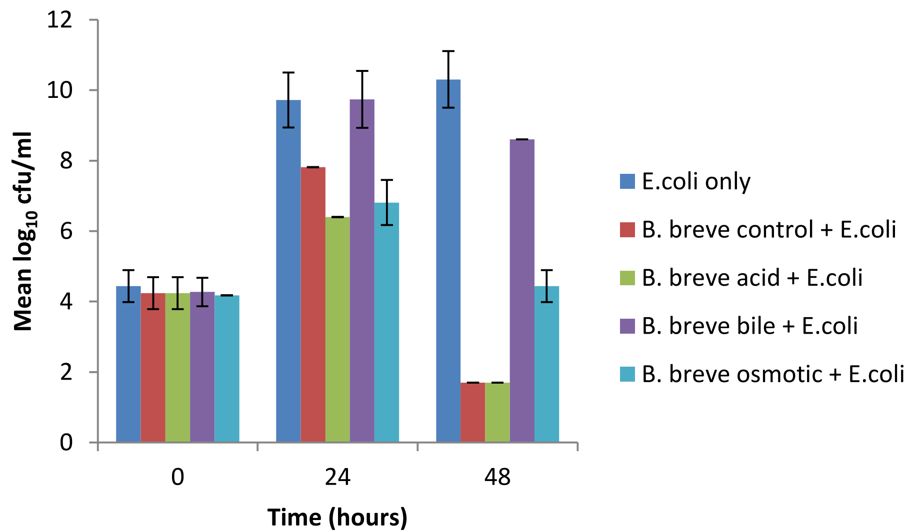

Fig. 3.8b Enumeration on VRBG agar of *E. coli* in co-culture with *B. breve* treatments at 0, 24 and 48 hours. Error bars represent standard error of the mean (SEM)

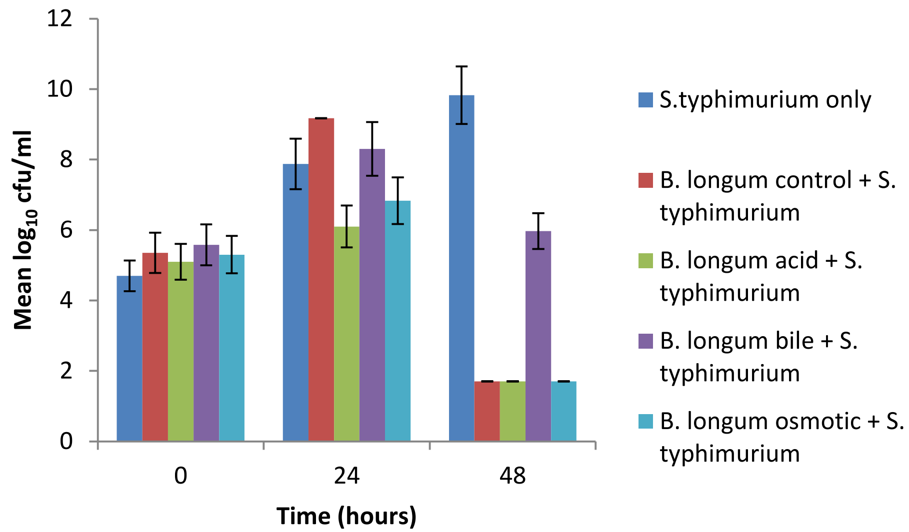

Fig. 3.9a Enumeration on VRBG agar of *S.* Typhimurium in co-culture with *B. longum* treatments at 0, 24 and 48 hours. Error bars represent standard error of the mean (SEM)

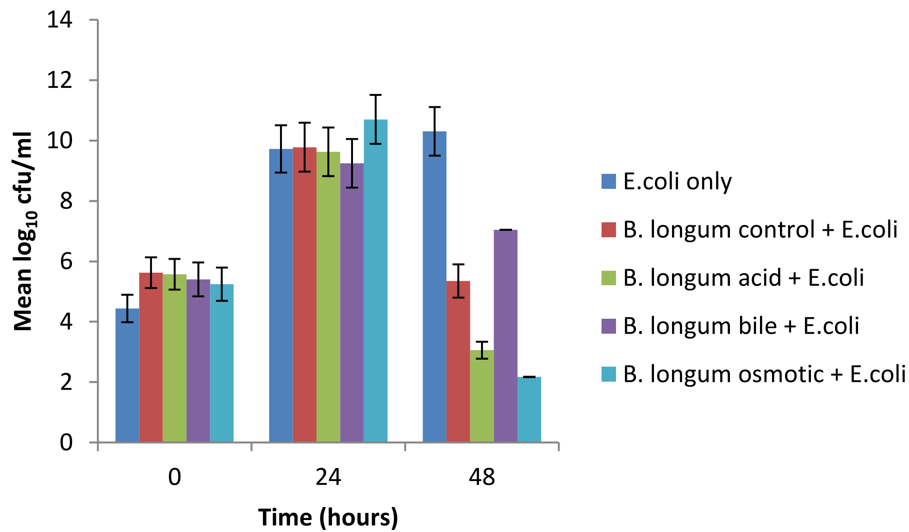

Fig. 3.9b Enumeration on VRBG agar of *E. coli* in co-culture with *B. longum* treatments at 0, 24 and 48 hours. Error bars represent standard error of the mean (SEM)

3.4 Discussion

This study was aimed at investigating the effect of exposure to stress on the *in vitro* antimicrobial activity of bifidobacteria. This was initially assessed by agar overlay (spot test), and then, agar well diffusion. Whereas the spot test involves the presence the *Bifidobacterium* colonies, and therefore, direct cell-to-cell contact with the indicator organisms, the well diffusion method involves only cell-free supernatants. It was carried out to confirm whether inhibition required direct cell contact, or if it was due to metabolites/substances released into the medium. The fact that inhibition occurred in the well diffusion method may suggest that inhibition is due to the latter.

According to the sizes of inhibition zones in the unstressed state, the antimicrobial activities of the four bifidobacteria studied could be ranked, from highest to lowest, as *B. breve*, *B. longum*, *B. animalis* ssp. *lactis* (C) and *B. animalis* ssp. *lactis* (D). This pattern was the same in both agar overlay and agar well diffusion methods. The study of Tejero-Sarinena *et al.* (2012) similarly found that the *B. breve* strain used in their study showed the most potent antagonistic effect against pathogenic bacteria, among the bifidobacteria used, although no *B. animalis* ssp. *lactis* strain was included.

It is interesting that the unstressed *B. animalis* ssp. *lactis* strains in this present study showed lower inhibitory activity than the unstressed *B. breve* and *B. longum* strains, although *B. animalis* ssp. *lactis* is considered as a strong acid producer. A possible explanation could be that it produced more lactic acid than acetic acid, as observed in the *B. animalis* ssp. *lactis* Bb12 used in the study of Hutt *et al.* (2006), and lactic acid is considered less

inhibitory than acetic acid, because acetic acid has a higher pKa, allowing it to diffuse across the cell membrane at higher pH (Fliss *et al.* 2010). Acetic acid has a low molecular weight and greater liposolubility, which allows it to penetrate bacterial membranes faster than lactic acid (Fernandez *et al.* 2009). Nonetheless, lactic acid makes the outer membrane of Gram negative bacteria more permeable to other inhibitory compounds (Makras *et al.* 2006).

As *B. animalis* ssp. *lactis* is highly used commercially, it may be suggested that one of the reasons for its wide use is that it does not produce as much acetic acid, which is known to result in off-flavours and off-odours in fermented dairy products (Margolles and Sanchez 2012). However, it is not clear whether the *B. longum* and *B. breve* strains used in this study produce lactic acid and acetic acid in different quantities to the *B. animalis* ssp. *lactis* strains. Quantities of lactic and acetic acid produced have been shown to differ with strain type (Makras and De Vuyst 2006).

In contrast to the explanation that acetic acid is more inhibitory than lactic acid, the study of Tejero-Sarinena *et al.* (2012) showed that higher production of lactic acid was correlated with greater pH reduction and consequently increased inhibition, whereas higher production of acetic acid did not influence pH and inhibition significantly. The strains of bifidobacteria used in their study, i.e. *B. longum*, *B. bifidum* and *B. breve*, all produced significantly higher amounts of lactic acid than acetic acid, except *B. infantis*, which produced more acetic acid than lactic acid, and also showed the least potent inhibitory activity against the pathogens used in the study.

Therefore it may be possible that other inhibitory substances, apart from organic acids, were produced by unstressed *B. breve* and *B. longum*, which may account for the higher inhibition in comparison to *B. animalis* ssp. *lactis* observed in this present study. However, this was not investigated. If it were investigated, it would have involved the neutralisation of the cell-free supernatants and then assessing inhibition by well diffusion. The presence of inhibition zones would be suggestive of other antimicrobial substances such as bacteriocins and BLIS (Bevilacqua *et al.* 2003). Furthermore, the contribution of other antimicrobial substances by bifidobacteria in inhibiting Gram negative pathogens, has been considered to be negligible (Makras and De Vuyst 2006). The higher inhibition observed by *B. breve* and *B. longum* in this study may simply be due to the production of more lactic acid.

In both agar overlay and agar well diffusion methods, there was inhibition of indicator organisms observed in stressed and unstressed bifidobacteria. However, in many cases, there were significantly smaller inhibition zone diameters in stressed treatments relative to the unstressed, and this may imply reduced antimicrobial activity after exposure to stress. It could be suggested that the metabolism of the cells may have been affected, such that less organic acid was produced, though it is not clear how their metabolism may be affected by stress, to then lead to change in organic acid production. It may also be plausible to suggest that exposure to stress caused extended lag phases, allowing for repair of injury, such that by the time the experiment was stopped, the amount of inhibitory substances released in the medium were not as much as in the unstressed state.

Shah and Ravula (2000) found that reduced water activity of probiotic yoghurt due to sugar (sucrose) addition (12-16%) resulted in increased fermentation times and decreased levels of acetic and lactic acid and increased pyruvic acid production (HPLC analysis). This may be an indication that stressful conditions could affect carbohydrate metabolism. Similar effects on lactic acid and acetic acid production with varying aeration (oxygen levels) were observed by Talwalkar and Kailasapathy (2003), Marianelli *et al.* (2010) and Ruiz *et al.* (2012). Furthermore, studies by Sanchez *et al.* (2005) on *B. longum* NCIMB 8809 reported up-regulation of the F6PPK enzyme activity and changes in the metabolic end products (lactate, acetate) in the presence of bile, with a reduction in acetate/lactate ratio. The implications this alteration in acid ratio may have on antimicrobial activity and its consequent effects *in vivo* are not certain.

To shed light on the impact of stress exposure on growth and metabolism rates, the acidification rate was studied. The rate at which the pH of the medium is lowered may be related to the growth/metabolism rate. In *B. animalis* ssp. *lactis* (C), the pattern of acidification did not differ much between the unstressed and stressed treatments, although the acid-stressed and bile-stressed treatments had slightly slower acidification, as the pH values were still higher at the 24-hour point. By the 48-hour point, the pH values were similar (Fig. 3.5a). The effect of stress on acidification rate was more clearly demonstrated in *B. breve*, where exposure to bile slowed down acidification considerably. Conversely, osmotic stress appeared to speed up acidification. However, by the 48-hour point as well, the pH values were similar (Fig 3.5b). In *B. longum*, the unstressed culture appeared to have a

slower acidification rate compared to the stressed, with the bile-stressed treatment showing the fastest acidification. The pH values at the 48-hour point were also similar, for the stressed treatments (Fig. 3.5c).

Relating the acidification patterns to the agar overlay and well diffusion methods, it may be suggested that the impact of stress on pH is more pronounced in the earlier stages, and this may corroborate the smaller inhibition zones observed in the well diffusion assay, since supernatants were obtained after 24 hours of growth of the stressed bifidobacteria. This may also be down to extended lag phases. However, the fact that the pH values after 48 hours were similar for stressed and unstressed treatments may suggest that there are differences in the quantities of organic acid in the medium, after exposure to stress, as corroborated by smaller inhibition zones observed in the agar overlay (spot test), where the bifidobacteria were incubated for a total of 48 hours. Lactic acid and acetic acid are both produced during the exponential phase and the stationary phase of growth of bifidobacteria, but more in the exponential phase (Jalili *et al.* 2009).

The effect of exposure to stress on the pH in the earlier stages of incubation may be further supported by results from the co-culture experiments. Although there were no consistent differences between stressed and unstressed cells, it could be noticed that there was usually growth of the *E. coli* or *S.* Typhimurium after 24 hours, and then decline after 48 hours. This may be due to the fact the probiotics have longer lag phases and generation times than *Salmonella* spp. and *E. coli*, such that they are able to grow before the bifidobacteria could express their antimicrobial activity (Marianelli *et al.* 2010). Better growth of *E. coli* or *S.* Typhimurium in co-culture with

stressed bifidobacteria, relative to those co-cultured with unstressed bifidobacteria, may suggest that the ability of the bifidobacteria to reduce pH, which consequently affects the growth of *E. coli* and *S.* Typhimurium, has been impeded, most likely due to the effect of stress on the lag phase.

Inhibition by bifidobacteria may be more due to the action of the undissociated organic acids themselves, than just the reduction in pH caused by organic acid production. Organic acids have been shown to cause more inhibition of *S.* Typhimurium than hydrochloric acid (inorganic acid) at the same pH (Makras and De Vuyst 2006). The study by Alvarez-Ordonez *et al.* (2010) showed that, from highest to lowest, the order of acids in inhibiting *S.* Typhimurium was acetic, lactic, citric, hydrochloric. Organic acids, which are weak acids, penetrate cell membranes of bacteria as undissociated molecules. They dissociate intracellularly, causing a reduction in cytoplasmic pH, thereby affecting the metabolic activities of the cell (Pan *et al.* 2009; Alvarez-Ordonez *et al.* 2010).

It may also require mentioning that RCM, which was used for growing bifidobacteria for the well diffusion experiment, and also used for the co-culture experiments, contains sodium acetate (3 g/L). Sodium acetate, as a form of acetic acid, can be inhibitory to some bacteria. This was demonstrated by De Keersmaecker *et al.* (2006), who observed 20% inhibitory activity of sterile MRS (pH 4.5) against *S.* Typhimurium. This inhibitory activity was partially attributed to the sodium acetate content (60mM) of MRS.

3.5 Conclusion

Despite the apparent reduction in antimicrobial activity that can occur upon exposure of bifidobacteria to some stresses, there was still antimicrobial activity nonetheless. Reduction in antimicrobial activity of bifidobacteria exposed to stress appears to be most likely due to extended lag phases. It is not clear whether exposure to stress may cause genetic changes that could be translated to altered antimicrobial activity. Moreover, it is possible that effects of stress on antimicrobial activity observed *in vitro* may not be translated *in vivo*. More evidence would be required from further studies, as there is still a poor understanding of the mechanisms of probiotic action. This is the first known study looking directly at the effects of exposure to stress on the specific property of antimicrobial activity. It may be possible that in the future, assessment of antimicrobial activity of known stress-tolerant bifidobacteria would be conducted with prior exposure to stress, as this may paint a more representative picture of what applies in reality.

CHAPTER FOUR: EFFECTS OF STRESS ON ANTIBIOTIC SUSCEPTIBILITY PROFILES OF BIFIDOBACTERIUM SPP.

4.1 Introduction

Antibiotics have been widely used in the treatment of bacterial infections, since the discovery of penicillin in 1928 by Alexander Fleming. However, the overuse and misuse of antibiotics has led to a problem of antibiotic resistance, where bacteria become resistant to antibiotics which they were previously susceptible to. Bacteria can be intrinsically or naturally resistant to antibiotics, whereby the antibiotic targets are absent in the bacterial species, or there is low cell permeability, the presence of efflux mechanisms, or inactivation of the antibiotics. Some bacteria may acquire antibiotic resistance genes, thus contributing to the spread of the resistance problem (Saarela *et al.* 2000; Ammor *et al.* 2008b; EFSA 2008).

Bacteria which may be potentially used as probiotics are usually screened for the presence of antibiotic resistance genes, and the potential for spread to other bacteria, particularly pathogens. The fact that probiotic bacteria are added to various products makes them a potential source for the spread of antibiotic resistance genes (D'Aimmo *et al.* 2007).

Sensitivity to antibiotics may be considered as a desirable feature for probiotic microorganisms, as it is considered a safety concern, should they possess antibiotic resistance genes. Resistance genes are of concern when they are carried on mobile genetic elements (plasmids), which may be transferred between bacteria via horizontal gene transfer mechanisms such as conjugation, transduction and transformation. Intrinsic resistance is unlikely to be transmitted (Zhou *et al.* 2005; Ammor *et al.* 2008b; Gueimonde *et al.* 2010).

Antibiotic therapy has been known to disrupt the intestinal microbial balance, leading to antibiotic-associated diarrhoea. Probiotics with intrinsic antibiotic resistance may be useful for treatment of such conditions (Zhou *et al.* 2005; Hammad and Shimamoto 2010). Probiotics may be co-administered with antibiotics during treatment of intestinal infections, to prevent or alleviate antibiotic-associated diarrhoea and other related gastrointestinal symptoms, by restoring the intestinal microbial balance (Katz 2006; Saarela *et al.* 2007). Tolerance of probiotic microorganisms to antibiotics can be considered a useful trait in this regard, as the organisms would not be affected by antibiotic therapy (Yazid *et al.* 2000; Masco *et al.* 2006; Vernazza *et al.* 2006b).

The susceptibility or resistance of bacteria to antibiotics can be established qualitatively or quantitatively. Qualitative determination involves diffusion of the antibiotic from a disc into agar. Quantitative assessment is by the determination of the minimum inhibitory concentration (MIC). This can be determined by a series of two-fold dilutions of the antibiotic in broth or agar. The MIC is the lowest concentration of an antibiotic that can inhibit microbial growth (Masco *et al.* 2006; EFSA 2008).

Sub-lethal environmental stress has been demonstrated to alter antibiotic resistance in some food-related pathogens such as *Escherichia coli, Staphylococcus aureus* and *Cronobacter sakazakii* (McMahon *et al.* 2007b; Huang *et al.* 2009; Al-Nabulsi *et al.* 2011). The stress conditions bifidobacteria are exposed to may alter their physiological properties, as well as antibiotic susceptibility patterns. However, this has not been widely studied. Some studies on the effects of acid and bile on antibiotic

susceptibility patterns of lactobacilli have been documented (Charteris *et al.* 2000; Elkins and Mullis 2004; Kheadr 2006).

Studying possible modifications in susceptibility and resistance patterns due to exposure to stress would be useful in selecting probiotic microorganisms for prophylactic use (Kheadr *et al.* 2007). Therefore, this chapter examined whether exposure to stress conditions can affect the antibiotic resistance/susceptibility profiles of the bifidobacteria under study.

4.2 Materials and Methods

4.2.1 Bacterial cultures

Bifidobacterial cultures were namely *Bifidobacterium breve* NCTC 11815, *B. longum* NCTC 11818, *B. animalis* ssp. *lactis* strain C and *B. animalis* ssp. *lactis* strain D, as described in 2.2.1.

