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Stress responses in probiotic *Lactobacillus casei*

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Abstract

Survival in harsh environments is critical to both the industrial performance of lactic acid bacteria (LAB) and their competitiveness in complex microbial ecologies. Among the LAB, members of the *Lactobacillus casei* group have industrial applications as acid-producing starter cultures for milk fermentations and as specialty cultures for the intensification and acceleration of flavor development in certain bacterial-ripened cheese varieties. They are amongst the most common organisms in the gastrointestinal tract of humans and other animals, and have the potential to function as probiotics. Whether used in industrial or probiotic applications, environmental stresses will affect the physiological status and properties of cells, including altering their functionality and biochemistry. Understanding the mechanisms of how LAB cope with different environments is of great biotechnological importance, from both a fundamental and applied perspective: hence, interaction between these strains and their environment has gained increased interest in recent years. This article presents an overview of the important features of stress responses in *Lb. casei*, and related proteomic or gene expression patterns which may improve their use as starter cultures and probiotics.

Introduction

Bacteria, like all other organisms, are affected by their surrounding environment and they react dynamically to changes in external conditions. In their interaction with their surroundings, they use multiple systems for sensing and adapting to stressful conditions, which can include exposure to extreme temperatures or stresses generated by cell growth itself, such as production of acidic metabolites and nutrient depletion (starvation) (Jobin *et al.*, 1997; Ehrnsperger *et al.*, 1998). After exposure to a physical stress, it is important for the bacteria to maintain functional cellular physiology which will enable adaptation to the altered conditions. Any change in the environment can result in the induction of differentially regulated genes. The synthesis of the relevant proteins and pathways, which may be condition-specific or form part of a general stress response (Hecker *et al.*, 1996), allows cells to be protected from fatal damage and adapt to the new conditions (Gottesman, 1984).

Lactobacillus is the largest genus within the lactic acid bacteria (LAB) which plays a crucial role in the development a unique organoleptic profile and good hygienic quality of fermented products, particularly in the dairy industry. These strains are exposed to various stresses during most of their lifecycle because of various growth-limiting conditions used in manufacture (Hecker *et al.*, 1996). Among lactobacilli, *Lb. casei* strains are widely used in the dairy industry and fermentation processes. In addition, some of the *Lb. casei* strains exhibit probiotic effects and act as health-promoting live cultures, particularly is yoghurt and fermented drinks. It is clear that the ability of individual strains to adapt efficiently to stress is important for these beneficial

strains to survive, compete with other microflora and express functional traits aligned to delivering health benefits.

There are number of publications on stress response studies in the *Lb. casei/paracasei* group. These reports include studies on cold shock in *Lb. casei* (Beaufils *et al.*, 2007); osmotic stress (Piuri *et al.*, 2003; Piuri *et al.*, 2005), heat shock (Broadbent *et al.*, 1997), starvation (Hussain *et al*., 2009a, 2009b) and acid stress (Broadbent *et al.*, 2010, Hosseini Nezhad *et al*., 2010; Hosseini Nezhad *et al*., 2012). This article provides an overview of both the *Lb. casei* group and their applications in fermented dairy foods, plus a description of the known interactions between these strains and their surroundings. The general aim of this review is to discuss the environmental factors that may influence the growth, survival and functionality of the *Lb. casei* group and the phenomic characterization of stress responses in these probiotic bacteria.

A brief overview of the genus *Lactobacillus*

Lactobacillus is the largest and one of the most important genera of the LAB. According to *Taxonomic Outline of the Prokaryotes* (Garrity *et al.*, 2004), the genus *Lactobacillus* belongs to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, family *Lactobacillaceae*. The genus is diverse and consists of numerous different species, with more than 140 validly described species at present, most of which are of industrial importance (Singh *et al*., 2009), and demonstrate a wide variety of phenotypic, biochemical and physiological traits (Coeuret *et al.*, 2003; Felis and Dellaglio, 2007).

