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Exploring the relationship between exposure to technological and gastrointestinal stress and probiotic functional properties of lactobacilli and bifidobacteria

O.D. Amund

School of Life Sciences, Faculty of Health and Life Sciences, Coventry University, Priory Street, Coventry CV1 5FB, United Kingdom

Email: ac1139@coventry.ac.uk
Abstract

Strains of *Lactobacillus* and *Bifidobacterium* are considered probiotic because of their associated potential health benefits. Probiotics are commonly administered orally via incorporation into food products. Microorganisms for use as probiotics encounter stress conditions, which include acid, bile, osmotic, oxidative, heat and cold stresses. These can occur during processing and storage, and during passage through the gastrointestinal tract, and can affect viability. Probiotic bacteria have to remain viable in order to confer any health benefits. Therefore, the ability to withstand technological and gastrointestinal stresses are crucial probiotic selection criteria. Whilst the stress tolerance mechanisms of lactobacilli and bifidobacteria are largely understood, the impact of exposure to stressful conditions on the functional properties of surviving probiotic microorganisms is not clear. This review explores the potentially positive and negative relationships between exposure to stress conditions and probiotic functional properties such as resistance to gastric acid and bile, adhesion and colonization potential, and tolerance to antibiotics. Protective strategies can be employed to combat negative effects of stress on functional properties. However, further research is needed to ascertain synergistic relationships between exposure to stress and probiotic properties.

Key words: probiotics, gastrointestinal stress, technological stress, functional properties
Introduction

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. The most commonly used are strains of species of *Lactobacillus* and *Bifidobacterium*.

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).

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.

Research on pathogenic microorganisms suggest that exposure to stressful conditions might aid their virulence. Links have been found between the induction of stress-tolerance responses and increased virulence in pathogens such as *Listeria monocytogenes* and *Salmonella* (Gahan and Hill 1999; Begley et al. 2009; Alvarez-Ordonez et al. 2011).

To draw a parallel with probiotic microorganisms, it can be proposed that exposure to stress may aid probiotic microorganisms in their functionality, or
otherwise impede them. This review explores the potential influence of stress on beneficial probiotic properties of lactobacilli and bifidobacteria.

**Probiotic carrier products**

Probiotics are usually grown to high numbers in industrial growth medium, concentrated, and then frozen or dried by spray- or freeze-drying, to be added to specific carrier products and stored under suitable conditions (Mills et al. 2011; Tripathi and Giri 2014). Dairy products such as yoghurt and other fermented and non-fermented milks are the most common vehicles for delivering probiotics. This has been suggested to be due to their perceived healthy image historically, as well as consumer familiarity with their live microbial content (Heller 2001).

However, there has been development in the area of non-dairy probiotic products, due to increasing consumer demand because of changing consumer tastes and increasing vegetarianism, as well as limitations of dairy products, such as cholesterol content, lactose intolerance, allergies to milk proteins and the requirement for cold storage facilities (Granato et al. 2010b; Ranadheera et al. 2010).

Probiotics are now being incorporated into drinks such as fruit juices, and are being sold as supplements in tablet, capsule and powder form. There is also research into developing cereal/oat-based, soya-based, confectionery and meat products (Rivera-Espinoza and Gallardo-Navarro 2010; Tripathi and Giri 2014).
For beneficial effects to be achieved, it has been recommended that minimum levels of probiotics around $10^6$-$10^7$ CFU/ml should be present in the product by the time of consumption/end of shelf-life (Ozer and Kirmaci 2010). Daily probiotic intake has been suggested at $10^8$-$10^9$ CFU, with regular consumption of 100 g/day of product (Granato et al. 2010a; Tripathi and Giri 2014).

**Probiotic selection criteria**

Microorganisms intended for use as probiotics are required to meet certain criteria for selection, based on safety, technological and functional characteristics. 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 recognized as safe), and possess no transferable 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 microbiota, 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 are focused on suitability for large-scale production and incorporation into food products without loss of viability (Lacroix and Yildirim 2007). 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 requirements, which are initially determined \textit{in vitro}, include resistance to acid/gastric acid, resistance to bile, adhesion to the intestinal epithelium and ability to transiently colonize the gut, stimulation of the immune system, and antagonistic activity against pathogens (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 colonization 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 also directly or indirectly influence the immune system (Shah 2007). Nevertheless, whilst in vitro functional properties may be indicators of potential health benefits in humans, such health benefits of candidate probiotic strains should always be demonstrated by well-designed human trials (Makinlen et al. 2012).