4.2.2 Stress treatment

The bifidobacteria (*B. animalis* ssp. *lactis* C and D, *B. breve* and *B. longum*) were exposed to acid, bile, osmotic and oxidative stress as described in 3.2.2.1 – 3.2.2.4. Unstressed cells were prepared as described in 3.2.2.5.

4.2.3 Determination of minimum inhibitory concentration (MIC)

Two-fold (doubling) dilutions of antibiotics were prepared in Reinforced Clostridial Agar (RCA) using guidelines by the National Committee for Clinical Laboratory Standards (NCCLS 2000). Antibiotics (Sigma) and their concentrations used included tetracycline (0.25 – 64 µg/ml), chloramphenicol (0.3125 – 2 µg/ml), ampicillin (0.3125 – 2 µg/ml), vancomycin (0.3125 – 2 µg/ml) and erythromycin (0.3125 – 2 µg/ml). Stock solutions of the antibiotics, at concentration of 1280 µg/ml, were prepared in appropriate solvents (detail below) and the dilution series were prepared in sterile distilled water. Stock solutions of tetracycline (tetracycline hydrochloride), ampicillin (sodium salt) and vancomycin (vancomycin hydrochloride) were prepared in sterile distilled water, while stock solutions of erythromycin and chloramphenicol were prepared in ethanol. The subsequent dilution series were prepared in sterile

universal bottles, to concentrations ten times the desired final concentration in agar. Sterilized RCA was allowed to reach 55 °C in a water bath and 1 ml of each solution from the dilution series was added to 9 ml of molten agar and poured into sterile petri dishes, i.e. 1:10 dilution to reach desired final concentration in agar. The control plates contained no antibiotic. All agar plates were allowed to set at room temperature, and kept refrigerated until time of use (maximum two weeks).

The bacterial suspensions (0.5 McFarland turbidity) were diluted 1:10 in normal saline, to achieve a final concentration of approximately 10^7 cfu/ml. A 2 µl aliquot of each suspension was spotted on each agar dilution plate. The plates were allowed to dry at room temperature for 30 minutes and then incubated under anaerobic conditions at 37 °C for 48 hours. Experiments were carried out in duplicate. The minimum inhibitory concentration (µg/ml) was considered as the lowest concentration of each antibiotic that completely inhibited growth.

The bifidobacteria were classified as resistant or susceptible to the antibiotics studied, according to the microbiological breakpoints (cut-off values) defined by EFSA (2008). Breakpoints are defined by studying the distribution of MICs of an antibiotic within bacterial populations of a single genus or species. Parts of the population which deviate from the normal susceptible populations can be classified as resistant. This can be useful in identifying strains possessing acquired antibiotic resistance genes (Ammor *et al.* 2008b). Organisms were classified as susceptible to an antibiotic when inhibition occurred at breakpoint level of the specific antibiotic, and classified as resistant to an antibiotic when not inhibited at breakpoint level of the

antibiotic. Table 4.1 shows the EFSA (2008) microbiological breakpoints of the five antibiotics used in this study for *Bifidobacterium* spp.

Table 4.1 Microbiological breakpoints for *Bifidobacterium* spp.

Antibiotic	**Breakpoint (µg/ml)**
Ampicillin	2
Vancomycin	2
Erythromycin	0.5
Tetracycline	8
Chloramphenicol	4

4.2.4 Survival in the presence of antibiotics

Twenty ml of RCM containing tetracycline or chloramphenicol was inoculated with 200 µl aliquots of suspensions (0.5 McFarland turbidity) of *B. breve* or *B. animalis* ssp. *lactis* (strain C). *B. breve* suspensions were inoculated into RCM of 1 µg/ml tetracycline and 2 µg/ml chloramphenicol. *B. animalis* ssp. *lactis* (strain C) suspensions were inoculated into RCM of 32 µg/ml tetracycline and 2 µg/ml chloramphenicol. These were carried out in duplicate. Enumeration was done on RCA at 0, 3, 6, 24 and 48 hours. RCA plates were incubated under anaerobic conditions at 37°C for 48 hours.

4.2.5 Expression of tetracycline resistance gene *tet*(W) in *B. animalis* ssp. *lactis* (C) by quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Colonies from 48-hour cultures of *B. animalis* ssp. *lactis* (strain C) on RCA were suspended in 5 ml MRSc broth adjusted to acid, bile or osmotic stress conditions, i.e. pH 3, 1% (w/v) bile or 3% (w/v) NaCl, and 5 ml unadjusted MRSc broth as control. Suspensions were incubated under anaerobic conditions at 37 ^{o}C for 1 hour. Cells were harvested by centrifuging at 26,700 x *g* for 15 minutes and the supernatant discarded. Pellets were resuspended in normal saline and adjusted to turbidity equivalent to 2 McFarland (approximately 6 x 10^8 cfu/ml). The final volume of each suspension was 10 ml. Suspensions were stabilized using Qiagen RNAprotect Bacteria Reagent (Qiagen, UK) and RNA was then extracted by enzymatic lysis and mechanical disruption and purified using the Qiagen RNeasy Protect Bacteria Mini Kit, following the Qiagen RNAprotect Bacteria Reagent Handbook protocols. RNA integrity was assessed using the Agilent Bioanalyzer (Agilent, UK). RNA integrity number (RIN) > 9.3 for all samples indicated high RNA integrity. RNA purity and concentration were measured using a NanoDrop spectrophotometer (Thermo Scientific, UK). The A260/280 ratio of >2 for all samples suggested high purity and A260/230 ratio of >1.8 for all samples suggested high RNA concentration. Reverse transcription of RNA into complementary DNA (cDNA) with removal of genomic DNA (gDNA) contamination was carried out using the Qiagen QuantiTect Reverse Transcription Kit, following the QuantiTect Reverse Transcription Handbook protocol. Incubation was done using a Rotor-Gene 6000 cycler (Qiagen, UK).

In the quantitative polymerase chain reaction (qPCR), primers (Table 4.2) were used to amplify the tetracycline resistance gene *tet*(W) and reference genes *atp*D, *tuf*A and *ldh*. The expression of the gene in *B.* animalis ssp. *lactis* only was studied because resistance to tetracycline was only displayed by *B. animalis* ssp. *lactis* C and D used in this study (see results). Due to time and cost, it was not feasible to study the expression in both strains, therefore only strain C was selected, as there was a higher MIC of tetracycline against it, compared to strain D (see results). The *tet*(W) primers used for *B. animalis* ssp. *lactis* were as described in Gueimonde *et al.* (2010) and supplied by Sigma-Aldrich, UK. For accurate gene quantification, the expression of the gene of interest, i.e. *tet*(W), was normalised to the expression of the reference genes, whose expression does not change under the various experimental conditions. The reference genes were as used by Foroni *et al.* (2011). The stability of these reference genes was assessed using the geNorm software. Reference gene primers were designed by qStandard Ltd, UK. Two µl of cDNA were amplified in a 10 µl reaction using the Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent UK) with each primer at a final concentration of 500 nmol/L. Amplification was carried out in a Rotor-Gene 6000 cycler, under the following conditions: 1 cycle of 95 °C for 3 min followed by 40 cycles of 95 °C for 5 sec and 57 °C for 1 sec. Quantitative PCR was carried out in three replicates, and mean number of normalised copies per reaction was calculated.

Table 4.2 Primer sequences

Gene	Forward primer	Reverse primer
tet(W)	5'-GCCCGGCCACATGGAT-3'	5'-GCCCCATCTAAAACAGCCAAA-3'
*atp*D	5'-CTCCACCTCGCGAATCCT-3'	5'-GAAGTTCTGGCCGAGGAAC-3'
*tuf*A	5'-GAGTACGACTTCAACCAGATCG-3'	5'-ATGTTCTTCACGAAGTCGGC-3'
ldh	5'-CCGACATGGTCGTCATCAC-3'	5'-GGGTTGGTGATGAGCATGTA-3'

4.2.6 Statistical analysis

Gene expression data were analysed by Student's t-test to compare unstressed and stressed data groups, using Microsoft Excel 2007. Statistical significance was set at $P \leq 0.05$.

4.3 Results

4.3.1 MIC determination

The MICs of five antibiotics against bifidobacteria after exposure to acid, bile, osmotic and oxidative stress were determined by agar dilution, and compared against the MICs of the controls. Table 4.3 shows a summary of the MICs of the antibiotics used for the organisms and treatments tested, with indications of resistance (R) or susceptibility (S). All four bifidobacteria were sensitive to chloramphenicol, erythromycin, ampicillin and vancomycin. *B. breve* and *B. longum* were sensitive to tetracycline, while both *B. animalis* ssp. *lactis* strains were resistant to tetracycline.

Overall there were few differences between MICs of stressed and unstressed bifidobacteria, and where differences occurred, they were only by one two-fold dilution factor (higher or lower). Also, the stressed bifidobacteria remained in the same category (S or R) as the unstressed, where differences in MIC were observed.

Differences in the MIC of chloramphenicol were only observed in *B. breve* exposed to acid, bile and oxidative stress, which appeared to have a lower MIC of chloramphenicol, in comparison to the unstressed.

Differences in the MIC of erythromycin were observed in *B. animalis* ssp. *lactis* (C) exposed to osmotic and oxidative stress, which appeared to have a lower MIC of erythromycin and *B. longum* exposed to osmotic stress, which appeared to have a higher MIC of erythromycin, in comparison to the unstressed. Lower MICs were also observed in *B. breve* exposed to acid and bile stress.

Unstressed *B. animalis* ssp. *lactis* (C) appeared to have the lowest MIC of ampicillin of all four bifidobacteria studied. Higher MICs were observed in *B. animalis* ssp. *lactis* (C) exposed to osmotic and oxidative stress, in comparison to the unstressed. Higher MICs were also observed in *B. animalis* ssp. *lactis* (D) exposed to acid and osmotic stress, and *B. breve* exposed to acid stress, in comparison to the unstressed. Unstressed *B. longum* had the highest MIC, and when exposed to bile and oxidative stress, appeared to have lower MIC of ampicillin, in comparison to the unstressed.

Vancomycin showed a higher MIC against *B. animalis* ssp. *lactis* (C) exposed to osmotic stress and lower MIC against oxidatively stressed *B. animalis* ssp. *lactis* (C). A lower MIC was observed in *B. animalis* ssp. *lactis* (D) exposed to osmotic stress, and in *B. longum* exposed to acid stress.

Unstressed *B. animalis* ssp. *lactis* (C) appeared to have the highest MIC of tetracycline of all four bifidobacteria. Lower MIC was only observed in *B. animalis* ssp. *lactis* (C) exposed to oxidative stress. *B. animalis* spp. *lactis* (D) appeared to have higher MICs after exposure to bile and osmotic stress. *B. breve* and *B. longum* showed the lowest MIC of tetracycline, and only their acid-stressed treatments showed lower MICs of tetracycline.

Table 4.3 MICs (µg/ml) of five antibiotics against (1) *B. animalis* ssp. *lactis* (C) (2) *B. animalis* ssp. *lactis* (D) (3) *B. breve* and (4) *B. longum* after exposure to acid, bile, osmotic and oxidative stress

	Chloramphenicol					Erythromycin					Ampicillin					Vancomycin					Tetracycline				
	Control	Acid	Bile	Osmotic	Oxidative	Control	Acid	Bile	Osmotic	Oxidative	Control	Acid	Bile	Osmotic	Oxidative	Control	Acid	Bile	Osmotic	Oxidative	Control	Acid	Bile	Osmotic	Oxidative
1	1 (S)	1 (S)	1 (S)	1 (S)	1 (S)	0.125 (S)	0.125 (S)	0.125 (S)	0.0625 (S)	0.0625 (S)	0.0625 (S)	0.0625 (S)	0.0625 (S)	0.125 (S)	0.125 (S)	0.5 (S)	0.5 (S)	0.5 (S)	1 (S)	0.25 (S)	32 (R)	32 (R)	32 (R)	32 (R)	16 (R)
2	1 (S)	1 (S)	1 (S)	1 (S)	1 (S)	0.125 (S)	0.125 (S)	0.125 (S)	0.125 (S)	0.125 (S)	0.125 (S)	0.25 (S)	0.125 (S)	0.25 (S)	0.125 (S)	1 (S)	1 (S)	1 (S)	0.5 (S)	1 (S)	16 (R)	16 (R)	32 (R)	32 (R)	16 (R)
3	1 (S)	0.5 (S)	0.5 (S)	1 (S)	0.5 (S)	0.125 (S)	0.0625 (S)	0.0625 (S)	0.125 (S)	0.125 (S)	0.25 (S)	0.5 (S)	0.25 (S)	0.25 (S)	0.25 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	1 (S)	0.5 (S)	1 (S)	1 (S)	1 (S)
4	1 (S)	1 (S)	1 (S)	1 (S)	1 (S)	0.125 (S)	0.125 (S)	0.125 (S)	0.25 (S)	0.125 (S)	1 (S)	1 (S)	0.5 (S)	1 (S)	0.5 (S)	1 (S)	0.5 (S)	1 (S)	1 (S)	1 (S)	1 (S)	0.5 (S)	1 (S)	1 (S)	1 (S)

4.3.2 Survival in the presence of antibiotics

To further investigate the effects of exposure to stress on the susceptibility of bifidobacteria to antibiotics, *B. animalis* ssp. *lactis* (strain C) and *B. breve* were selected for enumeration in the presence of tetracycline and chloramphenicol at concentrations ≥ the MIC values of the unstressed, after exposure to acid, bile and osmotic stress. Figs. 4.1 and 4.2 show the survival of *B. animalis* ssp. *lactis* (strain C) and *B. breve* treatments in the presence of tetracycline (Fig. 4.1a and 4.2a) and chloramphenicol (Fig. 4.1b and 4.2b).

Overall, the survival patterns of the different treatments were similar in both organisms. There appeared to be better survival in tetracycline than in chloramphenicol. However, the survival of both *B. animalis* ssp. *lactis* (strain C) and *B. breve* exposed to osmotic stress, in the presence of chloramphenicol, appeared to be better than those exposed to acid and bile, or unstressed. Also, bile-treated *B. breve* counts at Time 0 were much lower than the Time 0 counts for *B. breve* unstressed or exposed to acid and osmotic stresses. It is not clear if this was due to poor survival of exposure to bile before inoculating into the antibiotic-containing RCM, or if it was due to interaction of bile-treated *B. breve* with the antibiotics.

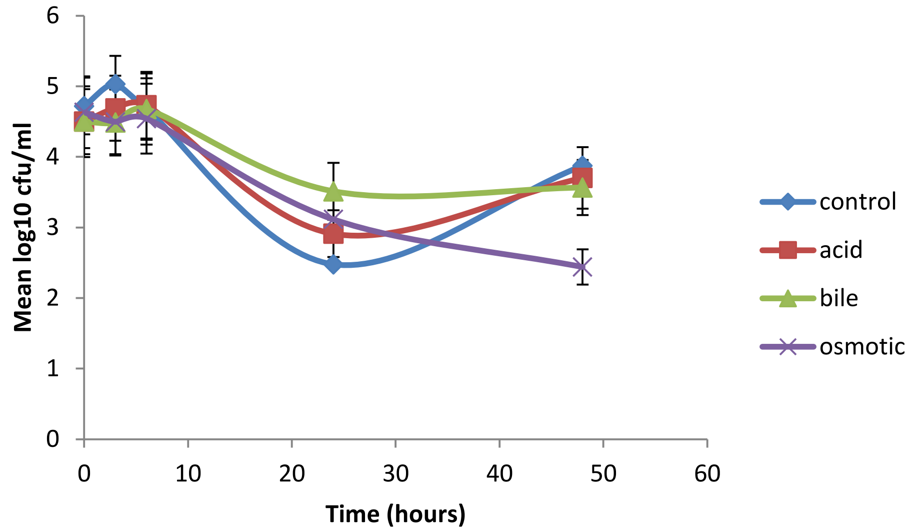

Fig. 4.1a Enumeration of *B. animalis* ssp. *lactis* (strain C) exposed to stress conditions in RCM of 32 µg/ml tetracycline, at 0, 3, 6, 24 and 48 hours. Error bars represent standard error of the mean (SEM)

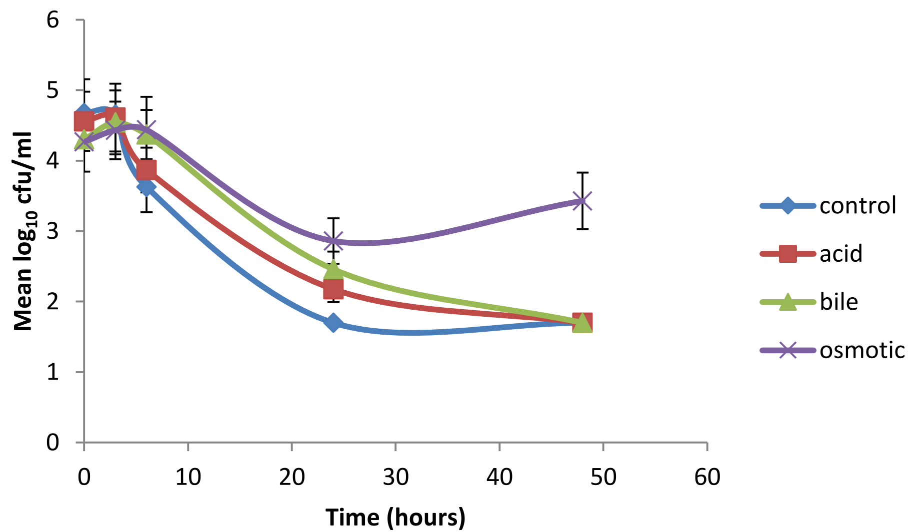

Fig. 4.1b Enumeration of *B. animalis* ssp. *lactis* (strain C) exposed to stress conditions in RCM of 2 µg/ml chloramphenicol, at 0, 3, 6, 24 and 48 hours. Error bars represent standard error of the mean (SEM)

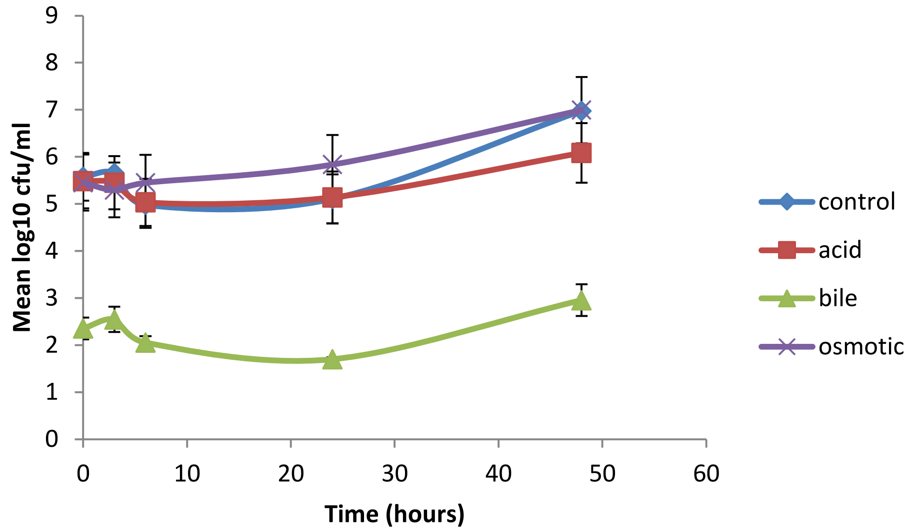

Fig. 4.2a Enumeration of *B. breve* exposed to stress conditions in RCM of 1µg/ml tetracycline, at 0, 3, 6, 24 and 48 hours. Error bars represent standard error of the mean (SEM)