Lactobacillus species are either naturally present in raw milk and dairy products or added intentionally for technological reasons or to generate health benefits. The genus is a group of common indigenous microorganisms, which are natural inhabitants of the gastrointestinal (GI) tract of mammals and considered as probiotics (Gorbach, 1990; Goldin and Gorbach, 1992; Schaafsma, 1996; de Roos and Katan, 2000; Brizuela *et al*., 2001). Several health-promoting effects attributed to the consumption of lactobacili strains have been demonstrated, including their control of undesirable microorganisms in the intestinal and urogenital tract and inhibition of pathogenic organisms (Wood, 1992; Sgouras *et al.,* 2004; Maragkoudakis *et al.,* 2006), beneficial effects on gut health (Fuller *et al.* 1992), reducing viral diarrhea (de Roos and Katan, 2000), preventing intestinal disorders (Ingrassia *et al.,* 2005), hypocholesteraemic effects, binding of mutagenic compounds, lowering the environmental pH and immune enhancement (Gorbach, 1990; Goldin and Gorbach, 1992; Schaafsma, 1996; de Roos and Katan, 2000; Brizuela *et al.,* 2001). There is also evidence of the ability of some lactobacilli strains to produce bacteriocins or hydrogen peroxide, which exert antimicrobial activity on other species, resulting in inhibited growth and cell death (Barefoot and Klaenhammer, 1983; Joerger and Klaenhammer, 1986; Callewaert and De Vuyst, 2000; Leal-Sanchez *et al.,* 2002; Cotter *et al.,* 2005).

General aspects of *Lactobacillus casei*

Lb. casei species are the predominant species of the *Lactobacillus* genus, with respect to their distribution in a large number of habitats and applications. *Lb. casei* is an aciduric, rod-shaped LAB that can be isolated from a variety of environments including raw and fermented milk and meat or plant products, as well as the oral, intestinal, and reproductive tracts of humans and

animals (Kandler and Weiss, 1986). Within the genus *Lactobacillus*, *Lb. casei* forms part of the facultatively heterofermentative species cluster which produce lactic acid from hexose sugars *via* the Embden-Meyerhof pathway and from pentoses by the 6-phosphogluconate/phosphoketolase pathway (Axelsson, 1998). Related strains of the *Lb. casei* group were firstly classified into three species, *Lb. casei*, *Lb. paracasei* and *Lb. rhamnosus* according to DNA-DNA relatedness by Collins *et al.* (1989). Further studies led to proposals that members of the *Lb. casei* group be divided into three species: *Lb. rhamnosus*, *Lb. zeae and Lb. casei* including the strains of *Lb. paracasei* and *Lb. casei* with neotype strain of ATCC 334 for the latter species (Collins *et al.*, 1989; Dellaglio *et al.*, 1991; Dicks *et al.*, 1996; Mori *et al.*, 1997; Chen *et al.*, 2000). However, Dellaglio *et al.* (2002) proposed that the strains *Lb. casei* and *paracasei* species form a single taxon, and therefore should be united with the name *Lb. casei* and rejected the name *Lb. paracasei*, a proposed later supported by Dobson *et al.* (2004).

It is obvious that strains *Lb. casei* and *Lb. paracasei* form a closely related taxonomic group within the lactobacilli and these two species are very similar, in certain analyses almost identical. According to the latest taxonomy (Felis and Dellaglio, 2007), the *Lb. casei* group should include *Lb. casei*, *Lb. paracasei*, *Lb. rhamnosus* and *Lb. zeae*, which are ordered here on the basis of the number of species included in each species. *Lb. casei* has a G+C content of 45-47%, and the proposed neotype strain, ATCC 334, has a genome size previously estimated at 2.2 Mbp (Tynkkynen *et al.*, 1999).

Industrial applications

Lb. casei is grouped in the thermophilic LAB, which are well-known for their biotechnological importance. The most common industrial application of *Lb. casei* strains is in dairy products and processing (Kosin and Rakshit, 2006). *Lb. casei/paracasei* are the dominant species of non-starter LAB (NSLAB) in several semi-hard cheese varieties. NSLAB are not deliberately added during manufacture but survive pasteurization in low numbers and grow in the cheese matrix during ripening: they are important because they are responsible for producing flavours and aromas that are distinctive to a region or manufacturing plant (Crow *et al.,* 2001; McSweeney *et al.*, 1993). *Lb. casei/paracasei* produce aminotransferase (AT) enzymes with specificities similar to those of the starter *Lactococcus* strains, but with a larger variation in activities and specificities among strains (Hansen *et al.,* 2002; Williams *et al.,* 2002; Thage *et al*., 2004a; Thage *et al.,* 2004b). This is evident from the production of 2- and 3-methylbutanal by *Lb. paracasei subsp. paracasei* and *Lb. casei* which could not be attributed exclusively to the catabolism of the corresponding amino acids (Kieronczyk *et al.,* 2004); AT activity specific for isoleucine, valine and leucine was detected in *Lb. paracasei* (Hansen *et al.,* 2002). *Lb. paracasei* also showed AT activity for Asp and produces the α-keto acid oxaloacetate, which may be catabolised into diacetyl, acetoin and 1,3-butanediol (Law and Tamime, 2010), all known flavour compounds in matured hard cheeses.