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 colonization resistance and modulation of immune resistance.

The consumption of probiotics, and the transient colonisation which occurs, can influence the composition and activity of the natural gut microbiota 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).

The growth and metabolism of probiotic microorganisms can alter the intestinal environment such that colonization 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).

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 recognized by host cells which are sensitive to them, e.g. toll-like receptors, 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).

**Stress and probiotic microorganisms**

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 gastrointestinal tract (Ruiz et al. 2011). These include acid, bile, osmotic, oxidative, heat and cold stresses which can affect viability and functionality (Zomer et al. 2009) (Table 1).
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). Acidic conditions are also encountered in fruit juices (Perricone et al. 2014).

Low environmental pH and weak organic acids pose negative implications for bacterial cells, and could cause severe damage. Exposure to low pH conditions, such as strong acid in the stomach, causes a reduction in intracellular pH due to intracellular accumulation of protons (H⁺ ions) from the environment, and also affects transmembrane pH. Undissociated organic acids, including lactic and acetic acid, can diffuse across the cell membrane and dissociate to form H⁺, thus lowering cytoplasmic pH. These alter the proton motive force, 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. Thus, acid resistance is one of the criteria for probiotic selection (Corcoran et al. 2008; Wesche et al. 2009).

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.

Probiotics are commonly grown to high numbers before undergoing drying processes to produce powders which can be added to probiotic products. During spray-drying, the bacteria are exposed to high temperatures for a short time, such as inlet temperatures of up to 200 °C and outlet temperatures in the range of 70-100 °C, which can disrupt the integrity of viable bacterial cells (Golowczyc et al. 2010; 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 destabilization of non-covalent interactions at high temperatures (Corcoran et al. 2008; Champomier-Verges et al. 2010).

Lactobacilli and bifidobacteria are generally microaerophilic and obligate anaerobes respectively. Probiotic bacteria can be exposed to oxidative stress at different stages in their production, such as during fermentation, drying and storage, as well as in the gastrointestinal tract (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 from incomplete reduction of oxygen, which can cause damage by reacting with proteins, lipids and DNA (Corcoran et al. 2008; Li et al. 2010). Furthermore, in dried cells, oxidative stress can occur due to oxidation of cell components, such as membrane lipid oxidation (Teixeira et al. 1996). Spray-dried probiotics have been shown to be more susceptible to oxidative stress due to cellular injuries that occur during dehydration (Behboudi-Jobbehdar et al. 2013). Anaerobes lack the enzymes catalase and superoxide dismutase which can decompose and detoxify ROS (Li et al. 2010). Tolerance to oxygen and ROS is therefore a desirable characteristic for probiotic microorganisms.

Probiotics are exposed to low temperatures prior to consumption, during production and storage, which can result in cold shock (Corcoran et al. 2008; Wesche et al. 2009). Probiotic cultures can be cryogenically frozen, stored and then thawed for use, or freeze-dried, where the cells are frozen at temperatures around -196 °C and dried by sublimation under high vacuum, to produce powders (Maus and Ingham 2003; Mills et al. 2011). Probiotic dairy products are also usually stored at refrigeration temperatures of 4-5 °C, and low storage temperature has been considered as a reason for loss in viability (Tripathi and Giri 2014). While viability of dried probiotics is maximized at much lower temperatures up to -18 °C, storage of food products at low refrigeration temperatures can cause membrane damage and affect replication, transcription and translation in probiotics (Champomier-Verges et al. 2010). Cryotolerance (i.e. cold tolerance) is thus a desirable feature in probiotic bacteria.
Osmotic stress may occur during spray-drying and freeze-drying, and may also occur due to the presence of high concentration of salts or other solutes (such as sugars) in food products (Wesche et al. 2009; Hosseini Nezhad et al. 2015). Drying processes reduce water activity ($a_w$), thereby leading to an increase in osmotic stress (Prasad et al. 2003). In the gastrointestinal tract, osmotic stress in probiotics can result from the low $a_w$ in the upper small intestine (high osmolarity equivalent to 0.3M NaCl) (Sheehan et al. 2007; Alvarez-Ordóñez et al. 2011), and due to fluctuations in diet (De Dea Lindner et al. 2007). Osmotic stress results from shifts in external osmolarity, which leads to movement of water into or 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 turgor during osmotic upshift (De Angelis and Gobbetti 2004; Corcoran et al. 2008).