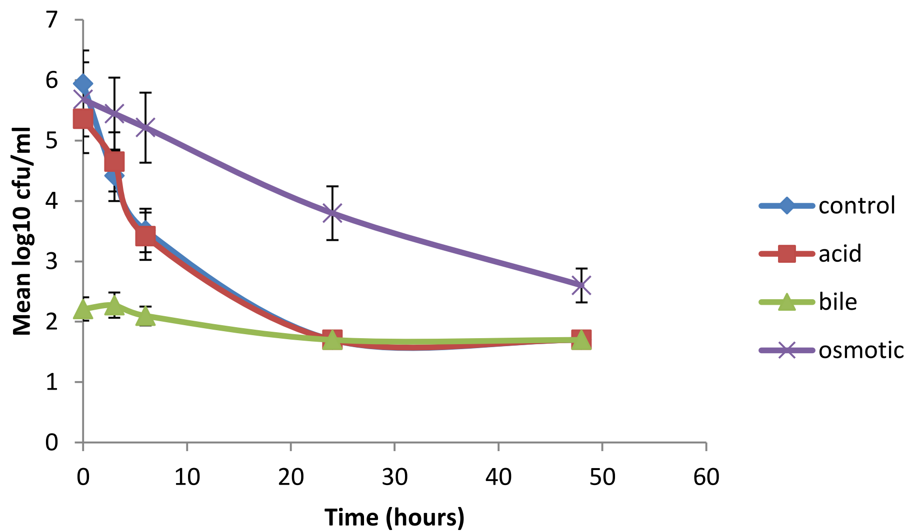

Fig. 4.2b Enumeration of *B. breve* exposed to stress conditions in RCM of 2 µg/ml chloramphenicol, at 0, 3, 6, 24 and 48 hours. Error bars represent standard error of the mean (SEM)

4.3.3 Tetracycline resistance gene *tet*(W) expression

Expression of *tet*(W), a gene involved in tetracycline resistance, was measured in *B. animalis* ssp. *lactis* exposed to acid, bile and osmotic stress, by qRT-PCR. There appeared to be significantly higher expression of *tet*(W) in *B. animalis* ssp. *lactis* (C) exposed to acid, bile and osmotic stress conditions, than in the unstressed (Fig. 4.3). The highest expression relative to the control was in the osmotically-stressed *B. animalis* ssp. *lactis* (C).

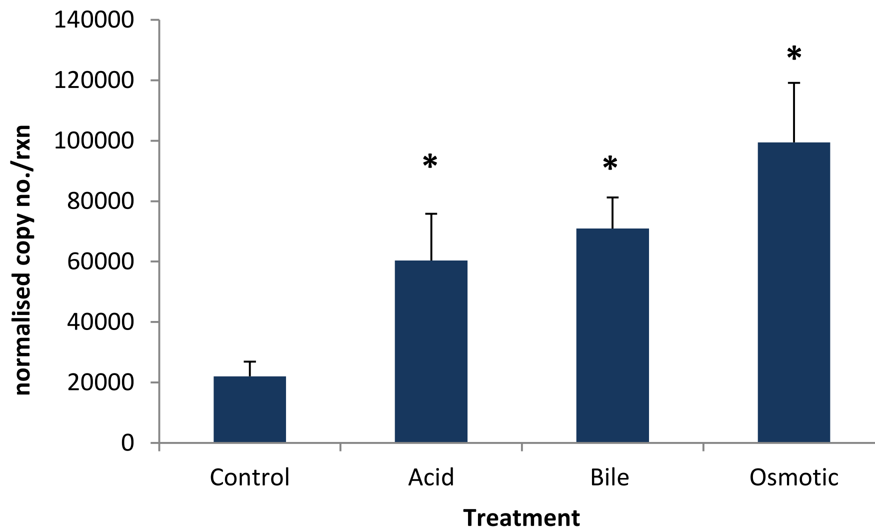

Fig. 4.3 Expression of *tet*(W) under control, acid, bile and osmotic conditions. Error bars represent standard error of the mean (SEM). Asterisks represent significant differences in comparison to the control ($P \leq 0.05$)

4.4 Discussion

Bifidobacteria are generally very susceptible to Gram-positive spectrum antibiotics such as macrolides, erythromycin, teicoplanin, novobiocin, vancomycin; broad spectrum antibiotics such as rifampicin, chloramphenicol; and beta-lactams such as penicillin, ampicillin, amoxicillin, imipenem. Most *Bifidobacterium* spp. are resistant to Gram-negative spectrum antibiotics such as fusidic acid, nalidixic acid and polymyxin B; and aminoglycosides such as neomycin, kanamycin, streptomycin and gentamicin. They have also been found to show variable susceptibility to tetracycline and some cephalosporins (Lim *et al.* 1993; Zhou *et al.* 2005; Ammor *et al.* 2007).

In this study, five antibiotics were used, each representing a different class/mode of action. Ampicillin, a penicillin derivative, belongs to the β-lactam group, and the mode of action is via inhibition of cell wall synthesis (Mayo *et al.* 2010). Susceptibility to ampicillin and other penicillin derivatives in bifidobacteria is suggested to be due to the lack of β-lactamase production (Moubareck *et al.* 2005; Masco *et al.* 2006). Any resistance is most likely to be due to cell wall impermeability (Charteris *et al.* 1998). The organisms used in this present study were all susceptible to ampicillin (MIC ≤ 2 μg/ml) and exposure to stress did not make them resistant or more susceptible to ampicillin.

Vancomycin is a glycopeptide, which acts by inhibition of cell wall synthesis, though at a different site than β-lactams (Mayo *et al.* 2010). Vancomycin is one of the last broadly effective antibiotics against infections caused by multi-drug resistant pathogens, and resistance to vancomycin is therefore a concern. Varying levels of susceptibility to vancomycin have been reported in

bifidobacteria, though this has been suggested to be due to the type of method used for assessment (Zhou *et al.* 2005). However, the organisms in this study appeared to be susceptible to vancomycin (MIC ≤ 2 µg/ml), and this was consistent after exposure to stress.

Erythromycin is a macrolide antibiotic, which inhibits ribosomal protein synthesis (Mayo *et al.* 2010). Bifidobacteria are highly susceptible to erythromycin (MIC ≤ 0.5 µg/ml) and the organisms used in this study displayed this, irrespective of stress exposure.

Phenicol antibiotics such as chloramphenicol interfere with protein synthesis. Bifidobacteria are usually sensitive to chloramphenicol (Mayo *et al.* 2010). MICs > 4 µg/ml are considered to indicate resistance (EFSA 2008). The organisms in this study were all susceptible to chloramphenicol, and this did not change with exposure to stress.

Tetracyclines are also inhibitors of ribosomal protein synthesis and bifidobacteria show variable levels of susceptibility (Mayo *et al.* 2010). MICs > 8 µg/ml are considered to indicate resistance to tetracycline (EFSA 2008). In this present study, both *B. animalis* ssp. *lactis* strains were resistant to tetracycline, and this was maintained after exposure to stress. *B. breve* and *B. longum* were susceptible to tetracycline (MIC < 8 µg/ml) and this remained the case when exposed to stress.

Tetracycline resistance is the most common antibiotic resistance in bifidobacteria, and the *tet* genes, such as *tet*(O), *tet*(M) and *tet*(W), which code for ribosomal protection proteins, are most commonly responsible for this trait. The most commonly detected gene in bifidobacteria is *tet*(W), which

is located on the bacterial chromosome (Kazimierczak *et al.* 2006; Gueimonde *et al.* 2010). The *tet* genes in *Bifidobacterium* are known to protect ribosomes from the action of tetracyclines (Gueimonde *et al.* 2013). Tetracycline-susceptible bifidobacteria have also been found to possess chromosomally encoded *tet*(W) genes (Ammor *et al.* 2008a).

In this study, higher expression of *tet*(W) in *B. animalis* ssp. *lactis* after exposure to acid, bile and osmotic stress conditions was observed. Gueimonde *et al.* (2010) also observed slight induction of *tet*(W) in *B. animalis* ssp. *lactis* upon bile exposure. It could be suggested that this up-regulation in the expression of *tet*(W) observed in this present study may be a protective mechanism, in response to stress conditions that can affect ribosomes in bacterial cells, since *tet* genes code for ribosomal protection proteins. This increased expression of *tet*(W) in *B. animalis* ssp. *lactis* did not however manifest as increased tetracycline resistance.

Whilst the bifidobacteria used in this study were not demonstrated to possess any antibiotic resistance genes which are borne on mobile genetic elements (plasmids, transposons), it could be suggested that exposure of probiotic bacteria to stress may increase the transmission of antibiotic resistance plasmids, thereby contributing to the antibiotic resistance problem. For instance, the *tet*(W) gene of *Bifidobacterium*, though integrated in the bacterial chromosome, may often be surrounded by transposase target sequences, i.e. genes coding for transposases. Transposases are enzymes that catalyse the movement of DNA fragments between different locations, by recognising specific target sequences. This may suggest that under

adequate conditions, the *tet*(W) gene may be transferred (Kazimierczak *et al.* 2006; Ammor *et al.* 2008a; Gueimonde *et al.* 2010).

McMahon *et al.* (2007a) reported that under sublethal environmental stress conditions (pH, osmotic, high/low temperature), the horizontal transmission rates of the two plasmids studied (R386 and TP307), between plasmid-bearing *Escherichia coli* donor cultures and recipient *E. coli* and *Salmonella typhimurium* strains, were significantly increased in comparison to control conditions. This may further justify the need to screen potential probiotic bacteria for the presence of antibiotic resistance genes, and the potential of transmission of those genes to pathogens, with the additional consideration of environmental stress. However, *in vitro* transferability may not necessarily imply transmission *in vivo*. There is currently no evidence to suggest that bifidobacteria can transfer antibiotic resistance to other enteric bacteria (Gueimonde *et al.* 2013).

One group of antibiotics not included in the study is the aminoglycosides, which include gentamicin, kanamycin, neomycin and streptomycin. Aminoglycosides are also inhibitors of ribosomal protein synthesis, but bifidobacteria are generally intrinsically resistant to aminoglycosides, because, being anaerobes, they lack cytochrome-mediated drug transport (Mayo *et al.* 2010). It has been observed that exposure to treatments which affect the lipid bilayer of the cell membrane, i.e. detergents such as ox-bile, can increase susceptibility to aminoglycosides (Charteris *et al.* 2000).

The study by Kheadr *et al.* (2007) reported increased susceptibility of acid stressed bifidobacteria to ampicillin, vancomycin, aminoglycosides, chloramphenicol and erythromycin in varying levels. Bile-stressed bifidobacteria showed particularly increased susceptibility to aminoglycosides. This was considered to be due to enhanced cell wall permeability facilitated by ox-bile. Increased susceptibility to chloramphenicol, and erythromycin to some extent, was observed. Also, bile stress conferred resistance to tetracycline in most of the strains studied. Oxidatively stressed bifidobacteria showed increased susceptibility cell-wall directed β-lactams, chloramphenicol, erythromycin and tetracycline. This was attributed to alterations in membrane properties and disruption of membrane-bound proteins, as a result of attack of polyunsaturated fatty acids in the cell membranes by free radicals.

Bacteria for probiotic use would preferably exhibit stress tolerant properties. It is possible that these stress tolerance mechanisms may interact with their resistance or susceptibility to antibiotics. Noriega *et al.* (2005) observed that bile-adapted strains of *B. animalis* and *B. longum* showed increased resistance to tetracyclines, though the mechanism by which this occurred was unclear. Similarly, Collado and Sanz (2007) observed that some acid-resistant strains of *Bifidobacterium* were more resistant to antibiotics like ampicillin, tetracycline, penicillin and rifampicin. It was suggested that general modifications in cell permeability and surface properties may be responsible.

While the bifidobacteria used in this study showed *in vitro* susceptibility to the antibiotics tested, it is possible that they would be able to survive better *in vivo* than *in vitro*, since survival is dependent on the antibiotic concentration.

Bifidobacteria are mainly located in the colon, and since antibiotics are mainly absorbed in the ileum, the therapeutic dosage which reaches the colon might be lower than the initial ingested dose, thereby allowing survival (Yazid *et al.* 2000; Moubareck *et al.* 2005).

4.5 Conclusion

This study examined the effect of stress exposure on antibiotic susceptibility patterns of bifidobacteria. In general, there was no change from susceptibility to resistance, or vice-versa, according to the EFSA breakpoints. Changes in MIC were only by one dilution factor above or below, relative to the control. It is possible that these are fluctuations which may be due to interference from media, growth conditions and inoculum volume (EFSA 2008). The expression of the intrinsic tetracycline resistance gene in *B. animalis* ssp. *lactis* appeared to be higher after exposure to stress conditions. This however did not necessarily translate into higher MIC of tetracycline. It is possible that the stress tolerance mechanisms overlap with antibiotic susceptibility/resistance. However, if increased susceptibility to antibiotics occurs upon exposure to environmental stress, particularly where resistance to an antibiotic is intrinsic, this should be looked at further, to understand the mechanisms and implications for prophylactic use of probiotics against side effects of antibiotic therapy.

CHAPTER FIVE: EFFECTS OF STRESS ON BIOFILM FORMATION POTENTIAL OF BIFIDOBACTERIUM SPP.

5.1 Introduction

Bifidobacteria are of intestinal origin, being one of the earliest colonizers of the gastrointestinal tract after birth, and predominate the intestinal microflora of breast-fed infants (O'Grady and Gibson 2005). Their human origin makes them good potential probiotic candidates, as they would be more likely to colonise the gut. After surviving transit through the gastrointestinal tract, probiotic microorganisms need to be able to colonise the gut temporarily.

Adhesion to the intestinal epithelial cells (enterocytes) is considered as necessary for probiotic microorganisms to colonise the large intestine, and colonisation is important for beneficial health effects such as modulation of the immune system to be observed (Tuomola *et al.* 2001). By attaching to the epithelium, probiotics can compete with enteric pathogens and prevent their attachment to the epithelium i.e. competitive exclusion (Oelschlaeger 2010; Wohlgemuth *et al.* 2010). Bifidobacteria have been shown to adhere very well to intestinal cells, which in turn can enable them to colonise the large intestine (Fliss *et al.* 2010).

An outcome of adhesion may be the formation of biofilms. A biofilm is an aggregation of microorganisms within an extracellular polymeric matrix, usually polysaccharide, on a biotic or abiotic surface (Costerton *et al.* 1995). Biofilms are ubiquitous, being found in a range of different natural environments, including the human gastrointestinal tract (Probert and Gibson, 2002).

Biofilm formation is a complex process generally consisting of three stages: attachment, maturation and dispersion (Bjarnsholt *et al.* 2013). Biofilm-

associated organisms are considered to exhibit very different properties from planktonic (unaggregated) organisms (Shemesh *et al.* 2007). Organisms within a biofilm are usually more resistant to adverse environmental conditions and antibiotics (Stepanovic *et al.* 2007; Xu *et al.* 2011).

Most adherent bacteria occur in nature in biofilms, including the microorganisms in the gut (Lebeer *et al.* 2007). In the human gut, bacteria can exist as biofilms on the colonic epithelium, the mucus layer covering it, as well as on the surface of food particles (Probert and Gibson 2002). Bacteria which form biofilms on food particles are more likely to be involved in food digestion, which may give them an advantage when competing for available nutrients (Macfarlane and Macfarlane 2006).

Biofilm formation in pathogens has been widely studied because of their implications for health. These studies have been carried out to understand the nature of biofilm formation in these pathogens, in order to devise suitable measures of controlling them (Xu *et al.* 2011). Pathogens which have been studied include *Listeria monocytogenes* (Begley *et al.* 2009; Sandasi *et al.* 2010; Xu *et al.* 2011), *Actinobacillus actinomycetemcomitans* (Perez *et al.* 2006), enterococci (Extremina *et al.* 2011), *Streptococcus mutans* (Shemesh *et al.* 2007), *Salmonella* (Speranza *et al.* 2011) and staphylococci (Stepanovic *et al.* 2007).

Biofilm formation has been suggested to be influenced by nutrient availability, environmental conditions, as well as strain type. Such conditions include those found in the gastrointestinal environment, namely low pH, high osmolarity and the presence of bile (Lebeer *et al.* 2007). A common method

for quantifying biofilms *in vitro* is the crystal violet assay for cultures grown in microtitre plates (Stepanovic *et al.* 2007).

The cell surface of bifidobacteria and other Gram-positive bacteria have been shown to have pili or fimbriae, which are hair-like appendages suggested to be involved in the attachment and colonisation of host tissues, and the development of biofilms (Foroni *et al.* 2011; Juge 2012). Many lactic acid bacteria and bifidobacteria produce exopolysaccharides (EPS), which are extracellular carbohydrate polymers. Suggested roles of EPS in bifidobacteria include cell recognition, adhesion to surfaces, formation of biofilms to enhance colonisation of various ecosystems, protection against host defences such as phagocytosis, and protection against osmotic stress (Ruas-Madiedo *et al.* 2010; Russell *et al.* 2011).

Exposure to the stress conditions of the gastrointestinal tract may alter the metabolic, physiological and surface properties of potential probiotic microorganisms, thereby affecting the production of colonisation factors such as EPS, such that their ability to adhere to the intestinal epithelium is affected (Collado *et al.* 2006). Modification to adhesion properties may lead to alteration in probiotic capacity (Tuomola *et al.* 2001).

The study of effects of gastrointestinal stresses on the ability of bifidobacteria and other potential probiotic microorganisms to adhere to the intestinal epithelium may be carried out by assessing biofilm formation, as well as production of EPS. Therefore, this chapter was aimed at examining possible effects of exposure of bifidobacteria to stress conditions on their potential for *in vitro* biofilm formation and EPS production.

5.2 Materials and Methods

5.2.1 Bacterial cultures

Bifidobacterial cultures were namely *Bifidobacterium breve* NCTC 11815, *B. longum* NCTC 11818, *B. animalis* ssp. *lactis* strain C and *B. animalis* ssp. *lactis* strain D, as described in 2.2.1.

5.2.2 Stress treatment

The bifidobacteria (*B. animalis* ssp. *lactis* C and D, *B. breve* and *B. longum*) were exposed to acid, bile, osmotic and oxidative stress as described in 3.2.2.1 – 3.2.2.4. Unstressed cells were prepared as described in 3.2.2.5.