Lb. casei species are commonly isolated from high quality cheese varieties and have the potential to be used as adjunct cultures, which are added to the cheese milk for acceleration or intensification of flavour development (Wijesundera *et al.,* 1997; Marti´nez-Cuesta *et al.,* 2001;

Antonsson *et al.*, 2003; Kask *et al.*, 2003; McSweeney, 2004; Broadbent and Steele, 2005; Ong *et al.*, 2007; Law and Tamime, 2010), and to control unwanted detrimental microbial activities caused by clostridia and gas-forming lactobacilli (Christiansen *et al.*, 2005). *Lb. casei* group is present in cheese at low numbers post-manufacture: however, as ripening progresses, it becomes the dominant species representing up to 96% of the NSLAB population at the end of ripening. It was found that dominant strains of *Lb. casei* in 6-month-old cheeses appeared to be affected more by adjunct treatment and not cheese variety (Law and Tamime, 2010).

Although these bacteria are mainly used in cheese manufacturing, they are of potential utility in other industrial applications, such as L-lactic acid production (Panesar *et al.*, 2007) and water-soluble soy-containing-fermented beverages (Granato *et al*., 2010). Patra *et al.*, 2009 reported the development of a recombinant strain of *Lb. casei* which, when pre-grown on lactose, were able to synthesize sorbitol from glucose. Sorbitol, the most commonly used polyol in the United States, is the standard sweetener in several sugar-free products and it is claimed to have important health-promoting effects. Inactivation of the L-lactate dehydrogenase gene led to an increase in sorbitol production (Nissen *et al.*, 2005). A sorbitol producing *Lb. casei* strain might be of considerable interest in the food industry and is one example of the growing interest in this species as an industrial microbe.

Probiotic potential of *Lb. casei*

A term “probiotic” is used for bacterial strains that colonize the GI tract and exert a beneficial health effect. Schrezenmeir and de Vrese (2001) published a comprehensive definition for

probiotics: ‘a preparation of or a product containing viable, defined microorganims in sufficient numbers, able to alter microflora, by implantation or colonization, in a compartment of host and by that, exert beneficial effects on the host health’. Among lactobacilli, strains belonging to species of *Lb. acidophilus* and the *Lb. casei* complexes are the most frequently used as probiotics (Klaenhammer and Kullen, 1999; Mercenier *et al.*, 2003, Granato *et al*., 2010) whereas several strains of *Lb. casei* with potential probiotic traits, as well as satisfying stringent technological characteristics, have already been identified (Guerin-Danan *et al.*, 1998; Spanhaak *et al.*, 1998; Crittenden *et al.*, 2002). Several health benefits have been reported for these strains including inhibition of pathogenic organisms (Hudault *et al.*, 1997), increasing immune responses (Matsuzaki *et al.*, 1998) and enhancing the immune system in the digestive tract (Paubert-Braquet *et al.*, 1995; Peluso *et al.*, 2007). These strains have been implicated in numerous other health benefits, such as reduction of diarrhea (Isolauri *et al*., 1991; Guerin-Danan *et al.*, 1998) and they have also been found to modify the digestive microflora (Djouzi *et al.*, 1997). Furthermore, bacteriocin production has been documented for such strains (Caridi, 2002; Avonts *et al.*, 2004). The effectiveness of *Lb. casei* in improving murine chronic inflammatory bowel disease is associated with down-regulation of proinflammatory cytokines, such as IL-6 and IFN-γ, in lamina propria mononuclear cells (Matsumoto *et al.*, 2005).