**Probiotic stress response mechanisms**

Candidate probiotic microorganisms should be able to survive these various stress conditions. Lactic acid bacteria and bifidobacteria have been shown to possess defence mechanisms which aid their survival during exposure to various stress conditions (Corcoran et al. 2008) (Table 1).

Lactobacilli and bifidobacteria have been demonstrated to possess an acid tolerance response, 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 alkanization 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. 2010; Ruiz et al. 2011).

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. 2010).

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 enzyme, which is responsible for bile salt deconjugation, though its role is not clear (Sanchez et al. 2010; Ruiz et al. 2011).

Heat shock has been widely studied in lactobacilli and bifidobacteria. They can utilize 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, 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).

Enzymes such as NADH oxidase and NADH peroxidase are required by anaerobic lactobacilli and bifidobacteria to scavenge environmental oxygen and hydrogen peroxide respectively. NADH oxidases and peroxidases are commonly found in lactic acid bacteria and bifidobacteria, enabling the use of oxygen or hydrogen peroxide as external electron acceptors during metabolism. The activity of NADH oxidases is induced by oxygen, and results in altered fermentation end-products in homofermentative lactobacilli and bifidobacteria (Axelsson 2004; Ruiz et al. 2012). 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 hydrogen peroxide, which prevents the overabundance of free iron that leads to oxidative damage (Wesche et al. 2009; Yamamoto et al. 2011).

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. In addition, 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). Furthermore, protective compounds known as compatible solutes, such as betaine and trehalose, are accumulated in probiotic strains. These stabilize protein structure and function at low temperatures (Sleator and Hill 2007).

Lactobacilli and bifidobacteria are unable to accumulate sufficiently high concentrations of Na\(^+\) or K\(^+\) to maintain turgor, and they synthesize 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).

Potential influence of stress on probiotic properties

Technological stress

Lactobacilli of the same strain isolated from different product formulations
have been shown to vary in their probiotic properties such as tolerance to
gastrointestinal conditions, inhibition of pathogens and adhesion to intestinal
cells. Grzeskowiak et al. (2011) reported differences in the in vitro properties
of Lactobacillus rhamnosus GG isolates from different sources. 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 Lb. 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).

Resistance of probiotic Lactobacillus strains to gastrointestinal conditions
has been demonstrated to be influenced by the type of carbohydrate used as
carbon source in the medium the lactobacilli were grown on (Hernandez-
None of the aforementioned studies have explicitly linked any of these differences with specific stress conditions. However, Fayol-Messaoudi et al. (2005) showed that the temperature at which *Lb. rhamnosus* GG was grown *in vitro* had an influence on its production of antimicrobial substances. A significant reduction in the killing activity of cell-free culture supernatants against *Salmonella enterica* ser. Typhimurium was observed when *Lb. rhamnosus* GG was grown at 32 °C, compared to when grown at 37 °C, despite no significant differences in growth patterns and lactic acid production between both temperatures. This report may suggest a negative effect of temperature stress on the production of bacteriocins or bacteriocin-like inhibitory substances by probiotics during food manufacture, such as in fermented milk products where probiotics are involved in fermentation. This may affect the potential for health benefit, as health benefits may occur not just from the action of ingested probiotics in the gut, but also from the ingestion of metabolites released into the food product during fermentation (Ranadheera et al. 2010; Tripathi and Giri 2014). This could be feasible if such antimicrobial peptides can withstand conditions of the gastrointestinal tract, as demonstrated by Dicks et al. (2010).

Similarly, potential probiotic strains of *Lb. kefir* which had been spray-dried showed significantly decreased capacity for adhesion to the intestinal epithelium and protection against *Salmonella* invasion. This was despite the absence of detectable membrane damage due to spray-drying, suggesting a possible detrimental effect of heat stress on some cell-surface adhesion structures (Golowczyc et al. 2011). Ninomiya et al. (2009) reported decreased growth and EPS production of a *B. longum* strain decreased with
dissolved oxygen concentrations above 0.05 ppm. Decreased EPS production during culture may have an impact on the ability to adhere to the intestinal epithelium (Ninomiya et al. 2009). In contrast, the study of Qian et al. (2011) on some *Bifidobacterium* spp. demonstrated that cells grown in non-reducing media, and therefore under oxidative stress, showed greater intracellular granule production, in response to oxidative stress, when compared to those grown in reducing media. Furthermore, those grown under oxidative stress showed higher exopolymer production, acid tolerance and cell surface hydrophobicity, which has been positively correlated with adhesion ability to host cells.