5.2.3 Crystal violet assay

Biofilm formation was assessed by the crystal violet assay method described by Stepanovic *et al.* (2007), with modifications. Twenty µl of bacterial suspension was added to 180 µl of Reinforced Clostridial Medium (RCM) contained in flat-bottomed 96-well polystyrene microtitre plates (Sterilin Ltd UK) and incubated under anaerobic conditions at 37 °C for 48 hours. Negative control was 200 µl RCM. After incubation, the medium was removed and wells were washed three times with 200 µl of phosphate buffered saline (PBS, BR0014, Oxoid) to remove non-adherent and loosely adherent cells. After washing, the remaining adherent bacterial cells were heat-fixed by exposing to hot air at 60 °C for 1 hour. The fixed cells were stained with 150 µl of 1% crystal violet (Sigma) for 15 minutes at room temperature, after which, the wells were washed by placing under running

tap water until plates were free of the stain. The microtitre plates were air dried at room temperature and 200 µl of dimethyl sulfoxide (Sigma) was added to each well. Plates were incubated at room temperature for 1.5 hours to solubilise the crystal violet. One hundred µl of the resulting solutions were transferred into a fresh microtitre plate for optical density (OD) measurement (Fig. 5.1). The OD of each well was measured at 595 nm in an Omega Fluostar plate reader (BMG Labtech Ltd, UK). Experiments were done in three replicates and mean OD calculated.

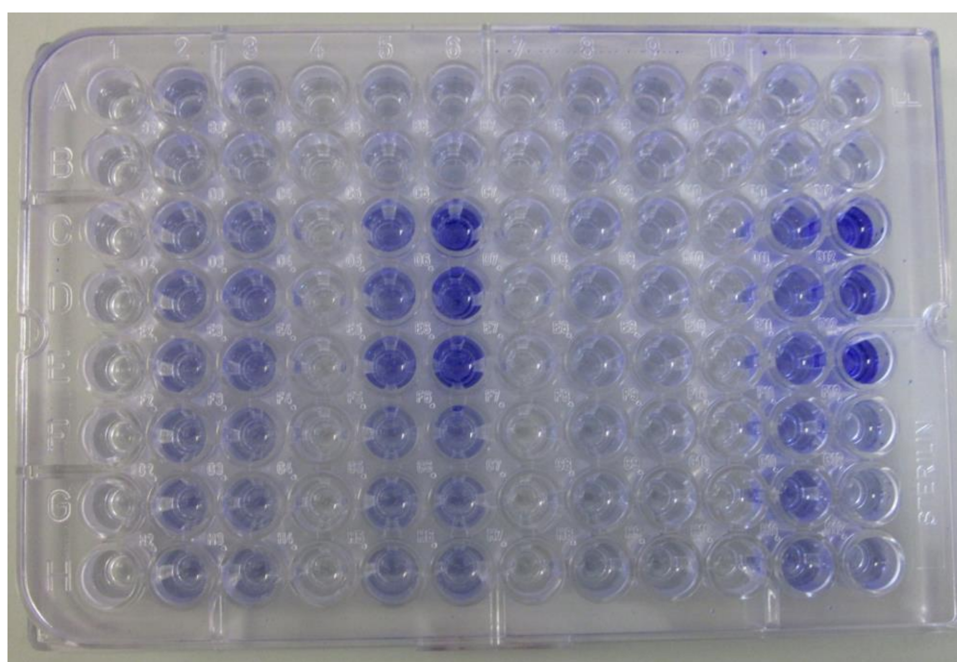

Fig. 5.1 96-well microtitre plate showing wells containing solubilised crystal violet for before measuring the optical density to assess biofilm formation

5.2.4 Expression of *gtf*01207 EPS-synthesis gene in *B. animalis* ssp. *lactis* (C) by quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Samples were prepared and qRT-PCR analysis was carried out as described in 4.2.5. The primers used to amplify the *gtf*01207 gene, which is involved in exopolysaccharide (EPS) synthesis, and reference genes *atp*D, *tuf*A and *ldh* are listed in Table 5.1. The expression of the gene in *B.* animalis ssp. *lactis* only was studied because *gtf*01207 has only been associated with *B. animalis* ssp. *lactis* in previous literature, and the available primer sequences were therefore specific to it (Ruas-Madiedo *et al.* 2009). Due to time and cost, it was not feasible to check for suitable primers for *B. breve* and *B. longum*. The primers used for *B. animalis* ssp. *lactis* were as described in Ruas-Madiedo *et al.* (2009) and supplied by Sigma-Aldrich, UK. For accurate gene quantification, the expression of the gene of interest, i.e. *gtf*01207, was normalised to the expression of the reference genes, whose expression does not change under the various experimental conditions. The reference genes were as used by Foroni *et al.* (2011). The stability of these reference genes was assessed using the geNorm software. Reference gene primers were designed by qStandard Ltd, UK. Quantitative PCR was carried out in three replicates, and mean number of normalised copies per reaction was calculated.

Table 5.1 Primer sequences

Gene	Forward primer	Reverse primer
*gtf*01207	5’-CGTGCTGAGTCGAAAGAATCG-3’	5’-TTGTAGAACGTGATCGGCTCA-3’
*atp*D	5’-CTCCACCTCGCGAATCCT-3’	5’-GAAGTTCTGGCCGAGGAAC-3’
*tuf*A	5’-GAGTACGACTTCAACCAGATCG-3’	5’-ATGTTCTTCACGAAGTCGGC-3’
ldh	5’-CCGACATGGTCGTCATCAC-3’	5’-GGGTTGGTGATGAGCATGTA-3’

5.2.5 Statistical analysis

Data were analysed by Student’s t-test to compare unstressed and stressed data groups, using Microsoft Excel 2007. Statistical significance was set at $P \leq 0.05$.

5.3 Results

5.3.1 Quantitation of biofilm formation by crystal violet assay

Biofilm formation in the tested bifidobacteria was quantified by the crystal violet assay. Effects of exposure to acid, bile, osmotic and oxidative stress on the potential for biofilm formation were also assessed. Comparisons of mean optical densities for the tested organisms exposed to stress were made, relative to the control. Optical densities represent the amount of released crystal violet, providing an estimation of the biofilm formation.

There appeared to be low biofilm formation in all four strains tested, as the optical density values were generally rather low (<0.1). Among the unstressed bifidobacteria, the relative order of biofilm formation, from highest to lowest, was *B. breve*, *B. animalis* ssp. *lactis* (C), *B. animalis* ssp. *lactis* (D) and *B. longum*. The effects of exposure to acid, bile, osmotic and oxidative stresses on the the biofilm formation of these organisms are summarised in Table 5.2.

Table 5.2 Effects of exposure to stress on biofilm formation relative to unstressed cultures

	Acid	Bile	Osmotic	Oxidative
B. animalis ssp. *lactis* (C)	0	+	+	+
B. animalis ssp. *lactis* (D)	0	+	0	+
B. breve	-	-	-	-
B. longum	0	-	0	0

(+ = higher biofilm; 0 = no difference; - = lower biofilm)

Acid stress appeared to result in no significant difference in biofilm formation in both *B. animalis* ssp. *lactis* strains and *B. longum*, while there was a significantly less biofilm formation in *B. breve* (Fig. 5.2a).

There appeared to be significant difference in all four bifidobacteria exposed to bile stress, with significantly higher biofilm formation observed in both *B. animalis* ssp. *lactis* strains and significantly lower biofilm formation observed in *B. breve* and *B. longum* (Fig. 5.2b).

Osmotic stress appeared to have no significant effect on *B. animalis* ssp. *lactis* (D) and *B. longum*, while biofilm formation in *B. animalis* ssp. *lactis* (C) and *B. breve* appeared to be significantly higher and lower, respectively (Fig. 5.2c).

Significantly higher biofilm formation in oxidatively stressed cultures were observed in both *B. animalis* ssp. *lactis* strains, while there appeared to be significantly lower biofilm formation in *B. breve* and no significant difference in *B. longum* (Fig. 5.2d).

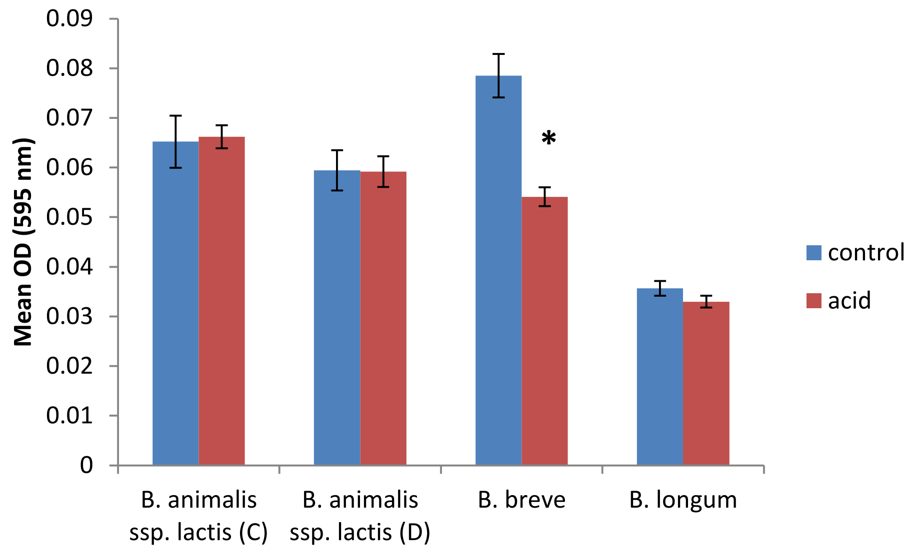

Fig. 5.2a Biofilm formation in bifidobacteria after exposure to acid stress. Error bars represent standard error of the mean (SEM). Significant differences are represented by asterisks ($P \leq 0.05$)

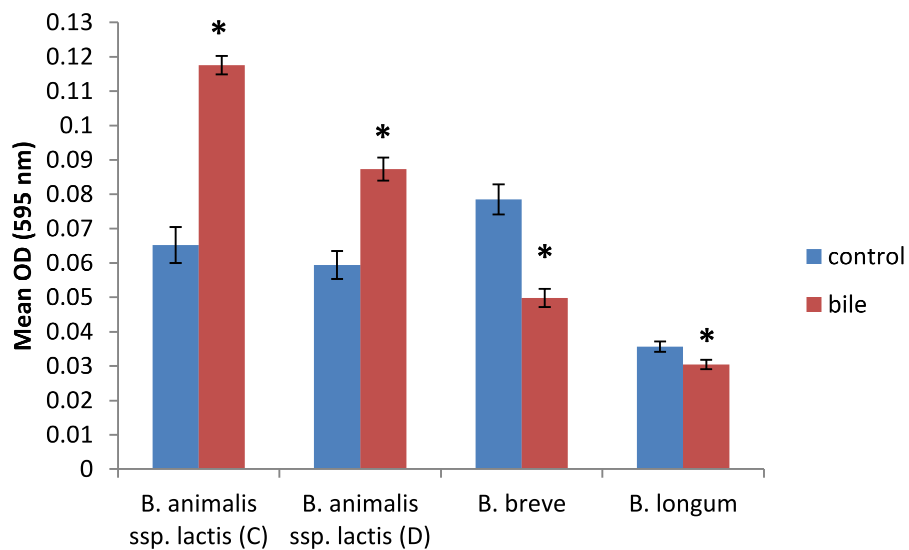

Fig. 5.2b Biofilm formation in bifidobacteria after exposure to bile stress. Error bars represent standard error of the mean (SEM). Significant differences are represented by asterisks ($P \leq 0.05$)

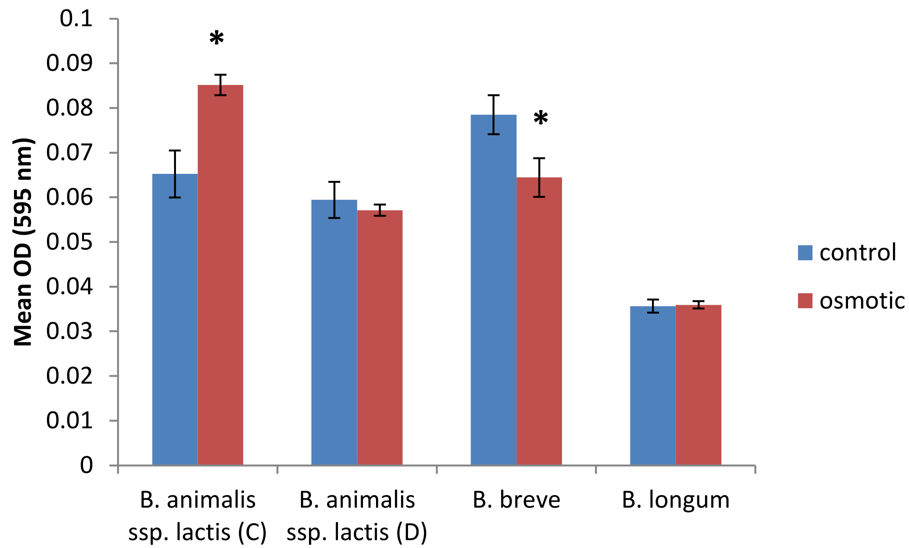

Fig. 5.2c Biofilm formation in bifidobacteria after exposure to osmotic stress. Error bars represent standard error of the mean (SEM). Significant differences are represented by asterisks ($P \leq 0.05$)

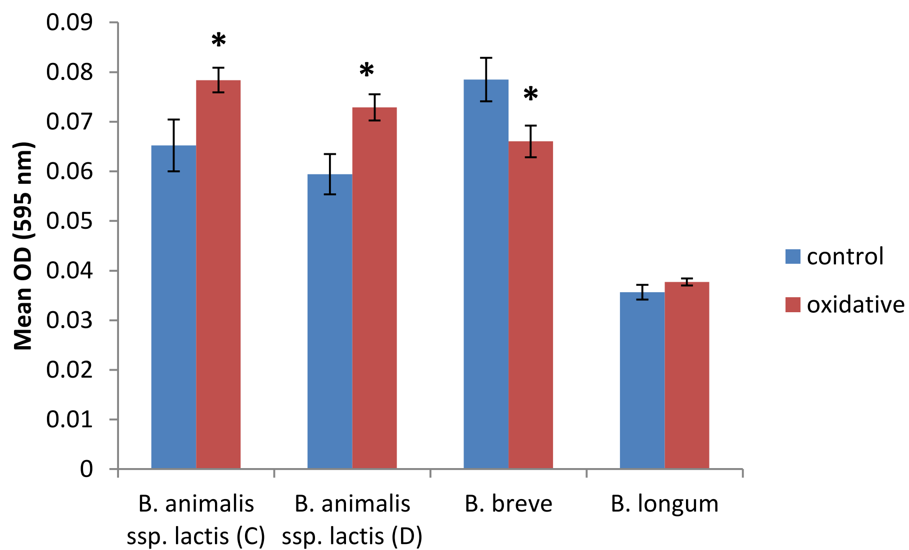

Fig. 5.2d Biofilm formation in bifidobacteria after exposure to oxidative stress. Error bars represent standard error of the mean (SEM). Significant differences are represented by asterisks ($P \leq 0.05$)

5.3.2 Expression of *gtf*01207 EPS-synthesis gene

Expression of *gtf*01207, a gene involved in EPS synthesis, was measured in *B. animalis* ssp. *lactis* (C) exposed to acid, bile and osmotic stress, as well as unstressed, by qRT-PCR. *B.* There appeared to be higher expression of *gtf*01207 in *B. animalis* ssp. *lactis* (C) exposed to acid, bile and osmotic stress conditions, in comparison to the unstressed, with the osmotically stressed culture showing significantly higher expression than the unstressed (Fig. 5.3).

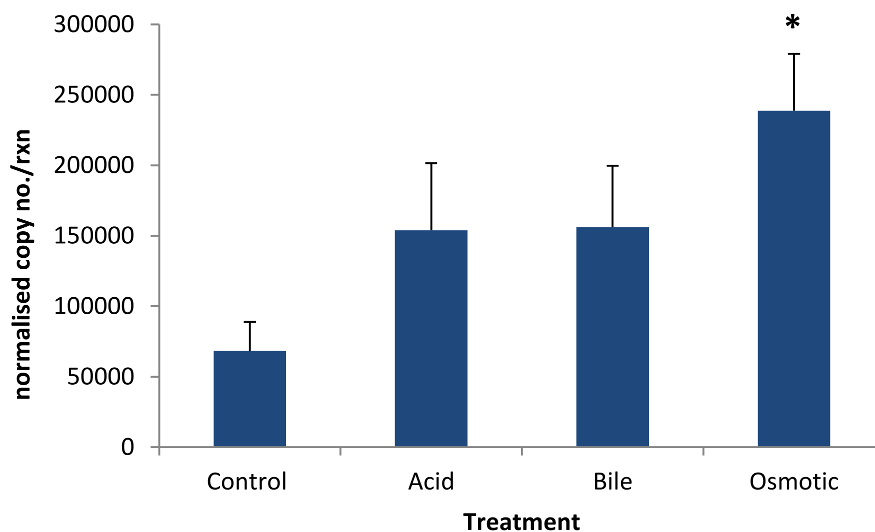

Fig. 5.3 Expression of *gtf*01207 under control, acid, bile and osmotic conditions. Error bars represent standard error of the mean (SEM). Asterisks represent significant differences in comparison to the control ($P \leq 0.05$)

5.4 Discussion

This present study was aimed at investigating the possible effect of exposure to stress on the ability of bifidobacteria to form biofilms. This was under the assumption that stress may lead to modifications in the cell surface, as suggested by previous studies. Ruiz *et al.* (2007) reported morphological changes, as studied by transmission electron microscopy (TEM) and changes in membrane protein: phospholipid ratio in *B. animalis* ssp. *lactis* in response to bile, though no indication was given about any possible effect on adhesion capability or biofilm formation. Ahn *et al.* (2001) also observed morphological changes, as studied by scanning electron microscopy (SEM) and changes in protein and fatty acid profiles of bifidobacteria under oxygen stress.

Furthermore, the study by Guglielmetti *et al.* (2009) on the adhesion of *B. bifidum* MIMBb75 to human intestinal cell lines showed that adhesion was reduced by bile salts as well as low pH. It was suggested that this reduction in adhesion may be due to modifications in the cell surface properties. Lebeer *et al.* (2007) in their study of biofilm formation by *Lactobacillus rhamnosus* GG found that low concentrations of bile (0.05%, 0.1%, 0.2%) stimulated biofilm formation, though it reduced with increasing concentration, and was greatly reduced at higher concentrations (1.5%). Low pH was also found to significantly reduce biofilm formation in *Lb. rhamnosus* GG. It was also suggested that alterations in the cell surface by acid and bile may influence biofilm development.

This present study differed from that of Lebeer *et al.* (2007) in that whereas Lebeer *et al.* assessed biofilm formation with the stress agent present in the

medium, this study assessed biofilm formation in normal medium, after exposing the organisms to the stress agent. This was done on the basis that probiotic microorganisms would be exposed to and have to survive stress in the upper part of the gut, before reaching the colon, which provides a favourable condition for their growth.