General stress responses in bacteria

The response of microorganisms to any major change in environmental conditions - including uncontrollable environmental factors (*e.g*., radiation and dry air), natural stresses like acidity and sometimes starvation, and the deliberate application of preservation factors encountered during

food processing, such as heat, pressure, electric pulses, ultrasonic, acids and salts, use of disinfection or cleaning agents, rapid chilling or freezing - is known as the stress response (Yousef and Juneja, 2003). Once microorganisms sense a stress, specific metabolic processes are altered, such as induction of new metabolic pathways or formation of other translation products, resulting in increased production of specific stress metabolites or other mechanisms which aid survival under the deleterious conditions (Yousef and Juneja, 2003; Foster 2005; Boor, 2006). Exploration of stress responses is motivated by curiosity-driven reasons and also due to the importance in industry and safety aspects in food microbiology (Beales, 2004). The specific metabolic processes of microorganism in response to any external disturbance include changes of transcription rates, translation products and/or metabolism (Cotter and Hill, 2003; Yousef and Juneja, 2003; De Angelis and Gobbetti, 2004). Some of these are global, coordinated regulation systems that drastically change the pattern of gene expression in the cell and the cellular processes, resulting in the general stress response (Storz and Zheng, 2000). This system alters different cellular processes including cell division, DNA metabolism, housekeeping functions, membrane composition, transport, *etc*. (Storz and Zheng, 2000) and the resulting regulation may lead to the synthesis of general stress proteins that cope with the imposed stress and provide increased tolerance to deleterious conditions (Hussain *et al*., 2009a). Other mechanisms are stress-specific so that responses are tailored to certain stress conditions (van de Guchte *et al.*, 2002; Cotter and Hill, 2003; Yousef and Juneja, 2003; De Angelis and Gobbetti, 2004).

Microbial response to stress may produce these outcomes (adapted from Yousef and Juneja, 2003):

1. Induction of a series of proteins that repair damage, maintain the cell, or eliminate the stress agent;
2. Transient increase in resistance or tolerance to deleterious factors;
3. Cell transformation to a dormant state, *i.e.*, spore formation or passage to a viable but not cultivable state;
4. Evasion of host organism defenses;
5. Adaptive mutations.

Part of the response may also include: changes in cell size and shape, changes in fatty acid profiles, decrease in protein synthesis and production of distinct sets of proteins (Hartke, 1997).

Interaction of the *Lb. casei* group with the environment

The intestine is a complex ecological system and the normal interactions between the microbiota of the GI tract and the host is a symbiotic relationship from which both partners benefits (Hooper *et al.*, 2002). *Lb. casei* group have been detected by molecular approaches in the human GI tract (Satokari *et al.*, 2003; Wall *et al.*, 2007). The environmental conditions in the stomach are destructive to a number of microorganisms because of the presence of bile salts, acids and enzymes. The gastric juice contains hydrochloric acid, which creates an extreme acid stress on the transiting bacteria. The microbes are also affecting each other by competing for available nutrients, production of antimicrobial compounds, or by metabolic cooperation and vitamin excretion. Therefore survival during passage through the GI tract is of the particular importance for probiotics to preserve their expected health-promoting effects (Elli *et al.*, 2006). Prior

exposure to stressors will be important in determining the subsequent competitiveness of probiotic strains in the GI tract.

The survival and growth of NSLAB including *Lb. casei* species in cheese making and maturation is an excellent example of a bacterial stressful life. NSLAB are exposed to a variety of stresses, as the temperature is elevated in the cheese cooking process and the addition of NaCl increases osmolarity during the manufacturing of cheeses, while the maturation environmental conditions provide a number of factors that are potentially stressful to growth. It provides sub-optimal growth conditions or conditions that cells can tolerate but where growth is impaired or suspended. Heat treatment during cheese manufacture may not eliminate NSLAB but heat shock may contribute toward their adaptability to further extreme conditions. Predisposal to heat stress has been shown to protect against further heat shock or other stressing conditions in multiple bacterial species (Hecker *et al.,* 1996; Pichereau *et al.,* 2000; Desmond *et al.,* 2001). Salt content and water activity (a_w) are interrelated and their correlation co-efficient in cheese is determined as 0.997 (Cogan and Beresford, 2005). Salt, water loss by evaporation and hydrolysis of proteins combine to cause depression in a_w during cheese ripening, which has inhibitory effects on microbial growth. The salt concentrations in cheese range from 0.7-7%, which corresponds to a_w values of between 0.99-0.95. These conditions expose NSLAB to osmotic stress during early growth phases. Optimum pH for NSLAB growth is normally >5.8, while cheese curd pH drops from ~6.0 during acidification processes (due to starter culture growth) and reaches post-manufacture pH in the range of 4.5-5.3. These conditions will favour survival of acid tolerant NSLAB strains which will then be a part of cheese ripening flora. Before the