**Gastrointestinal stress**

Acid and bile are the key stresses probiotic microorganisms would be exposed to during gastrointestinal transit. Although they may survive these stresses, exposure to such conditions may have an impact on other probiotic properties. From various studies, there could be evidence to suggest synergistic as well as deleterious relationships between exposure to gastrointestinal stress and functional properties of probiotic microorganisms. Tolerance of probiotics to therapeutic doses of antibiotics may be desirable when probiotics are co-administered with antibiotics, to prevent antibiotic-associated diarrhoea (Amund et al. 2014). Few studies have examined the effects of exposure to gastrointestinal stress on the susceptibility of lactobacilli and bifidobacteria to antibiotics. Acid-stressed and bile-stressed lactobacilli and bifidobacteria have shown modified susceptibilities to antibiotics. Increased or reduced susceptibilities have been observed,
varying with type of stress, species/strain and type of antibiotic (Charteris et al. 2000; Elkins and Mullis 2004; Kheadr 2006; Kheadr et al. 2007).

Strains of *Bifidobacterium* spp. showing acid resistance and bile resistance exhibited greater resistance to antibiotics including tetracycline, ampicillin, rifampicin and penicillin (Noriega et al. 2005; Collado and Sanz 2007). This may be due to modified surface properties and cell permeability.

Amund et al. (2014) reported higher expression of tetracycline resistance gene *tet(W)* in *B. animalis* ssp. *lactis* after exposure to acid, bile and osmotic stress conditions. Similarly, a slight induction of *tet(W)* in *B. animalis* ssp. *lactis* upon exposure to bile was reported by Gueimonde et al. (2010). Higher expression of *tet(W)* in *B. animalis* ssp. *lactis* after exposure to stresses did not however manifest as greater tetracycline resistance (Amund et al. 2014).

*Tet* genes code for proteins that protect the ribosomes, which are the target for tetracyclines (Gueimonde et al. 2010). Therefore up-regulation in the expression of *tet(W)* observed in these studies may be a protective response to stress conditions affecting ribosomes in bacterial cells, which may consequently enhance tolerance to therapeutic doses of tetracycline.

Adhesion to the intestinal epithelial cells is considered as necessary for probiotic microorganisms to colonize the large intestine, and colonization is important for beneficial health effects such as modulation of the immune system to be observed (Tuomola et al. 2001). Modifications which occur in the presence of stress may be stress tolerance or response mechanisms, which can in turn impact on the adhesion and colonization potential of probiotic bifidobacteria and lactobacilli. Morphological changes and changes in protein and fatty acid profiles of bifidobacteria under oxygen and bile
stress have been reported (Ahn et al. 2001; Ruiz et al. 2007). 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. Similar findings were reported by Haddaji et al. (2015) on the effect of acid stress on membrane properties and adhesion ability of *Lactobacillus casei*.

Biofilms, i.e. microbial communities embedded in an extracellular polymeric matrix, may develop as a result of adhesion to the intestinal epithelium (Ambalam et al. 2014). Bile was reported to stimulate biofilm formation in strains of bifidobacteria and lactobacilli, owing to enhanced cell surface hydrophobicity (Ambalam et al. 2012; Ambalam et al. 2014). Studies of biofilm formation in *Lactobacillus rhamnosus* GG and *Lb. reuteri* strains have shown that lower concentrations of bile (0.05-0.3%) stimulated biofilm formation, while biofilm formation was greatly reduced at increased bile concentrations (<1%). In addition, low pH was found to significantly reduce biofilm formation in *Lb. rhamnosus* GG while it stimulated biofilm formation in *Lb. reuteri* strains (Lebeer et al. 2007; Slizova et al. 2015). Biofilm development may be influenced by alterations in the cell surface due to acid and bile (Lebeer et al. 2007). Furthermore, 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).