The results of the biofilm study showed optical density values which could be considered as very low (<0.1). This could be due to culture medium used. Media composition has been shown to have a major influence on *in vitro* biofilm formation (Lebeer *et al.* 2007; Stepanovic *et al.* 2007). Reinforced Clostridial Medium (RCM) was used to culture the bifidobacteria in this study. It is a semi-solid medium, i.e. it contains some agar, which makes it suitable for cultivating obligately anaerobic bacteria like *Bifidobacterium* spp. It also contains L-cysteine (as cysteine hydrochloride), which is a reducing agent, lowering the redox potential to further encourage anaerobic conditions in the medium. The amino acid cysteine is also an important nitrogen source for bifidobacteria (Roy 2003).

It could be suggested that while RCM is good for growth of bifidobacteria, it may not be suitable for *in vitro* biofilm formation. The presence of agar in the medium may have resulted in less adherence of the bacteria to the walls of the microtitre plates, and more adherence to the agar particles. It is not clear what the influence of other components of the medium may have had on biofilm formation. The presence of glucose as a carbon source in MRS medium was found to have a negative effect on biofilm formation by *Lb. rhamnosus* GG, though this effect was not observed in other *Lb. rhamnosus*

and *Lb. casei* strains studied (Lebeer *et al.* 2007). RCM also contains glucose (5g/L), but at a lower proportion to MRS (20g/L).

Another possible explanation for the low biofilm formation could be the presence of a reducing agent in RCM. The study of Qian *et al.* (2011) on some *Bifidobacterium* spp. demonstrated that cells grown in MRS which is without cysteine, showed reduced intracellular granule and exopolymer production compared to those grown in MRS with cysteine and RCM. MRS-grown cells also showed higher cell surface hydrophobicity; cell surface hydrophobicity has been positively correlated with adhesion ability to host cells (Perez *et al.* 1998; Pan *et al.* 2006). On the other hand, it may be due to the anaerobic incubation condition. Ninomiya *et al.* (2009), in their study of exopolysaccharide (EPS) production in a strain of *B. longum*, showed that a CO_2 concentration of ≥20% in the anaerobic gas mixture was necessary for substantial EPS production. In this present study, the anaerobic gas mixture contained 10% CO_2. This may have had an impact on the biofilm formation.

The highest biofilm formation in the unstressed cultures was observed in *B. breve*, followed by both commercial *B. animalis* ssp. *lactis* strains, and then *B. longum*. Biofilm formation potential may be dependent on the bacterial strain, but also on the medium used. Therefore it is not clear if this order in biofilm formation would be the same, had another culture medium been used.

Differences in biofilm formation between stressed and unstressed bifidobacteria were observed. In *B. breve*, there was lower biofilm formation in the cultures exposed to each of the four stresses studied. In both commercial *B. animalis* ssp. *lactis* strains, acid-stressed cultures showed no

difference in biofilm formation relative to the unstressed. Bile, osmotic and oxidatively stressed *B. animalis* ssp. *lactis* showed higher biofilm formation relative to the unstressed. *B.* breve, which produced the highest amount of biofilm in the unstressed state, appeared to be negatively affected by exposure to stress, whereas both commercial *B. animalis* ssp. *lactis* strains produced less biofilm in the unstressed state and appeared to be positively affected by exposure to stress. *B. longum* showed the least potential for biofilm formation, and was largely unaffected by exposure to stress.

It may be suggested that biofilm formation in *B. animalis* ssp. *lactis* can be stimulated by exposure to stresses, and that biofilm formation in the *B. breve* strain studied can be suppressed by exposure to stresses. However, there is no clear explanation for how these changes in biofilm potential might occur, as well as how exposure to the individual stresses affects biofilm formation. It is possible that the effects of stress on the lag phase of bacterial cells (i.e. extended lag phases) may have an implication on the capacity to form biofilms (Kroukamp *et al.* 2010). This may provide an explanation for the *B. breve* observations, i.e. exposure of *B. breve* to stresses causes injury, which results in an extended lag to allow repair, and which ultimately affects biofilm formation. On this basis, it could be implied that *B. animalis* ssp. *lactis* is more resistant to stress, such that its lag phase is not highly affected by exposure to stress.

Exopolysaccharide (EPS) production may be related to biofilm formation potential in bifidobacteria. As such, effects of stress on biofilm production may be linked to effects of stress on EPS production. *Bifidobacterium* spp. have been screened for EPS production and EPS has been quantified and

characterised (Ruas-Madiedo *et al.* 2007; Audy *et al.* 2010; Leivers *et al.* 2011; Fanning *et al.* 2012; Lopez *et al.* 2012; Prasanna *et al.* 2012).

In this study, EPS production was not quantified. However, the expression of *gtf*01207, an EPS-synthesis gene in *B. animalis* ssp. *lactis*, was studied after exposure to stress conditions. Ruas-Madiedo *et al.* (2009) observed enhanced production of EPS by *B. animalis* ssp. *lactis* in the presence of bile, as studied by cryo-SEM, as well as increased expression of *gtf*01207, a gene which codes for a priming glycosyltransferase (p-GTF) involved in EPS synthesis. The p-GTF catalyses the transfer of a sugar-1-phosphate to a lipophilic carrier molecule anchored in the cell membrane, which is the first step in the assembly of the repeating unit which builds the polysaccharide (Ruas-Madiedo *et al.* 2007).

This present study showed higher expression of *gtf*01207 in *B. animalis* ssp. *lactis* after exposure to acid, bile and osmotic stresses, though only significantly for osmotic stress. There was also some correlation between the biofilm results and the EPS gene expression results. This may suggest that EPS production is stimulated by multiple stresses as a protective response, which also can enhance biofilm formation.

This suggestion may be justified by the study of Vieira *et al.* (2004) on the *bolA* gene in *Escherichia coli*. The *bolA* gene, which is a stress response gene that causes round morphology when over-expressed, was found to be involved in biofilm development. Over-expression of the gene induced biofilm development, while deletion of the gene decreased biofilm formation. Under stress conditions, *bolA* was expressed, and consequently biofilm formation

induced. This observation led to the suggestion that biofilm is a protective mechanism against harsh environmental conditions.

These modifications which occur in the presence of stress may be stress tolerance or response mechanisms, which in turn, impact, positively or negatively, on the adhesion and colonisation potential of bifidobacteria and other probiotics. It may be suggested that bifidobacteria which can show increased or higher biofilm formation and EPS production in response to stress would be good candidates for probiotic use, as demonstrated by *B. animalis* ssp. *lactis* in this study. For instance, Collado *et al.* (2006) observed that acid-resistant strains of bifidobacteria showed great adhesion and pathogen displacement capacity. Similarly, Candela *et al.* (2010) reported that stress-related proteins such as DnaK and enolase, which are suggested to play roles in the binding of *B. animalis* ssp. *lactis* to human epithelial cells, were induced in response to bile.

5.5 Conclusion

Adhesion and colonisation of the intestine is important for any probiotic effect to be seen. As such, in light of the fact that probiotics are exposed to various stress conditions before reaching the large intestine, it may be advisable to assess the *in vitro* biofilm formation and EPS production of candidate probiotics after exposure to stress. This may be more representative of the situation *in vivo*, and may reveal new candidate probiotic strains. Culture media and culture conditions may need to be considered carefully when studying biofilm formation in bifidobacteria. The higher biofilm formation and *gtf*01207 expression shown by *B. animalis* ssp. *lactis* after exposure to stress, in this study, may be used to justify its use as a potential probiotic.

CHAPTER SIX: GENERAL DISCUSSION

6.1 Scope of research

The aim of this study was to examine whether bifidobacteria, and by extension, other potentially probiotic microorganisms, can exhibit changes in their desired *in vitro* probiotic functional properties when exposed to stress conditions similar to those they can be exposed to during food production, and particularly during gut transit. This was based on the hypothesis that since various changes occur in bacterial cells in response to environmental stress, perhaps there would be an impact on the functional properties of probiotic microorganisms as a result. Another hypothesis this study attempted to test was that perhaps stress response mechanisms did not only aid survival and viability of probiotic microorganisms, but that they were also integral to the beneficial properties of probiotic microorganisms.

The idea was that if potentially probiotic microorganisms were exposed to stress before assessing their functional properties, the outcomes of such assessment would give a more realistic insight into the an organism's actual potential to provide health benefit. The research was also a means of further understanding the mechanisms of probiotic action, by particularly studying the interaction between stress and functionality.

6.2 Selection of stress conditions

The effects of acid, bile, osmotic and oxidative stresses on antimicrobial activity, antibiotic susceptibility and biofilm formation, were examined. The particular conditions used for each stressor were selected on the basis of identifying conditions which resulted in no growth after 24 hours of exposure,

thus indicative of stress, but which also resulted in negligible reduction in cell numbers after one hour of exposure (see Chapter 2). This was important because comparisons were being made with unstressed controls, and it was necessary to ensure that any differences observed were as a result of effects of stress on the bacterial cells, rather than on the bacterial cell numbers.

One hour exposure was considered as sufficient exposure time, as this was demonstrated by Zomer *et al.* (2009) and Zomer and van Sinderen (2010), where one hour exposure of *B. breve* UCC2003 to various stresses revealed changes in the regulation of various genes in response to the stresses. Also, as gut transit time can range from <1 – 4 hours (Li *et al.* 2010), one hour exposure was considered appropriate to represent exposure to conditions in the gut.

Among the stress conditions chosen, more extreme conditions were generally used to elicit stress in the commercial *B. animalis* ssp. *lactis* strains, in comparison to those used to elicit stress in *B. breve* and *B. longum*, which are bifidobacteria of human origin. This further demonstrated the reported greater stress tolerance shown by *B. animalis* ssp. *lactis*, which makes it favoured commercially, among other bifidobacteria (Raeisi *et al.* 2013).

6.3 Effects of stress on probiotic functionality

Potential probiotic microorganisms are required to meet various functional criteria, which are usually determined *in vitro*. These properties have a basis in the proposed mechanisms of probiotic action (see Chapter 1). This study assessed the effects of stress on antimicrobial activity, antibiotic

susceptibility and biofilm formation, as described in Chapters 3, 4 and 5, respectively.

The antimicrobial activities of bifidobacteria exposed to each of the stresses were maintained, although there appeared to be lower antimicrobial activity, on the basis of smaller inhibition zones observed, relative to the unstressed bifidobacteria. This can suggest that stressed bifidobacteria released less inhibitory substances, mainly organic acids, into the surrounding medium, thereby resulting in less inhibition of the indicator bacteria. The explanation for this may be that stress affects the lag phase, extending it, such that by the time the experiments were stopped, there were less inhibitory substances released. Another possible explanation, which cannot however be supported by the methods used in this study, may be the impact of stress on the transcriptome of the bifidobacteria.

An over-expression of genes involved in carbohydrate metabolism was observed in *B. longum* NCIMB 8809 exposed to bile. There was also an enhanced formation of lactic acid and acetic acid, and a decrease in the acetate/lactate ratio, suggesting more lactic acid was produced than usual (Sanchez *et al.* 2005). It can therefore be suggested that the lower antimicrobial activity observed in this study could be the result of altered sugar metabolism, leading to higher lactic acid production, and lactic acid is less inhibitory than acetic acid. Changes in the regulation of genes involved in carbohydrate metabolism were also observed after the exposure of *B. breve* UCC2003 to stress conditions (Zomer *et al.* 2009).

Antibiotic susceptibility patterns of bifidobacteria in this study remained generally unaffected by exposure to stress. Higher expression of the tetracycline resistance gene *tet*(W) was observed in *B. animalis* ssp. *lactis* (C) exposed to stress, although this did not correlate to higher MIC of tetracycline against *B. animalis* ssp. *lactis* (C). These results may imply that exposure to stress does not affect the ability of probiotics to tolerate therapeutic doses of antibiotics, since the bifidobacteria in this study did not become more sensitive to the antibiotics tested after exposure to stress.

The results of the biofilm formation study suggested that the effect of stress on biofilm formation may depend on the species of bifidobacteria, whereby some showed enhanced biofilm formation and others showed reduced biofilm formation. On this basis, a distinction could be made between commercial strains of bifidobacteria (*B. animalis* ssp. *lactis* strains C and D) and non-commercial strains (*B. breve* NCTC 11815 and *B. longum* NCTC 11818). However it was not clear whether the lower biofilm formation observed in the non-commercial bifidobacteria was as a result of the effect of stress on the lag phase or on genes involved in biofilm formation, since *B. animalis* ssp. *lactis* is more resistant to stress. Higher expression of the EPS-synthesis gene *gtf*01207 in *B. animalis* ssp. *lactis* (C) exposed to stress, particularly osmotic stress, may allude to a link between stress and biofilm formation, whereby stress is necessary to facilitate this property, and hence probiotic benefit. Overall, based on the results of individual experiments, it is not clear if there may be an effect of stress on functionality *in vivo*.

6.4 Implications of the study

There has been recent controversy over the European Food Safety Authority (EFSA) health claims regulation, which rejected numerous health claims for probiotics, and the effective ban on the descriptive usage of the term 'probiotic' as it implies health benefit. The reason for this has been the unsatisfactory dossiers of evidence available to back such health claims (Katan 2012; Salminen and van Loveren 2012; Binnendijk and Rijkers 2013).

Various questions have been raised about whether the bacteria in probiotic products actually remain viable and whether they actually provide any benefit. A lot of research has gone into devising ways of shielding the bacteria from adverse environmental conditions, such as microencapsulation and delivery through tablets and other formulations (Champagne *et al.* 2005; Ross *et al.* 2005). To some extent, this study has attempted to shed light on what could happen to probiotic bacteria which are not protected, and possibly contribute to the case for delivering probiotics through such protective means. On the other hand, some results, i.e. biofilm formation in *B. animalis* ssp. *lactis*, may facilitate the suggestion that some probiotic bacteria need to be exposed to stress for their functional properties to be enhanced, and that stress-tolerance is not only for the purpose of ensuring survival and viability, but also for beneficial properties. This would however need to be further explored.

Another possible consequence from this study may be the inclusion of a stress step, where candidate probiotic microorganisms are exposed to relevant shocks before a functional property is assessed, in a bid to better represent what can happen in reality. This might even reveal candidates that

would have been previously unlikely, or conversely, discount some established candidates. From the results of this study, it could be suggested that *B. animalis* ssp. *lactis* is justified in its use as a probiotic, while the *B. breve* and *B. longum* strains used in this study would need to be protected from stress in order to increase the likelihood of obtaining benefit from their antimicrobial and biofilm formation properties.

6.5 Limitations of the study

Most of the investigations in this study involved transferring the stress-exposed cultures into fresh media. Whilst consistency was maintained, and there was comparison with an unstressed control, it can be argued whether the stressed cultures were truly stressed, considering that they could have undergone repair of any injury caused by the stresses they were exposed to, such that it would be difficult to observe any clear differences in the properties being studied. Furthermore, any differences may have been as a result of extension of the lag phase to allow repair of injury. Therefore, it may be more appropriate to study the cells directly, rather than by means involving culture media.

The study of gene and protein expression may be suitable in this regard. However, in this study, only two genes were studied. In reality, several genes may be responsible for potential probiotic microorganisms to exhibit certain functional properties. Thus, it may be better to explore the relationship of various genes and proteins to certain functional properties, and how changes in their expression due to stress affect the associated functional properties.

However, the experimental design may be justified on the basis that it better represents the scenario *in vivo*, whereby the probiotics would have to first go through stressful conditions, before finally reaching the large intestine, which is their site of action, and which is a suitable environment of their growth. Further to this, it may have been appropriate to additionally study the gene expression in bacteria which had recovered after exposure to short-term or prolonged stress. This may have given an indication of whether the changes in the expression of certain genes related to a functional property persist when the stress is no longer there. This could also have been applied to the methods used for assessing the functional properties, i.e. functional properties should have been assessed after clear recovery from the stress, such that it would demonstrate whether exposure to stress can result in changes in heritable phenotypes.

In this regard, the concept of epigenetics, which studies modifications to DNA, which do not involve DNA mutation (Kasuga and Gijzen 2013), could be explored. It could be suggested that the exposure of the bifidobacteria to stress may result in epigenetic changes which are passed on to the next generation. However the design of this study was not able to support the investigation of this theory.

CHAPTER SEVEN: CONCLUSION AND SUGGESTIONS FOR FUTURE WORK

This study comparatively assessed some functional properties of commercial and non-commercial bifidobacteria which were exposed to stress conditions and unstressed. The aim was to evaluate whether the functional properties of probiotic microorganisms, which can be indicators of the potential to provide health benefit, are affected positively or negatively, when exposed to stress conditions. This study was considered necessary because of the gaps in knowledge about the exact mechanisms by which probiotics can exert their health benefits. Studying the effects of stress on *in vitro* functional properties was considered as a means of understanding what may pertain in reality.

Candidate probiotic microorganisms are assessed in a ‘native’ state, for their ability to tolerate stressful conditions of the gut, and also for other properties that suggest they would be beneficial, such as production of antimicrobial substances. However, there have been no studies on bifidobacteria to demonstrate that their probiotic beneficial properties are not changed after exposure to inevitable stress conditions. Therefore, in this study, the microorganisms were exposed short-term to individual stress conditions and then assessed for their functional properties.

The study of antimicrobial activity suggested that the main effect of stress is on the growth rate of bifidobacteria, which may affect the amount of inhibitory substances present, although this is only relevant with the time at which the measurement is carried out. Antimicrobial activity was not lost as a result of exposure to stress. However it is not clear what implications a slower growth rate (extended lag phase) may have for actual probiotic benefit in a real sense.

The study of antibiotic susceptibility revealed no clear effect of stress, suggesting that probiotic microorganisms are neither more sensitive nor more resistant to antibiotics because of stress. An absence of increased sensitivity implies that they can be co-administered with antibiotics, and that their potential to provide probiotic benefit is not diminished.

The study of biofilm formation showed that stress may stimulate biofilm formation, but in a strain or species-dependent manner. The apparent stimulation of biofilm formation by stress in the commercial strains could possibly justify the prevalence of certain probiotic strains over others. It could imply that some beneficial properties require exposure to stress in order to be exhibited. This idea may be crucial to the understanding of how probiotics work.

To better understand the relationship between stress response and other probiotic functional properties, a molecular approach would be better placed to study this. Genes and proteins which could act as biomarkers for certain functional properties would need to be identified, and changes in their expression under different stress conditions would be useful as an indicator of the influence of stress on functionality.

In addition, whilst this research looked at stress conditions individually, future work could study the probiotic microorganisms after exposure to consecutive stresses or combinations of stress conditions. This may be more representative of what could occur in reality, and may also reveal differences because of exposure to a previous stress.

Further to this, the study of the impact of conditions encountered during processing and propagation of probiotic cultures may be helpful to understand if technological stress conditions have a role in strain differences and also on the relationship between subsequent gastrointestinal stress and beneficial functional properties.