beginning of the ripening process, added salt, acids produced during acidification by starter cultures (lactic, acetic and propionic) and an exhausted carbohydrate source (lactose) convert cheese blocks into a stressful environment for the evolving ecosystem. As the majority of NSLAB are mesophilic (optimum temperature ~30^{o}C) and the ripening temperature is generally around 13^{o}C (noting that Cheddar cheeses are ripened at 6-8°C, which is exceptionally low), exposure to long-term sub-optimal conditions further challenges cells in the cheese matrix. Moreover, the redox (Eh) of cheese is about –250mV, which makes the environmental conditions suitable for obligatory or facultatively anaerobic organisms and is another important stress factor (oxidative stress), so that only selected groups of bacteria are able to establish in the resident microbial community (Parente and Cogan, 2004). This suggests that there is high competition between species and strains that will be able to grow on limited nutrients, survive, adapt and compete in a challenging and changing environment during cheese maturation (Shakeel-Ur-Rehman *et al.*, 2004), so that growth and dominance of strains with high tolerance towards a diverse range of stresses may be favoured.

Survival of *Lb. casei* during the manufacturing process of Cheddar cheese, and the influence of proteolytic patterns and production of organic acids, was studied by Ong *et al*. (2006) in context of adding probiotic strains into the Cheddar cheese as a delivery system. The results demonstrated that the added probiotic organisms, *Lb. casei* and *Lb. paracasei*, survived the manufacturing process at a high level without needing to alter the cheese making process. Furthermore, these authors demonstrated that the probiotics remained viable at a count of >7.5 $\log_{10}$ cfu g^{-1} at the end of ripening period of 6 months at 4^{o}C. The addition of the probiotic

strains as adjuncts did not alter the chemical composition (salt, fat, moisture and protein content) of the cheese, but acetic acid concentration was higher in probiotic cheeses, demonstrating a shift in metabolic products. This study demonstrated that an acceptable cheese could be manufactured with known probiotic strains added as adjuncts at the start of manufacture, which were able to survive and compete in the cheese matrix.

Under the stressful environments encountered by NSLAB, the strains that survive and contribute to dairy product quality must develop different mechanisms of coping with multiple stressors. The response of *Lb. casei/paracasei* to suboptimal conditions is reviewed briefly in the following sections.

Heat shock

A stress factor that has been extensively examined in lactobacilli is heat stress (Jeffery *et al.*, 1997; Teixeira *et al.*, 1997; Jordan and Cogan, 1999; Jolly and Morimoto, 2000; Prasad *et al.*, 2003; De Angelis *et al.*, 2004; Spano *et al.*, 2004; Christiansen *et al.*, 2006; Di Cagno *et al.*, 2006; Tao *et al.*, 2006). The effect of heat shock (sudden up-shift in growth temperature) and the induction of a stress response in *Lb. casei* LC301 was studied by Broadbent *et al*. (1997) using two-dimensional electrophoresis (2-DE). This showed that 15 proteins were induced in *Lb. casei* LC301 by moderate heat shock and that this corresponded to a 5-fold increase in survival of exponential-phase cells following a challenge at high temperature for 20 min (Broadbent *et al*., 1997).

Christiansen *et al.* (2006) studied heat resistance of *Lb. paracasei* isolated from semi-hard cheese and demonstrated the potential of *Lb. paracasei* strains to survive the pasteurization temperature of cheese milk when grown in milk rather than laboratory media. Induction of genes corresponding to heat shock proteins (HSPs) was observed at a transcriptional level as well as up-regulation in their synthesis when *Lb. paracasei* NFBC 338 was incubated at a higher temperature than normal (Desmond *et al.*, 2004): 2-DE revealed that GroEL expression was increased under heat adaptation conditions (52°C for 15 min). Desmond *et al*. (2004) also showed that when the *groESL* operon of *Lactococcus lactis (pGR01)* was transferred into *Lb. paracasei (pGR02)* or *Lc. lactis*, after induction with nisin the GroEL protein was over-expressed and was observed to be 15 to 20% of the total cellular protein for each strain. Heat shock treatment of the lactococci (at 54°C) and lactobacilli (at 60°C) strains showed that the heat-adapted cultures maintained a higher level of viability (~5-log increase).