Relationships may also exist between stress-tolerant traits and colonization potential. Acid-resistant strains of bifidobacteria showed great adhesion and pathogen displacement capacity (Collado et al. 2006). DnaK and enolase,
which are stress-related proteins that play roles in binding to human epithelial cells, were induced in \textit{B. animalis} ssp. \textit{lactis} in response to bile (Candela et al. 2010). Alp and Aslim (2010) reported a correlation between production of EPS and tolerance to bile and low pH in strains of \textit{Bifidobacterium}. Bifidobacteria which produced high levels of EPS were more resistant to acid and bile. Similar findings for a strain of \textit{Lb. paracasei} expressing an EPS gene were reported by Stack et al. (2010). Studies have also demonstrated positive relationships between exposure to gastrointestinal stress conditions and EPS production/gene expression. Enhanced EPS production and increased expression of an EPS-synthesis gene \textit{gtf01207}, was observed in \textit{B. animalis} ssp. \textit{lactis} in the presence of bile (Ruas-Madiedo et al. 2009). Amund et al. (2014) also showed higher expression of \textit{gtf01207} in \textit{B. animalis} ssp. \textit{lactis} after exposure to acid, bile and osmotic stresses, though only significantly for osmotic stress.

**Enhancing the stability of probiotic microorganisms**

**Exposure to sublethal stress**

Stress responses of probiotic microorganisms can be exploited for the enhancement of survival by pre-exposure to sublethal stress. This allows adaptation to specific stresses by inducing shock proteins, so that more successful stress responses can be mounted in the presence of more lethal levels of the stress, thus enhancing survival and viability. Cross-protection to other stresses has also been demonstrated. The effects of stress pretreatments on enhanced stress tolerance of lactobacilli and bifidobacteria have been reviewed by Corcoran et al. (2008), Mills et al. (2011), Ruiz et al.
(2011) and Sanchez et al. (2013). However, it is important that stress-adapted probiotics are reassessed for their functional properties, as some probiotic traits may be positively or negatively affected as a result (Sanchez et al. 2013).

**Encapsulation**

Microencapsulation involves encasing the probiotics in a protective outer coating (Ranadheera et al. 2015). Microencapsulation can enhance probiotic survival during processing, storage and gastrointestinal transit by segregating the cells from harsh environmental conditions. Polymers such as chitosan, alginate, carrageenan, starch, whey proteins, dextrans and inulin are used for encapsulation of probiotic cells (Nazzaro et al. 2012; Tripathi and Giri 2014). Encapsulation may not only enhance probiotic survival in terms of numbers, but may preserve functionality by protecting bioactive or effector molecules on the cell envelope of probiotic microorganisms, which are necessary for probiotic effects (de Vos et al. 2010; Lee et al. 2013).

**Food matrix**

Food carriers, such as yoghurt, can aid the survival of probiotics by buffering probiotic microorganisms through the gastrointestinal tract. They may also contain functional ingredients, such as prebiotics, which can interact with probiotics to alter their functionality (Ranadheera et al. 2010). Lactic acid bacteria strains have been shown to survive *in vitro* digestion models better in fermented milk than in culture media (Faye et al. 2012; Burns et al. 2014). Furthermore, the *apf* gene (aggregation-promoting factor), which was highly
expressed during the growth of a *Lb. acidophilus* strain in milk, has been demonstrated to play roles in bile tolerance, gastrointestinal survival and interactions with epithelial cells (Baugher and Klaenhammer 2011). This may suggest that dairy carrier foods have an influence on probiotic functional properties, as well as survival.

Different food formulations have also been shown to influence probiotic survival and adhesion ability differently *in vitro* (Ranadheera et al. 2012). Viability of a *Lb. reuteri* strain in fruit juices was influenced by the type of fruit juice (Perricone et al. 2014). Therefore, the type of food matrix must be taken into careful consideration. However, the potential beneficial effect of the food matrix may be lost where probiotics are delivered in non-food forms such as capsules (Ranadheera et al. 2010).