The study documented here was focused on some members of *Bifidobacterium* spp. Future studies could see an expansion to other bifidobacteria, as well as other probiotic groups such as *Lactobacillus* spp., *Lactococcus* spp., etc. More *in vitro* functional properties could be studied as well, and the studies of gastrointestinal stress could be carried out by using *in vitro* gut models in order to give an even more realistic representation of how gastrointestinal stress could affect functional properties. The influence of the food matrix and cold storage on probiotic functional properties could also be studied. Functional properties could be studied at different lengths of storage in the food carrier, using a molecular approach to assess whether certain genes are induced or repressed by components of the food products, and if this has any impact on functional properties, and in particular, after subsequent exposure to gastrointestinal stress. Furthermore, it could be useful to study the impact of host factors and other gut microbiota on the functional properties of probiotic microorganisms. These studies could contribute to the understanding of probiotic functionality and the interaction between these properties and different conditions encountered by probiotics.

REFERENCES

Abe, F., Muto, M., Yaeshima, T., Iwatsuki, K., Aihara, H., Ohashi, Y. and Fujisawa, T. (2010) Safety evaluation of probiotic bifidobacteria by analysis of mucin degradation activity and translocation ability. *Anaerobe*, **16:** 131-136

Ahn, J. B., Hwang, H. –J. and Park, J. –H. (2001) Physiological responses of oxygen-tolerant anaerobic *Bifidobacterium longum* under oxygen. *Journal of Microbiology and Biotechnology*, **11(3):** 443-451

Al-Nabulsi, A. A., Osaili, T. M., Elabedeen, N. A. Z., Jaradat, Z. W., Shaker, R. R., Kheirallah, K. A. *et al.* (2011) Impact of environmental stress desiccation, acidity, alkalinity, heat or cold on antibiotic susceptibility of *Cronobacter sakazakii*. *International Journal of Food Microbiology*, **146:** 137-143

Alvarez-Ordonez, A., Begley, M., Prieto, M., Messens, W., Lopez, M., Bernardo, A. and Hill, C. (2011) *Salmonella* spp. survival strategies within the host gastrointestinal tract. *Microbiology*, **157:** 3268-3281

Alvarez-Ordonez, A., Fernandez, A., Bernardo, A. and Lopez, M. (2010) Acid tolerance in *Salmonella typhimurium* induced by culturing in the presence of organic acids at diferente growth temperatures. *Food Microbiology*, **27:** 44-49

Ammor, M. S., Florez, A. B. and Mayo, B. (2007) Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. *Food Microbiology*, **24:** 559-570

Ammor, M. S., Florez, A. B., Alvarez-Martin, P., Margolles, A. and Mayo, B. (2008a) Analysis of tetracycline resistance *tet*(W) genes and their flanking sequences in intestinal *Bifidobacterium* species. *Journal of Antimicrobial Chemotherapy*, **62:** 688-693

Ammor, M. S., Florez, A. B., van Hoek, A. H. A. M., de los Reyes-Gavilan, C. G., Aarts, H. J. M., Margolles, A. and Mayo, B. (2008b) Molecular characterization of intrinsic and acquired antibiotic resistance in lactic acid bacteria and bifidobacteria. *Journal of Molecular Microbiology and Biotechnology*, **14:** 6-15

Archer, D. L. (1996) Preservation microbiology and safety: evidence that stress enhances virulence and triggers adaptive mutations. *Trends in Food Science and Technology*, **7:** 91-95

Arunachalam, K., Gill, H. S. and Chandra, R. K. (2000) Enhancement of natural immune function by dietary consumption of *Bifidobacterium lactis* (HN019). *European Journal of Clinical Nutrition*, **54:** 263-267

Ataie-Jafari, A., Larijani, B., Alavi Majd, H. and Tahbaz, F. (2009) Cholesterol-lowering effect of probiotic yogurt in comparison with ordinary yogurt in mildly to moderately hypercholesterolemic subjects. *Annals of Nutrition and Metabolism*, **54:** 22-27

Audy, J., Labrio, S., Roy, D. and LaPointe, G. (2010) Sugar source modulates exopolysaccharide biosynthesis in *Bifidobacterium longum* subsp. *longum* CRC 002. *Microbiology*, **156:** 653-664

Aureli, P., Capurso, L., Castellazzi, A. M., Clerici, M., Giovannini, M., Morelli, L. *et al.* (2011) Probiotics and health: an evidence-based review. *Pharmacological Research*, **63:** 366-376

Baker, H. C., Tran, D. N. and Thomas, L. V. (2009) Health benefits of probiotics for the elderly: a review. *Journal of Foodservice*, **20:** 250-262

Bakhtiar, S., LeBlanc, J. G., Salvucci, E., Ali, A., Martin, R., Langella, P. *et al.* (2013) Implications of the human microbiome in inflammatory bowel disease. *FEMS Microbiology Letters*, **342:** 10-17

Bao, Y., Zhang, Y., Zhang, Y., Liu, Y., Wang, S., Dong, X. *et al.* (2010) Screening of potential probiotic properties of *Lactobacillus fermentum* isolated from traditional dairy products. *Food Control*, **21:** 695-701

Barberis, C. M., Cittadini, R. M., Almuzara, M. N., Feinsilberg, A., Famiglietti, A. M., Ramirez, M. S. and Vay, C. A. (2012) Recurrent urinary infection with *Bifidobacterium scardovii*. *Journal of Clinical Microbiology*, **50(3):** 1086-1088

Baugher, J. L. and Klaenhammer, T. R. (2011) Application of omics tools to understanding probiotic functionality. *Journal of Dairy Science*, **94:** 4753-4765

Begley, M., Kerr, C. and Hill, C. (2009) Exposure to bile influences biofilm formation by *Listeria monocytogenes*. *Gut Pathogens*, **1:** 11

Behnsen, J., Deriu, E., Sassone-Corsi, M. and Raffatellu, M. (2013) Probiotics: Properties, examples, and specific applications. *Cold Spring Harbor Perpectives in Medicine*, **3:** a010074

Ben Amor, K., Breeuwer, P., Verbaarschot, P., Rombouts, F. M., Akkermans, A. D. L., De Vos, W. M. and Abee, T. (2002) Multiparametric flow cytometry and cell sorting for the assessment of viable, injured and dead *Bifidobacterium* cells during bile salt stress. *Applied and Environmental Microbiology*, **68(11):** 5209-5216

Bevilacqua, L., Ovidi, M., Di Mattia, E., Trovatelli, L. D. and Canganella, F. (2003) Screening of *Bifidobacterium* strains isolated from human faeces for antagonistic activities against potentially bacterial pathogens. *Microbiological Research*, **158:** 179-185

Biavati, B., Vescovo, M., Torriani, S. and Bottazzi, V. (2000) Bifidobacteria: History, ecology, physiology and applications. *Annals of Microbiology*, **50:** 117-131

Binnendijk, K. H. And Rijkers, G. T. (2013) What is a health benefit? An evaluation of EFSA opinions on health with reference to probiotics. *Beneficial Microbes*, **4(3):** 223-230

Birk, T., Takamiya Wik, M., Lametsch, R. and Knochel, S. (2012) Acid stress response and protein induction in *Campylobacter jejuni* isolates with different acid tolerance. *BMC Microbiology*, **12:** 174

Bjarnsholt, T., Alhede, M., Alhede, M., Eickhardt-Sorensen, R., Moser, C., Kuhl, M. *et al.* (2013) The *in vivo* biofilm. *Trends in Microbiology*, **21(9):** 466-474

Candela, M., Centanni, M., Fiori, J., Biagi, E., Turroni, S., Orrico, C. (2010) DnaK from *Bifidobacterium animalis* subsp. *lactis* is a surface-exposed

human plasminogen receptor upregulated in response to bile salts. *Microbiology*, **156:** 1609-1618

Chadwick, R., Henson, S., Moseley, B., Koenen, G., Liakopoulos, M., Midden, C., Palou, A., Rechkemmer, G., Schroder, D. and von Wright, A. (2003) *Functional Foods*. New York: Springer-Verlag Berlin Heidelberg

Champagne, C. P., Gardner, N. J. and Roy, D. (2005) Challenges in the addition of probiotic cultures to foods. *Critical Reviews in Food Science and Nutrition*, **45(1):** 61-84

Champomier-Verges, M., Zagorec, N. and Fadda, S. (2010) Proteomics: A tool for understanding lactic acid bacteria adaptation to stressful environments. In: Mozzi, F., Raya, R. R. and Vignolo, G. M. (eds.) *Biotechnology of Lactic Acid Bacteria: Novel Applications*. Iowa: Blackwell Publishing, pp: 57-72

Charteris, W. P., Kelly, P. M., Morelli, L. and Collins, J. K. (1998) Antibiotic susceptibility of potentially probiotic *Bifidobacterium* isolates from the human gastrointestinal tract. *Letters in Applied Microbiology*, **26:** 333-337

Charteris, W. P., Kelly, P. M., Morelli, L. and Collins, J. K. (1998) Ingredient selection criteria for probiotics microorganisms in functional fairy foods. *International Journal of Dairy Technology*, **51(4):** 123-136

Charteris, W. P., Kelly, P. M., Morelli, L. and Collins, J. K. (2000) Effect of conjugated oxgall salts on antibiotic susceptibility of oxgall salt-tolerant *Lactobacillus* and *Bifidobacterium* isolates. *Journal of Food Protection*, **63(10):** 1369-1376

Cheikhyoussef, A., Pogori, N., Chen, H., Tian, F., Chen, W., Tiang, J. and Zhang, H. (2009) Antimicrobial activity and partial characterization of bacteriocin-like inhibitiory substances (BLIS) produced by *Bifidobacterium infantis* BCRC 14602. *Food Control*, **20:** 553-559

Cheikhyoussef, A., Pogori, N., Chen, W. and Zhang, H. (2008) Antimicrobial proteinaceous compunds obtained from bifidobacteria: From production to their application. *International Journal of Food Microbiology*, **125:** 215-222

Chou, L. and Weimer, B. (1999) Isolation and characterization of acid- and bile-tolerant isolates from strains of *Lactobacillus acidophilus*. *Journal of Dairy Science*, **82:** 23-31

Chouraqui, J. P., Van Egroo, L. D. and Fichot, M. C. (2004) Acidified milk formula supplemented with *Bifidobacterium lactis*: impact on infant diarrhea in residential care settings. *Journal of Pediatric Gastroenterology and Nutrition*, **38:** 288-292

Collado, M. C. and Sanz, Y. (2007) Induction of acid resistance in *Bifidobacterium*: a mechanism for improving desirable traits of potentially probiotic strains. *Journal of Applied Microbiology*, **103:** 1147-1157

Collado, M. C., Gueimonde, M., Sanz, Y. and Salminen, S. (2006) Adhesion properties and competitive pathogen exclusion ability of bifidobacteria with acquired acid resistance. *Journal of Food Protection*, **69(7):** 1675-1679

Collado, M. C., Hernandez, M. and Sanz, Y. (2005) Production of bacteriocin-like compounds by human fecal *Bifidobacterium* strains. *Journal of Food Protection*, **68:** 1034-1040

Corcoran, B. M., Stanton, C., Fitzgerald, G. and Ross, R. P. (2008) Life under stress: The probiotic stress response and how it may be manipulated. *Current Pharmaceutical Design*, **14:** 1382-1399

Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R. and Lappin-Scott, H. M. (1995) Microbial biofilms. *Annual Reviews in Microbiology*, **49:** 711-745

D'Aimmo, M. R., Modesto, M. and Biavatti, B. (2007) Antibiotic resistance of lactic acid bacteria and *Bifidobacterium* spp. Isolated from dairy and pharmaceutical products. *International Journal of Food Microbiology*, **115:** 35-42

De Angelis, M. and Gobbetti, M. (2004) Environmental stress responses in *Lactobacillus*: a review. *Proteomics*, **4:** 106-122

De Dea Lindner, J., Canchaya, C., Zhang, Z., Neviani, E., Fitzgerald, G. F., van Sinderen, D. and Ventura, M. (2007) Exploiting *Bifidobacterium* genomes: the molecular basis of stress response. *International Journal of Food Microbiology*, **120:** 13-24

De Keersmaecker, S. C. J., Verhoeven, T. L. A., Desair, J., Marchal, K., Vanderleyden, J. and Nagy, I. (2006) Strong antimicrobial activity of *Lactobacillus rhamnosus* GG against *Salmonella typhimurium* is due to accumulation of lactic acid. *FEMS Microbiology Letters*, **259:** 89-96

Drago, L., Gismondo, M. R., Lombardi, A., de Haen, C. and Gozzini, L. (1997) Inhibition of in vitro growth of enteropathogens by new *Lactobacillus* isolates of human intestinal origin. *FEMS Microbiology Letters*, **153:** 455-463

Dunne, C., O'Mahony, L., Murphy, L., Thornton, G., Morrissey, D., O'Halloran, S. *et al.* (2001) *In vitro* selection criteria for probiotic bacteria of human origin: correlation with *in vivo* findings. *American Journal of Clinical Nutrition*, **73(suppl):** 386S-392S

Elkins, C. A. and Mullis, L. B. (2004) Bile-mediated aminoglycoside sensitivity in *Lactobacillus* species likely results from increased membrane permeability attributable to cholic acid. *Applied and Environmental Microbiology*, **70(12):** 7200-7209

European Food Safety Authority (2008) Technical guidance: Update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance. *EFSA Journal*, **732:** 1-15

European Food Safety Authority (2012) Scientific opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed. *EFSA Journal*, **10(12):** 3020

Extremina, C. I., Costa, L., Aguiar, A. I., Peixe, L. and Fonseca, A. P. (2011) Optimization of processing conditions for the quantification of enterococci biofilms using microtitre plates. *Journal of Microbiological Methods*, **84:** 167-173

Fanning, S., Hall, L. J., Cronin, M., Zomer, A., MacSharry, J., Goulding, D. *et al.* (2012) Bifidobacterial surface-exopolysaccharide facilitates commensal-host interaction through immune modulation and pathogen protection. *PNAS*, **109(6):** 2108-2113

FAO/WHO (2002) *Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food*

Faye, T., Tamburello, A., Vegarud, G. E. and Skeie, S. (2012) Survival of lactic acid bacteria from fermented milks in an in vitro digestion model exploiting sequential incubation in human gastric juice and duodenum juice. *Journal of Dairy Science*, **95:** 558-566

Fayol-Messaoudi, D., Berger, C. N., Coconnier-Polter, M. -H., Lievin-Le Moal, V. and Servin, A. L. (2005) pH-, lactic acid-, and non-lactic acid-dependent activities of probiotic lactobacilli against *Salmonella enterica* serovar Typhimurium. *Applied and Environmental Microbiology*, **71:** 6008-6013

Fernandez, A., Alvarez-Ordonez, A., Lopez, M. and Bernardo, A. (2009) Effects of organic acids on termal inactivation of acid and cold stressed *Enterococcus faecium*. *Food Microbiology*, **26:** 497-503

Fliss, I., Ouwehand, A. C., Kheadr, E., Lahtinen, S. and Davids, S. J. (2010) Antimicrobial activity of the genus *Bifidobacterium*. In: Mayo, B. and van Sinderen, D. (eds.) *Bifidobacteria: Genomics and Molecular Aspects*. Norfolk: Caister Academic Press, pp: 125-144

Foroni, E., Serafini, F., Amidani, D., Turroni, F., He, F., Bottacini, F. *et al.* (2011) Genetic analysis and morphological identification of pilus-like structures in members of the genus *Bifidobacterium*. *Microbial Cell Factories*, **10(Suppl 1):** S16

Gahan, C. G. M. and Hill, C. (1999) The relationship between acid stress responses and virulence in *Salmonella typhimurium* and *Listeria monocytogenes*. *International Journal of Food Microbiology*, **50:** 93-100

Goderska, K. and Stanton, C. (2010) Viability and stability of bifidobacteria in commercial preparations. In: Mayo, B. and van Sinderen, D. (eds.) *Bifidobacteria: Genomics and Molecular Aspects*. Norfolk: Caister Academic Press, pp: 217-234

Gonzalez, R., Klaassens, E. S., Malinen, E., de Vos, W. M. and Vaughan, E. E. (2008) Differential transcriptional response of *Bifidobacterium longum* to human milk, formula milk, and galactooligosaccharide. *Applied and Environmental Microbiology*, **74(15):** 4686-4694

Granato, D., Branco, G. F., Cruz, A. G., Faria, J. A. F. and Shah, N. P. (2010a) Probiotic dairy products as functional foods. *Comprehensive Reviews in Food Science and Food Safety*, **9:** 455-470

Granato, D., Branco, G. F., Nazzaro, F., Cruz, A. G. and Faria, J. A. F. (2010b) Functional foods and nondairy probiotic food development: trends, concepts and products. *Comprehensive Reviews in Food Science and Food Safety*, **9:** 292-302

Grzeskowiak, L., Isolauri, E., Salminen, S. and Gueimonde, M. (2011) Manufacturing process influences properties of probiotic bacteria. *British Journal of Nutrition*, **105:** 887-894

Gueimonde, M., Florez, A. B., van Hoek, H. A. M., Stuer-Lauridsen, B., Stroman, P., de los Reyes-Gavilan, C. G. and Margolles, A. (2010) Genetic

basis of tetracycline resistance in *Bifidobacterium animalis* subsp. *lactis*. *Applied and Environmental Microbiology*, **76(10):** 3364-3369

Gueimonde, M., Sanchez, B., de los Reyes-Gavilan, C. G. and Margolles, A. (2013) Antibiotic resistance in probiotic bacteria. *Frontiers in Microbiology*, **4(202)**

Guglielmetti, S., Tamagnini, I., Minuzzo, M., Arioli, S., Carlo, P., Comelli, E. and Mora, D. (2009) Study of the adhesion of *Bifidobacterium bifidum* MIMBb75 to human intestinal cell lines. *Current Microbiology*, **59:** 167-172

Hammad, A. M. and Shimamoto, T. (2010) Towards a compatible probiotic-antibiotic combination therapy: assessment of antimicrobial resistance in the Japanese probiotics. *Journal of Applied Microbiology*, **109:** 1349-1360

He, T., Priebe, M. G., Zhong, Y., Huang, C., Harmsen, H. J., Raangs, G. C. *et al.* (2008) Effects of yogurt and bifidobacteria supplementation on the colonic microbiota in lactose-intolerant subjects. *Journal of Applied Microbiology*, **104:** 595-604

Heller, K. J. (2001) Probiotic bacteria in fermented foods: product characteristics and starter organisms. *American Journal of Clinical Nutrition*, **73(suppl):** 374S-379S

Hernandez-Hernandez, O., Muthaiyan, A., Moreno, F. J., Montilla, A., Sanz, M. L. and Ricke, S. C. (2012) Effect of prebiotic carbohydrates on the growth and tolerance of *Lactobacillus*. *Food Microbiology*, **30:** 355-361

Huang, Y. –M., Kan, B., Lu, Y. and Szeto, S. (2009) The effect of osmotic shock on RpoS expression and antibiotic resistance in *Escherichia coli*. *Journal of Experimental Microbiology and Immunology*, **13:** 13-17