A number of approaches have been tested with a view to improving culture viability during spray drying, a procedure used to concentrate and preserve adjunct and starter cultures for use in the dairy industry and to deliver probiotic bacteria in functional foods and pharmaceutical preparations (often health supplements). However, spray drying results in significant decreases in cell viability due to exposure to high temperatures at air outlets and survival rates vary between strains, reflecting differences in thermal tolerance (Gardiner *et al*., 2000; Kosin *et al*., 2006). Gardiner *et al*. (2000) demonstrated that the optimal air outlet temperature for drying *Lb. paracasei* NFBC 338 in skim milk was 80–85 °C, with 66% survival. Cells appeared stressed, based on physiological changes and increased sensitivity to NaCl, which is linked to cell

membrane damage in sub-lethally injured bacteria. Corcoran *et al.* (2006) compared the viability of dried GroESL-overproducing *Lb. paracasei* NFBC 338 with that of controls. Spray- and freeze-dried cultures overproducing GroESL exhibited approximately 10- and 2-fold better survival, respectively, demonstrating the impact of GroESL expression in thermal tolerance.

Desmond *et al*. (2001) emphasized the importance of preconditioning cells with homologous stressors, particularly heat shock, prior to spray drying of *Lb. paracasei* NFBC 338. These authors demonstrated that preconditioning by exposure to NaCl, hydrogen peroxide or bile also improved survival after spray drying, indicating cross-protection in thermal adaptation, but that this was not as effective as prior heat shock.

HSPs can also be induced upon exposure to other stressful conditions other than thermal stress. Proteomic analysis has indicated a direct relationship between heat shock and cold shock proteins in *Lb. casei* (Beaufils *et al.* 2007).

Cold shock

In lactobacilli, cold shock is reported to induce changes in cellular composition including protein profiles (Mayo *et al.*, 1997; Derzelle *et al.*, 2003; Scheyhing *et al.*, 2004; Beaufils *et al.*, 2007). Sauvageot *et al.* (2006) reported one *csp*-like gene in *Lb. casei*, named *cspA*, which was identified by an inverse polymerase chain reaction approach based on degenerate primers. This gene encodes a protein of 66 amino acid residues, CspA, which has at least 74% identity with Csp proteins of other members of the *Lactobacillus* genus and its expression is induced after a

temperature downshift from 37°C to 20°C. The transcriptional start site has been determined and is situated 98 bp upstream of the initiation codon. A *cspA* mutant strain was constructed and it showed reduced growth rate compared with the wild type at both optimal and low temperatures, demonstrating that CspA plays an important role in the physiology of *Lb. casei* relating to carbohydrate transport, carbon metabolism and cold shock.

Beaufils *et al.* (2007) compared the cold shock response of several carbon catabolite repression mutants of *Lb. casei* to that of the wild-type strain. Following a shift from 37°C to lower temperatures (20, 15 or 10°C), all mutants showed significantly reduced growth rates. Moreover, glucose-grown mutants unable to form P-Ser-HPr *(ptsH1, hprK)* exhibited drastically increased sensitivity to freeze/thaw cycles. However, when the same mutants were grown on ribose or maltose, resistance to freezing and thawing was similar to the wild-type strain. These results suggested a direct interaction of HPr, or one of its phospho-derivatives, with CspA and/or another undetected cold shock protein in *Lb. casei*.

Bile salts and osmotic stress

Bile salts secreted by mammals act as a natural antibacterial barrier and may serve as a component of innate immunity, as they have limited antagonistic effect against resident microflora, so survival in the GI requires tolerance to exposure to bile salts. *Lb. casei* strains are also exposed to osmotic stress when high concentrations of salts or sugars are added to a dairy product in their various applications. Salt is regularly used in cheese making for its flavour, preservative properties and as a controller of acid production by the starter culture, therefore

increasing the osmolality of the environment which results in the movement of water from the cell, impacting on internal osmotic pressure unless checked by adaptive responses.