**Conclusions**

Candidate probiotic microorganisms are assessed in a ‘native’ state, for their ability to tolerate stressful conditions of the gut, as well as for other properties that suggest they would be beneficial (Nivoliez et al. 2012). From the studies reviewed, there may be both negative and positive/synergistic effects of exposure to stress conditions on beneficial probiotic properties. Ultimately these vary with the type of stress and the species/strain. It may therefore be useful to assess this interaction when evaluating potential probiotics. Nevertheless, negative effects can be combatted by employing various strategies to protect the microorganisms from stress. Further research would be needed to demonstrate any synergistic effects of stress on functionality. While there may be negative effects of stress on properties,
the properties appear only to be diminished, not lost. This therefore highlights why probiotics should be available in as high a number as possible in the product, to compensate not only for losses in viable numbers during gastrointestinal transit (Vasiljevic and Shah 2008), but also for the diminished functional properties.

References


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


Table 1 Stress conditions faced by probiotic microorganisms and strategies of stress response

<table>
<thead>
<tr>
<th>Stress type</th>
<th>Stress source</th>
<th>Stress response strategies</th>
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<tbody>
<tr>
<td>Heat</td>
<td>• Extremes of temperature during spray drying</td>
<td>• Proper protein folding by molecular chaperones e.g. GroEL, GroES, GrpE, DnaJ, DnaK, ClpB, Hsp20 &lt;br&gt; • Degradation of misfolded proteins by proteases e.g. ClpC, ClpP, FtsH &lt;br&gt; • Regulatory network via transcriptional regulators e.g. HrcA, HspR, CglR &lt;br&gt; • Small heat shock proteins e.g. Hsp18.5, Hsp19.3, Hsp18.55</td>
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<tr>
<td>Cold</td>
<td>• Extremes of temperature during freeze drying &lt;br&gt; • Storage temperature of carrier product</td>
<td>• Cold shock proteins e.g. CspA, CspL &lt;br&gt; • Cold induced proteins e.g. ClpP, pyruvate kinase, glycoprotein endopeptidase</td>
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<td>Oxidative</td>
<td>• Oxygen exposure during fermentation, drying and storage &lt;br&gt; • Presence of oxygen in carrier product &lt;br&gt; • Mouth during consumption</td>
<td>• ROS-scavenging enzymes e.g. NADH oxidase, NADH peroxidase, Mn-superoxide dismutase &lt;br&gt; • DNA/RNA-protective proteins e.g. Dpr (DNA-binding ferritin-like protein), NrdA (ribonucleotide reductase), MutT1 (NTP phosphohydrolases) &lt;br&gt; • Oxygen stress-protective proteins e.g. AhpC (alkyl hydroperoxide reductase C22), PNDR (Pyridine nucleotidel-disulfide reductase)</td>
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<td>Osmotic</td>
<td>• High osmotic pressure and low water activity during cell dehydration</td>
<td>• Proper protein folding by molecular chaperones e.g. GroEL, GroES, Hsp20, DnaK, GrpE, DnaJ1, DnaJ2 &lt;br&gt; • Transport systems: ATP-binding cassettes (ABC) e.g. OpuA, BusA; ion motive-force driven transporters e.g. BetP, ProP</td>
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<td>Acid</td>
<td>• Presence of organic acids during cultivation &lt;br&gt; • Increased acidity (pH &lt; 5) in carrier product &lt;br&gt; • Stomach acid</td>
<td>• Proton extrusion by F_{0}F_{1}-ATPase &lt;br&gt; • Cytoplasm buffering/ammonia production by branched-chain amino acid production, glutamine synthetase &lt;br&gt; • Alteration of cell wall by esterases e.g. lr1516 &lt;br&gt; • Regulatory systems e.g. membrane-associated histidine kinase</td>
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<tr>
<td>Bile</td>
<td>• Bile in small intestine</td>
<td>• Bile salt/acid detoxification by multidrug transporters and bile efflux pumps (BetA, Ctr) &lt;br&gt; • Bile salt deconjugation by bile salt hydrolase (Bsh1) &lt;br&gt; • Alteration of cell surface by production of extracellular exopolysaccharides; changes in fatty acid composition; Changes in surface-associated proteins (enolase, oligopeptide binding proteins) &lt;br&gt; • Changes in energetic metabolism e.g. increase in ATP synthesis, changes in the ratios of final glycolytic products &lt;br&gt; • Modification of redox status by methionine synthetase, peroxidase &lt;br&gt; • Proper protein folding by molecular chaperones e.g. ClpB, HrcA, GrpE, GroES &lt;br&gt; • Degradation of misfolded proteins by proteases e.g. ClpC</td>
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