Hutt, P., Shchepetova, J., Loivukene, K., Kullisaar, T. and Mikelsaar, M. (2006) Antagonistic activity of probiotic lactobacilli and bifidobacteria against entero- and uropathogens. *Journal of Applied Microbiology*, **100:** 1324-1332

Ishibashi, N. and Yamazaki, S. (2001) Probiotics and safety. *American Journal of Clinical Nutrition*, **73(suppl):** 465S-470S

Isolauri, E. and Salminen, S. (2000) Functional foods and acute infections: probiotics and gastrointestinal disorders. In: Gibson, G. R. and Williams, C. M. (eds.) *Functional Foods: Concept to Product*. Boca Raton: Woodhead Publishing Limited, pp. 167-180

Jalili, H., Razavi, S. H., Safari, M. and Malcata, F. X. (2009) Enhancement of growth rate and β-galactosidase activity, and variation in organis acid profile of *Bifidobacterium animalis* subsp. *lactis* Bb 12. *Enzyme and Microbial Technology*, **45:** 469-476

Jancovic, I., Sybesma, W., Phothirath, P., Ananta, E. and Mercenier, A. (2010) Applications of probiotics in food products – challenges and new approaches. *Current Opinion in Biotechnology*, **21:** 175-181

Jayamanne, V. S. and Adams, M. R. (2006) Determination of survival, identity and stress resistance of probiotic bifidobacteria in bio-yoghurts. *Letters in Applied Microbiology*, **42:** 189-194

Jayamanne, V. S. and Adams, M. R. (2009) Modelling the effects of pH, storage temperature and redox potential (E_h) on the survival of bifidobacteria in fermented milk. *International Journal of Food Science and Technology*, **44:** 1131-1138

Jia, L., Shigwedha, N. and Mwandembele, O. D. (2010) Use of D_{acid}-, D_{bile}-, z_{acid}-, and z_{bile}- values in evaluating bifidobacteria with regard to stomach pH and bile salt sensitivity. *Journal of Food Science*, **75(1):** M14-M18

Jiang, T. A., Mustapha, A. and Savaiano, D. A. (1996) Improvement of lactose digestion in humans by ingestion of unfermented milk containing *Bifidobacterium longum*. *Journal of Dairy Science*, **79:** 750-757

Juge, N. (2012) Microbial adhesins to gastrointestinal mucus. *Trends in Microbiology*, **20(1):** 30-39

Kasuga, T. and Gijzen, M. (2013) Epigenetics and the evolution of virulence. *Trends in Microbiology*, **21(11):** 575-582

Katan, M. B. (2012) Why the European Food Safety Authority was right to reject health claims for probiotics. *Beneficial Microbes*, **3(2):** 85-89

Katz, J. A. (2006) Probiotics for the prevention of antibiotic-associated diarrhea and *Clostridium difficile* diarrhea. *Journal of Clinical Gastroenterology*, **40(3):** 249-255

Kazimierczak, K. A., Flint, H. J. and Scott, K. P. (2006) Comparative analysis of sequences flanking *tet*(W) resistance genes in multiple species of gut bacteria. *Antimicrobial Agents and Chemotherapy*, **50:** 2632-2639

Kheadr, E. E. (2006) Impact of acid and oxgall on antibiotic susceptibility of probiotic lactobacilli. *African Journal of Agricultural Research*, **1(5):** 172-181

Kheadr, E., Dabour, N., Le Lay, C., Lacroix, C. and Fliss, I. (2007) Antibiotic susceptibility profile of bifidobacteria as affected by oxgall, acid and hydrogen peroxide stress. *Antimicrobial Agents and Chemotherapy*, **51(1):** 169-174

Klijn, A., Mercenier, A. and Arigoni, F. (2005) Lessons from the genomes of bifidobacteria. *FEMS Microbiology Reviews*, **29:** 491-509

Kroukamp, O., Dumitrache, R. G. and Wolfaardt, G. M. (2010) Pronounced effect of the nature of the inoculums on biofilm development in flow systems. *Applied and Environmental Microbiology*, **76(18):** 6025-6031

Lacroix C. and Yildirim, S. (2007) Fermentation technologies for the production of probiotics with high viability and functionality. *Current Opinion in Biotechnology*, **18:** 176-183

Lahtinen, S. J., Jalonen, L., Ouwehand, A. C. and Salminen, S. J. (2007) Specific *Bifidobacterium* strains isolated from elderly subjects inhibit growth of *Staphylococcus aureus*. *International Journal of Food Microbiology*, **117:** 125-128

Lebeer, S., Vanderleyden, J. and De Keersmaecker, S. C. J. (2008) Gene and molecules of lactobacilli supporting probiotic action. *Microbiology and Molecular Biology Reviews*, **72(4):** 728-764

Lebeer, S., Verhoeven, T. L. A., Vetez, M. P., Vanderleyden, J. and De Keersmaecker, S. C. J. (2007) Impact of environmental and genetic factors

on biofilm formation by the probiotic strain *Lactobacillus rhamnosus* GG. *Applied and Environmental Microbiology*, **73(21):** 6768-6775

Lee, H. Y., Park, J. H., Seik, S. H., Cho, S. A., Baek, M. W., Kim, D. J. *et al.* (2004) Dietary intake of various lactic acid bacteria suppresses type 2 helper T cell production in antigen-primed mice splenocyte. *Journal of Microbiology and Biotechnology*, **14:** 167-170

Lee, J. –H. And O'Sullivan, D. J. (2010) Genomic insights into bifidobacteria. *Microbiology and Molecular Biology Reviews*, **74(3):** 378-416

Lee, K., Lee, H. –G., Pi, K. and Choi, Y. J. (2008) The effect of low pH on protein expression by the probiotic bacterium *Lactobacillus reuteri*. *Proteomics*, **8:** 1624-1630

Lee, Y. –J., Yu, W. –K. and Heo, T. –R. (2003) Identification and screening for antmicrobial activity against *Clostridium difficile* of *Bifidobacterium* and *Lactobacillus* species isolated from healthy infant species. *International Journal of Antimicrobial Agents*, **21:** 340-346

Leivers, S., Hidalgo-Cantabrana, C., Robinson, G., Margolles, A., Ruas-Madiedo, P. and Laws, A. P. (2011) Structure of the high molecular weight exopolysaccharide produced by *Bifidobacterium animalis* subsp. *lactis* IPLA-R1 and sequence analysis of its putative *eps* cluster. *Carbohydrate Research*, **346:** 2710-2717

Le Leu, R. K., Hu, Y., Brown, I. L., Woodman, R. J. and Young, G. P. (2010) Synbiotic intervention of *Bifidobacterium lactis* and resistant starch protects against colorectal cancer development in rats. *Carcinogenesis*, **31:** 246-251

Li, Q., Chen, Q., Ruan, H., Zhu, D. and He, G. (2010) Isolation and characterisation of an oxygen, acid and bile resistant *Bifidobacterium animalis* subsp. *lactis* Qq08. *Journal of the Science of Food and Agriculture*, **90:** 1340-1346

Li, Y., Shimizu, T., Hosaka, A., Kaneko, N., Ohtsuka, Y. and Yamashiro, Y. (2004) Effects of *Bifidobacterium breve* supplementation on intestinal flora of low birth weight infants. *Pediatrics International*, **46:** 509-515

Lim, K. S., Huh, C. S. and Baek, Y. J. (1993) Antimicrobial susceptibility of bifidobacteria. *Journal of Diary Science*, **76:** 2168-2174

Lopez, P., Monteserin, D. C., Gueimonde, M., de los Reyes-Gavilan, C. G., Margolles, A., Suarez, A. and Ruas-Madiedo, P. (2012) Exopolysaccharide-producing *Bifidobacterium* strains elicit different *in vitro* responses upon interaction with human cells. *Food Research International*, **46:** 99-107

Macfarlane, S. and Macfarlane, G. T. (2006) Composition and metabolic activities of bacterial biofilms colonizing food residues in the human gut. *Applied and Environmental Microbiology*, **72(9):** 6204-6211

Mackey, B. M. (2000) Injured bacteria. In: Lund, A. M., Baird-Parker, T. C. and Gould, G. W. (eds.) *The Microbiological Safety and Quality of Food*. Gaithersburg: Aspen Publishers Inc., pp: 315-341

Makinen, K., Berger, B., Bel-Rhlid, R. and Ananta, E. (2012) Science and technology for the mastership of probiotic applictions in food products. *Journal of Biotechnology*, **162:** 356-365

Makras, L. and De Vuyst, L. (2006) The *in vitro* inhibition of Gram-negative pathogenic bacteria by bifidobacteria is caused by the production of organi acids. *International Dairy Journal*, **16:** 1049-1057

Makras, L., Triantafyllou, V., Fayol-Messaoudi, D., Adriany, T., Zoumpopoulou, G., Tsakalidou, E. *et al.* (2006) Kinetic analysis of the antibacterial activity of probiotic lactobacilli towards *Salmonella enterica* serovar Typhimurium reveals a role for lactic acid and other inhibitory compounds. *Research in Microbiology*, **157:** 241-247

Maragkoudakis, P. A., Zoumpopoulou, G., Miaris, C., Kalantzopoulos, G., Pot, B. and Tsakalidou, E. (2006) Probiotic potential of *Lactobacillus* strains isolated from dairy products. *International Dairy Journal*, **16:** 189-199

Margolles, A., Garcia, L., Sanchez, B., Gueimonde, M. and de los Reyes Gavilan, C. G. (2003) Characterisation of a *Bifidobacterium* strain with acquired resistance to cholate: a preliminary study. *International Journal of Food Microbiology,* **82:** 191-198

Margolles, A. and Sanchez, B. (2012) Selection of a *Bifidobacterium animalis* subsp. *lactis* strain with decreased ability to produce acetic acid. *Applied and Environmental Microbiology*, **78(9):** 3338-3342

Marianelli, C., Cifani, N. and Pasquali, P. (2010) Evaluation of antimicrobial activity of probiotic bacteria against *Salmonella enterica* subsp. *enterica* serovar typhimurium 1344 in a common medium under different environmental conditions. *Research in Microbiology*, **161:** 673-680

Marteau, P. R., de Vrese, M., Cellier, C. J. and Schrezenmeir, J. (2001) Protection from gastrointestinal diseases with the use of probiotics. *American Journal of Clinical Nutrition*, **73:** 430S-436S

Masco, L., Van Hoorde, K., De Brandt, E., Swings, J. and Huys, G. (2006) Antimicrobial susceptibility of *Bifidobacterium* strains from humans, animals and probiotic products. *Journal of Antimicrobial Chemotherapy*, **58:** 85-94

Maus, J. E. and Ingham, S. C. (2003) Employment of stressful conditions during culture production to enhance subsequent cold- and acid-tolerance of bifidobacteria. *Journal of Applied Microbiology*, **95:** 146-154

Mayo, B., Mayrhofer, S., Delgado, S. and Domig, K. J. (2010) Antibiotic resistance/susceptibility profiles of bifidobacteria and molecular analysis of atypical resistances. In: Mayo, B. and van Sinderen, D. (eds.) *Bifidobacteria: Genomics and Molecular Aspects*. Norfolk: Caister Academic Press, pp: 195-215

McMahon, M. A. S., Blair, I. S., Moore, J. E. and McDowell, D. A. (2007a) The rate of horizontal transmission of antibiotic resistance plasmids is increased in food preservation-stressed bacteria. *Journal of Applied Microbiology*, **103:** 1883-1888

McMahon, M. A. S., Xu, J., Moore, J. E., Blair, I. S. and McDowell, D. A. (2007b) Environmental stress and antibiotic resistance in food-related pathogens. *Applied and Environmental Microbiology*, **73(1):** 211-217

Mills, S., Stanton, C., Fitzgerald, G. F. and Ross, R. P. (2011) Enhancing the stress responses of probiotics for a lifestyle from gut to product and back again. *Microbial Cell Factories*, **10(Suppl 1):** S19

Molin, G. (2001) Probiotics in foods not containing milk or milk constituents, with special reference to *Lactobacillus plantarum* 299v. *American Journal of Clinical Nutrition*, **73(suppl):** 380S-385S

Mols, M. and Abee, T. (2011) *Bacillus cereus* responses to acid stress. *Environmental Microbiology*, **13(11):** 2835-2843

Moorhead, S. M. and Dykes, G. A. (2004) Influence of the *sigB* gene on the cold stress survival and subsequent recovery of two *Listeria monocytogenes* serotypes. *International Journal of Food Microbiology*, **91:** 63-72

Moubareck, C., Gavini, F., Vaugien, L., Butel, M. J. and Doucet-Populaire, F. (2005) Antimicrobial susceptibility of bifidobacteria. *Journal of Antimicrobial Chemotherapy*, **55:** 38-44

National Committee for Clinical Laboratory Standards (2000) *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically*; Approved standard – fifth edition M7-A5. National Committee for Clinical Laboratory Standards

Nazzaro, F., Fratianni, F., Nicolaus, B., Poli, A. and Orlando, P. (2012) The prebiotic source influences the growth, biochemical features and survival under simulated gastrointestinal conditions of the probiotic *Lactobacillus acidophilus*. *Anaerobe*, **18:** 280-285

Ninomiya, K., Matsuda, K., Kawahata, T., Kanaya, T., Kohno, M., Katakura, Y. *et al.* (2009) Effect of CO_2 concentration on the growth and exopolysaccharide production of *Bifidobacterium longum* cultivated under anaerobic conditions. *Journal of Bioscience and Bioengineering*, **107(5):** 535-537

Nivoliez, A., Camares, O., Paquet-Gachinat, M., Bornes, S., Forestier, C. and Veisseire, P. (2012) Influence of manufacturing processes on *in vitro* properties of the probiotic strain *Lactobacillus rhamnosus* Lcr35®. *Journal of Biotechnology*, **160:** 236-241

Noriega, L., de los Reyes-Gavilan, C. G. and Margolles, A. (2005) Acquisition of bile salt resistance promotes susceptibility changes in *Bifidobacterium*. *Journal of Food Protection*, **68(9):** 1916-1919

Noriega, L., Gueimonde, M., Sanchez, B., Margolles, A. and de los Reyes-Gavilan, C. G. (2004) Effect of the adaptation to high bile salts concentrations on glycosidic activity, survival at low pH and cross-resistance to bile salts in *Bifidobacterium*. *International Journal of Food Microbiology*, **94:** 79-86

O’Brien, J., Crittenden, R., Ouwehand, A. C. and Salminen, S. (1999) Safety evaluation of probiotics. *Trends in Food Science and Technology*, **10:** 418-424

Oelschlaeger, T. A. (2010) Mechanisms of probiotic actions – A review. *International Journal of Medical Microbiology*, **300:** 57-62

O'Grady, B. and Gibson, G. R. (2005) Microbiota of the human gut. In: Tamime, A. Y. (ed.) *Probiotic Dairy Products*. Oxford: Blackwell Publishing Ltd., pp: 1-15

Ohishi, A., Takahashi, S., Ito, Y., Ohishi, Y., Tsukamoto, K., Nanba, Y. *et al.* (2010) *Bifidobacterium* septicaemia associated with postoperative probiotic therapy in a neonate with omphalocele. *Journal of Pediatrics*, **156(4):** 679-681

Ouwehand, A. C., Kirjavainen, P. V., Shortt, C. and Salminen, S. (1999) Probiotics: mechanisms and established effects. *International Dairy Journal*, **9:** 43-52

Ozer, B. H. and Kirmaci, H. A. (2010) Functional milks and dairy beverages. *International Journal of Dairy Technology*, **63(1):** 1-15

Pan, W. –H., Li, P. –L. and Liu, Z. (2006) The correlation between surface hydrophobicity and adherence of *Bifidobacterium* strains from centenarians' faeces. *Anaerobe*, **12:** 148-152

Pan, X., Chen, F., Wu, T., Tang, H. and Zhao, Z. (2009) The acid, bile tolerance and antimicrobial proerty of *Lactobacillus acidophilus* NIT. *Food Control*, **20:** 598-602

Parvez, S., Malik, K. A., Ah Kang, S. and Kim, H. –Y. (2006) Probiotics and their fermented food products are beneficial for health. *Journal of Applied Microbiology*, **100:** 1171-1185

Perez, B. A., Planet, P. J., Kachlany, S. C., Tomich, M., Fine, D. H and Figurski, D. H. (2006) Genetic analysis of the requirement for *flp-2*, *tadV* and

rcpB in *Actinobacillus actinomycetemcomitans* biofilm formation. *Journal of Bacteriology*, **188(17):** 6361-6375

Perez, P. F., Minnaard, Y., Disalvo, E. A. and De Antoni, G. L. (1998) Surface properties of bifidobacterial strains of human origin. *Applied and Environmental Microbiology*, **64:** 21-26

Prado, F. C., Parada, J. L., Pandey, A. and Soccol, C. R. (2008) Trends in non-dairy probiotic beverages. *Food Research International*, **41:** 111-123

Prasad, J., Gill, H., Smart, J. and Gopal, P. K. (1998) Selection and characterisation of *Lactobacillus* and *Bifidobacterium* strains for use as probiotics. *International Dairy Journal*, **8:** 993-1002

Prasad, J., McJarrow, P. and Gopal, P. (2003) Heat and osmotic stress response of probiotic *Lactobacillus rhamnosus* HN001 (DR20) in relation to viability after drying. *Applied and Environmental Microbiology*, **69(2):** 917-925

Prasanna, P. H. P., Grandison, A. S. and Charalampopoulos, D. (2012) Screening human intestinal *Bifidobacterium* strains for growth, acidification, EPS production and viscosity potential in low-fat milk. *International Dairy Journal*, **23:** 36-44

Probert, H. M. and Gibson, G. R. (2002) Bacterial biofilms in the human gastrointestinal tract. *Current Issues in Intestinal Microbiology*, **3:** 23-27

Qian, Y., Borowski, W. J. and Calhoon, W. D. (2011). Intracellular granule formation in response to oxidative stress in *Bifidobacterium*. *International Journal of Food Microbiology*, **145:** 320-325

Raeisi, S. N., Ouoba, L. I. I., Farahmand, N., Sutherland, J. and Ghoddusi, H. B. (2013) Variation, viability and validity of bifidobacteria in fermented milk products. *Food Control*, **34:** 691-697

Ranadheera, R. D. C. S., Baines, S. K. and Adams, M. C. (2010) Importance of food in probiotic efficacy. *Food Research International*, **43:** 1-7

Ray, B. and Bhunia, A. (2008) Microbial stress response in the food environment. *Fundamental Food Microbiology*, 4th edition. Boca Raton: CRC Press, pp. 81-97

Reid, G. (2006) Safe and efficacious probiotics: what are they? *Trends in Microbiology*, **14(8)**

Rivera-Espinoza, Y. and Gallardo-Navarro, Y. (2010) Non-dairy probiotic products. *Food Microbiology*, **27:** 1-11

Roger, L. C., Costabile, A., Holland, D. T., Hoyles, L. and McCartney, A. L. (2010) Examination of faecal *Bifidobacterium* populations in breast- and formula-fed infants during the first 18 months of life. *Microbiology*, **156:** 3329-3341

Ross, R. P., Desmond, C., Fitzgerald, G. F. and Stanton, C. (2005) Overcoming the technological hurdles in the development of probiotic foods. *Journal of Applied Microbiology*, **98:** 1410-1417

Rowland, I. R., Rumney, C. J., Coutts, J. T. and Lievense, L. C. (1998) Effect of *Bifidobacterium longum* and inulin on gut bacterial metabolism and carcinogen-induced aberrant crypt foci in rats. *Carcinogenesis*, **19:** 281-285

Roy, D. (2003) Media for the detection and enumeration of bifidobacteria in food products. In: Corry, J. E. L., Curtis, G. D. W. and Baird, R. M. (eds.) *Handbook of Culture Media for Food Microbiology*. Amsterdam: Elsevier Science B. V., pp: 147-160

Ruas-Madiedo, P., Guiemonde, M., Arigoni, F., de los Reyes-Gavilan, C. G. and Margolles, A. (2009) Bile affects the synthesis of exopolysaccharides by *Bifidobacterium animalis*. *Applied and Environmental Microbiology*, **75(4):** 1204-1207

Ruas-Madiedo, P., Medrano, M., Salazar, N., de los Reyes-Gavilan, C. G., Perez, P. F. and Abraham, A. G. (2010) Exopolysaccharides produced by *Lactobacillus* and *Bifidobacterium* strains abrogate *in vitro* the cytotoxic effect of bacterial toxins on eukaryotic cells. *Journal of Applied Microbiology*, **109:** 2079-2086

Ruas-Madiedo, P., Moreno, J. A., Salazar, N., Delgado, S., Mayo, B., Margolles, A. and de los Reyes-Gavilan, C. G. (2007) Screening of exopolysaccharide-producing *Lactobacillus* and *Bifidobacterium* strains isolated from the human intestinal microbiota. *Applied and Environmental Microbiology*, **73(13):** 4385-4388

Ruiz, L., Gueimonde, M., Ruas-Madiedo, P., Ribbera, A., de los Reyes-Gavilan, C. G., Ventura, M. *et al.* (2012) Molecular cues to understand the aerotolerance phenotype of *Bifidobacterium animalis* subsp. *lactis*. *Applied and Environmental Microbiology*, **78(3):** 644-650

Ruiz, L., Ruas-Madiedo, P., Gueimonde, M., de los Reyes-Gavilan, C. G., Margolles, A. and Sanchez, B. (2011) How do bifidobacteria counteract environmental challenges? Mechanisms involved and physiological consequences. *Genes and Nutrition*, **6:** 307-318

Ruiz, L., Sanchez, B., Ruas-Madiedo, P., de los Reyes-Gavilan, C. G. and Margolles, A. (2007) Cell envelope changes in *Bifidobacterium animalis* ssp. *lactis* as a response to bile. *FEMS Microbiology Letters*, **274:** 316-322

Russell, D. A., Ross, R. P. Fitzgerald, G. F. and Stanton, C. (2011) Metabolic activities and probiotic potential of bifidobacteria. *International Journal of Food Microbiology*, **149:** 88-105

Saarela, M., Maukonen, J., von Wright, A., Vilpponen-Salmela, T., Patterson, A. J., Scott, K. P. *et al.* (2007) Tetracycline susceptibility of the ingested *Lactobacillus acidophilus* LaCH-5 and *Bifidobacterium animalis* subsp. *lactis* Bb-12 strains during antibiotic/probiotic intervention. *International Journal of Antimicrobial Agents*, **29:** 271-280

Saarela, M., Mogensen, G., Fonden, R., Matto, J. and Mattila-Sandholm, T. (2000) Probiotic bacteria: safety, functional and technological properties. *Journal of Biotechnology*, **84:** 197-215

Saavedra, J. M., Bauman, N. A., Oung, I., Perman, J. A. and Yolken, R. H. (1994) Feeding *Bifidobacterium bifidum* and *Streptococcus thermophilus* to infants in hospital for prevention of diarrhoea and shedding of rotavirus. *Lancet*, **344:** 1046-1049

Salminen, S. and van Loveren, H. (2012) Probiotics and prebiotics: health claim substantiation. *Microbial Ecology in Health and Disease*, **23:** 18568

Sanchez, B., Champomier-Verges, M. C., Anglade, P., Baraige, F., de los Reyes-Gavilan, C. G., Margolles, A. and Zagorec, M. (2005) Proteomic analysis of global changes in protein expression during bile salt exposure of *Bifidobacterium longum* NCIMB 8809. *Journal of Bacteriology*, **187:** 5799-5808

Sanchez, B., Champomier-Verges, M., del Carmen Collado, M., Anglade, P., Baraige, F., Sanz, Y. *et al.* (2007a) Low-pH adaptation and the acid tolerance response of *Bifidobacterium longum* biotype longum. *Applied and Environmental Microbiology*, **73(20):** 6450-6459

Sanchez, B., Champomier-Verges, M., Stuer-Lauridsen, B., Ruas-Madiedo, P., Anglade, P., Baraige, F. *et al.* (2007b) Adaptation and response of *Bifidobacterium animalis* subsp. lactis to bile: a proteomic and physiological approach. *Applied and Environmental Microbiology*, **73(21):** 6757-6767

Sanchez, B., Fernandez-Garcia, M., Margolles, A., de los Reyes-Gavilan, C. G. and Ruas-Madiedo, P. (2010a) Technological and probiotic selection criteria of a bile-adapted *Bifidobacterium animalis* subsp. *lactis* strain. *International Dairy journal*, **20:** 800-805

Sanchez, B., Ruiz, L., van Sinderen, D., Margolles, A. and Zomer, A. L. (2010b) Acid and bile resistance and stress response in bifidobacteria. In: Mayo, B. and van Sinderen, D. (eds.) *Bifidobacteria: Genomics and Molecular Aspects*. Norfolk: Caister Academic Press, pp: 71-96

Sanchez, B., Ruiz, L., Gueimonde, M., Ruas-Madiedo, P. and Margolles, A. (2012) Towrd improving technological and functional properties of probiotics in foods. *Trends in Food Science and Technology*, **26:** 56-63

Sandasi, M., Leonard, C. M. and Viljoen, A. M. (2010) The *in vitro* antibiofilm activity of selected culinary herbs and medicinal plants against *Listeria monocytogenes*. *Letters in Applied Microbiology*, **50:** 30-35

Schmidt, G. and Zink, R. (2000) Basic features of the stress response in three species of bifidobacteria: *B. longum*, *B. adolescentis*, and *B. breve*. *International Journal of Food Microbiology*, **55:** 21-25

Sekirov, I., Russell, S. L., Antunes, L. C. M. and Finlay, B. B. (2010) Gut microbiota in health and disease. *Physiological Reviews*, **90:** 859-904

Sela, D. A., Price, N. P. J. and Mills, D. A. (2010) Metabolism of bifidobacteria. In: Mayo, B. and van Sinderen, D. (eds.) *Bifidobacteria: Genomics and Molecular Aspects*. Norfolk: Caister Academic Press, pp: 45-70

Shah, N. P. (2007) Functional cultures and health benefits. *International Dairy Journal*, **17:** 1262-1277

Shah, N. P. and Ravula, R. R. (2000) Influence of water activity on fermentation, organic acids production and viability of yogurt and probiotic bacteria. *Australia Journal of Dairy Technology*, **55(3):** 127-131

Sheehan, V. M., Sleator, R. D., Hill, C. and Fitzgerald, G. F. (2007) Improving gastric transit, gastrointestinal persistence and therapeutic efficacy

of the probiotic strain *Bifidobacterium breve* UCC2003. *Microbiology*, **153:** 3563-3571

Shemesh, M., Tam, A. and Steinberg, D. (2007) Differrential gene expression profiling of *Streptococcus mutans* cultured under biofilm and planktonic conditions. *Microbiology*, **153:** 1307-1317

Shigwedha, N. and Jia, L. (2013) *Bifidobacterium* in human GI tract: Screening, isolation, survival and growth kinetics in simulated gastrointestinal conditions. In: Kongon, J. M. (ed.) *Lactic Acid Bacteria – R & D for Food, Health and Livestock Purposes*. InTech, pp. 281-308. Available on: www.intechopen.com/books/lactic-acid-bacteria-r-d-for-food-health-and-livestock-purposes

Simpson, P. J., Stanton, C., Fitzgerald, G. F. and Ross, R. P. (2005) Intrinsic tolerance of *Bifidobacterium* species to heat and oxygen and survival following spray drying and storage. *Journal of Applied Microbiology*, **99:** 493-501

Speranza, B., Corbo, M. R. and Sinigaglia, M. (2011) Effects of nutritional and environmental conditions on *Salmonella* sp. biofilm formation. *Journal of Food Science*, **76(1):** M12-M16

Stepanovic, S., Vukovic, D., Hola, V., Di Bonaventura, G., Djukic, S., Cirkovic, I. and Ruzicka, F. (2007) Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *APMIS*, **115:** 891-899

Stephens, P. J. and Mackey, B. M. (2003) Recovery of stressed microorganisms. In: Corry, J. E. L., Curtis, G. D. W. and Baird, R. M. (eds.) *Handbook of Culture Media for Food Microbiology*. Amsterdam: Elsevier Science B. V., pp: 25-48

Sybesma, W., Molenaar, D., van IJcken, W., Venema, K. and Kort, R. (2013) Genome instability in *Lactobacillus rhamnosus* GG. *Applied and Environmental Microbiology*, **79(7):** 2233-2239

Talwalkar, A. and Kailasapathy, K. (2003) Metabolic and biochemical responses of probiotic bacteria to oxygen. *Journal of Dairy Science*, **86:** 2537-2546

Talwalkar, A. and Kailasapathy, K. (2004) A review of oxygen toxicity in probiotic yogurts: Influence on the survival of probiotic bacteria and protective techniques. *Comprehensive Reviews in Food Science and Food Safety*, **3:** 117-124

Talwalkar, A., Miller, C. W., Kailasapathy, K. And Nguyen, M. H. (2004) Effect of packaging materials and dissolved oxygen on the survival of probiotic bacteria in yoghurt. *International Journal of Food Science and Technology*, **39:** 605-611

Tamime, A. Y., Marshall, V. M. E. and Robinson, R. K. (1995) Microbiological and technological aspects of milks fermented by bifidobacteria. *Journal of Dairy Research*, **62:** 151-187

Teitelbaum, J. E. and Walker, W. A. (2002) Nutritional impact of pre- and probiotics as protective gastrointestinal organisms. *Annual Reviews in Nutrition*, **22:** 107-138

Tejero-Sarinena, S., Barlow, J., Costabile, A., Gibson, G. R. and 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

Toure, R., Kheadr, E., Lacroix, C., Moroni, O. and Fliss, I. (2003) Production of antibacterial substances by bifidobacterial isolates from infant stool active against *Listeria monocytogenes*. *Journal of Applied Microbiology*, **95:** 1058-1069

Tuomola, E., Crittenden, R., Playne, M., Isolauri, E. and Salminen, S. (2001) Quality assurance criteria for probiotic bacteria. *American Journal of Clinical Nutrition*, **73(suppl):** 393S-398S

Vasiljevic, T. and Shah, N. P. (2008) Probiotics – From Metchnikoff to bioactives. *International Dairy Journal*, **18:** 714-728

Ventura, M., Turroni, F., Zomer, A., Foroni, E., Giubellini, V., Bottacini, F. *et al.* (2009) The *Bifidobacterium dentium* Bd1 genome sequence reflects its genetic adaptation to the human oral cavity. *PLOS Genetics*, **5(12):** e1000785

Ventura, M., Turroni, F., O'Connell Motherway, M., MacSharry, J. and van Sinderen, D. (2012) Host-microbe interactions that facilitate gut colonization by commensal bifidobacteria. *Trends in Microbiology*, **20(10):** 467-476

Vernazza, C. L., Gibson, G. R. and Rastall, R. A. (2006a) Carbohydrate preference, acid tolerance and bile tolerance in five strains of *Bifidobacterium*. *Journal of Applied Microbiology*, **100:** 846-853

Vernazza, C. L., Rabiu, B. A. and Gibson, G. R. (2006b) Human colonic morphology and the role of dietary intervention: introduction to prebiotics. In: Gibson, G. R. and Rastall, R. A. (eds.) *Prebiotics: Development and Application*. West Sussex: John Wiley and Sons Ltd, pp. 1-28

Vieira, H. L. A., Freire, P. and Arraino, C. M. (2004) Effect of *Escherichia coli* morphogene *bolA* on biofilms. *Applied and Environmental Microbiology*, **70(9):** 5682-5684

Vinderola, G., Capellini, B., Villarreal, F., Suarez, V., Quiberoni, A. and Reinheimer, J. (2008) Usefulness of a set of simple in vitro tests for the screening and identification of probiotic candidate strains for dairy use. *LWT – Food Science and Technology*, **41:** 1678-1688

Waddington, L., Cyr, T., Hefford, M., Truelstrup Hansen, L. and Kalmokoff, M. (2010) Understanding the acid tolerance response of bifidobacteria. *Journal of Applied Microbiology*, **108:** 1408-1420

Wang, C., Shoji, H., Sato, H., Nagata, S., Ohtsuka, Y., Shimizu, T. and Yamashiro, Y. (2007) Effects of oral administration of *Bifidobacterium breve* on fecal lactic acid and short-chain fatty acids in low birth weight infants. *Journal of Pediatric Gastroenterology and Nutrition*, **44:** 252-257

Wang, Y., Delettre, J., Guillot, A., Corrieu, G. and Beal, C. (2005) Influence of cooling temperature and duration on cold adaptation of *Lactobacillus acidophilus* RD758. *Cryobiology*, **50:** 294-307

Wesche, A. M., Gurtler, J. B., Marks, B. P. and Ryser, E. T. (2009) Stress, sublethal injury, resuscitation, and virulence of bacterial food pathogens. *Journal of Food Protection*, **72(5):** 1121-1138

Wohlgemuth, S., Loh, G. and Blaut, M. (2010) Recent developments and perspectives in the investigation of probiotic effects. *International Journal of Medical Microbiology*, **300:** 3-10

Wu, R., Sun, Z., Wu, J., Meng, H. and Zhang, H. (2010) Effect of bile salts stress on protein synthesis of *Lactobacillus casei* Zhang revealed by 2-dimensional gel electrophoresis. *Journal of Dairy Science*, **93:** 3858-3868

Xiao, J. Z., Kondo, S., Takahashi, N., Miyaji, K., Oshida, K., Hirmatsu, A. *et al.* (2003) Effects of milk products fermented by *Bifidobacterium longum* on blood lipids in rats and healthy adult male volunteers. *Journal of Dairy Science*, **86:** 2452-2461

Xiao, M., Xu, P., Zhao, J., Wang, Z., Zuo, F., Zhang, J. *et al.* (2011) Oxidative stress-related responses of *Bifidobacterium longum* subsp. *longum* BBMN68 at the proteomic level after exposure of oxygen. *Microbiology*, **157:** 1573-1588

Xu, H., Lee, H. –Y. and Ahn, J. (2011) Characteristics of biofilm formation by selected foodborne pathogens. *Journal of Food Safety*, **31:** 91-97

Yamamoto, Y., Gaudu, P. and Gruss, A. (2011) Oxidative stress and oxygen metabolism in lactic acid bacteria. In: Sonomoto, K. and Yokota, A. (eds.) *Lactic Acid Bacteria and Bifidobacteria: Current Progress in Advanced Research*. Norfolk: Caister Academic Press, pp: 91-102

Yazid, A. M., Ali, A. M., Shuhaimi, M., Kalaivaani, V., Rokiah, M. Y. and Reezal, A. (2000) Antimicrobial susceptibility of bifidobacteria. *Letters in Applied Microbiology*, **31:** 57-62

Yildirim, Z., Winters, D. K. and Johnson, M. G. (1999) Purification, amino acid sequence and mode of action of bifidocin B produced by *Bifidobacterium bifidum* NCFB 1454. *Journal of Applied Microbiology*, **86:** 45-54

Zhou, J. S., Pillidge, C. J., Gopal, P. K. and Gill, H. S. (2005) Antibiotic susceptibility profiles of new probiotic *Lactobacillus* and *Bifidobacterium* strains. *International Journal of Food Microbiology*, **98:** 211-217

Zomer, A., Fernandez, M., Kearney, B., Fitzgerald, G. F., Ventura, M. and van Sinderen, D. (2009) An interactive regulatory network controls stress response in *Bifidobacterium breve* UCC2003. *Journal of Bacteriology*, **191(22):**7039-7049

Zomer, A. and van Sinderen, D. (2010) Intertwinement of stress response regulons in *Bifidobacterium breve* UCC2003. *Gut Microbes*, **1(2):** 100-102

APPENDIX

Media and composition

Reinforced Clostridial Medium (RCM, CM0149, Oxoid Ltd)	g/l
Yeast extract	3
'Lab-Lemco' powder	10
Peptone	10
Glucose	5
Soluble starch	1
Sodium chloride	5
Sodium acetate	3
Cysteine hydrochloride	0.5
Agar	0.5

Reinforced Clostridial Agar (RCA, CM0151, Oxoid Ltd)	g/l
Yeast extract	3
'Lab-Lemco' powder	10
Peptone	10
Glucose	5
Soluble starch	1
Sodium chloride	5
Sodium acetate	3
Cysteine hydrochloride	0.5
Agar	15

De Man, Rogosa, Sharpe Broth (MRS, CM0359, Oxoid Ltd)	**g/l**
Peptone	10
'Lab-Lemco' powder	8
Yeast extract	4
Glucose	20
Tween 80	1
Dipotassium hydrogen phosphate	2
Sodium acetate $3H_2O$	5
Triammonium citrate	2
Magnesium sulphate $7H_2O$	0.2
Manganese sulphate $4H_2O$	0.05

Tryptone Soya Broth (TSB, CM0129, Oxoid Ltd)	**g/l**
Pancreatic digest of casein	17
Enzymatic digest of soya bean	3
Sodium chloride	5
Dipotassium hydrogen phosphate	2.5
Glucose	2.5

Nutrient Agar (NA, CM0003, Oxoid Ltd)	**g/l**
‘Lab-Lemco’ powder	1
Yeast extract	2
Peptone	5
Sodium chloride	5
Agar	15

Violet Red Bile Glucose Agar (VRBG, CM1082, Oxoid Ltd)	**g/l**
Enzymatic digest of animal tissues	7
Yeast extract	3
Bile salts No. 3	1.5
Sodium chloride	5
Neutral red	0.03
Crystal violet	0.002
Glucose	10
Agar	12

Phosphate Buffered Saline (PBS, BR0014, Oxoid Ltd)	**g/l**
Sodium chloride	8
Potassium chloride	0.2
Disodium hydrogen phosphate	1.15
Potassium dihydrogen phoshate	0.2