Wu et *al.* (2011) compared the growth and protein expression patterns of *Lb. casei* Zhang with and without bile salts. Analysis of the differentially expressed proteins showed that several pathways are involved with a complex physiological response under bile salts stress, particularly including cell protection (DnaK and GroEL), modifications in cell membranes (NagA, GalU, and PyrD), and key components of central metabolism (PFK, PGM, CysK, LuxS, PepC, and EF-Tu).

Piuri *et al.* (2005) found the growth of *Lb. casei* ATCC 393 in high salt resulted in modification of cell wall structural properties. Their results showed that resistance to lysis by hydrolases is related to the presence of O-acetyl groups in peptidoglycan (PG) that protects the PG strands from the hydrolytic activity of muramidase-type lysins. Nine penicillin-binding proteins (PBP), which carry out the glycotransferase and transpeptidase reactions in PG synthesis, were described for *Lb. casei* ATCC 393, three of which are considered to be the essential PBP (PBP1, PBP2a and PBP4b; MW 114, 95 and 62 kDa, respectively). Morphological changes in *Lb. casei* following growth in high NaCl resembled those changes described for cells of *B. subtilis* carrying mutations in PBP (Popham and Young, 2003). In another study on impact of hypertonic conditions (Machado *et al*., 2004), the hydrophobicity and the bile salt sensitivity of *Lb. casei* cells were increased while the glycolipid AcylH3DG was only present in membranes from cells grown in NaCl-containing medium. H4DG showed a significant incremental increase and H2DG a significant decrease. Fatty acid composition was also impacted, with an increase in

the saturated/unsaturated fatty acid ratio, with a corresponding rise in the fluidifying 11,12-methyleneoctadecanoic fatty acid (cyc 19:0).

Ge *et al.* (2011) recently reported the isolation of osmotic-tolerant mutants of *Lb. casei* that were able to survive high concentrations of glucose in high temperature fermentations for lactic acid production. The nature of the mutations was not further characterized.

Starvation

Survival of lactobacilli during starvation depends on their ability to utilize other energy sources. Starvation conditions were described as the decreased ability of organisms to synthesize ATP, generate proton motive force (PMF), and accumulate nutrients necessary to maintain viability over time (Kunji *et al.*, 1993). During dairy processing, the nutritional environment available to sustain growth and viability of the microflora varies considerably, but generally represents low carbohydrate status. Hussain *et al.* (2009a, 2009b) studied the ability of *Lb. casei* to adapt, survive and grow under carbohydrate starvation conditions similar to those found during cheese ripening. They compared growth and survival in a semi-defined synthetic medium supplemented with lactose of deprived of a carbohydrate source. Their results showed that *Lb. casei* remained viable after 30 days in a medium where fermentable carbohydrates were not present initially or were depleted following cell growth, indicating that the strain (originally isolated from cheese) has an ability to adapt to nutritional starvation conditions. Although a lower growth rate and final optical density were observed for lactose-free cultures relative to carbohydrate enriched cultures, survival was greatly enhanced when cells were grown without lactose, showing that the strain

was able to metabolise alternative energy sources, such as amino acids supplied in the form of tryptone. The profile of metabolites produced was consistent with amino acid degradation.

In response to starvation and nutritional stress, *Lb. casei* has been shown to rapidly and transiently express a characteristic set of proteins that aid survival and protect the cells from fatal damage (Hussain *et al.,* 2006). Most of the proteins were involved in glycolytic pathways, centering around pyruvate metabolism.

Alkali stress

Although many studies have examined the response of LAB to acid stress, very few studies have examined the stress response of LAB to alkaline environments. Sawatari and Yokota (2007) determined the maximum pH that allows growth (pHmax) for 34 strains of lactobacilli. Of these, *Lb. casei* NRIC 1917 and *Lb. paracasei* subsp. *tolerans* NRIC 1940 exhibited the highest alkali tolerance, with a pHmax of 8.9. Shift in the pH/glycolysis activity profile was also observed in *Lb. paracasei* subsp. *tolerans* NRIC 1940, which showed the highest alkali tolerance of the tested lactobacilli.

Acid stress responses

Acid stress has been described in different terms as "the combined biological effect of low pH and weak (organic) acids present in the environment" (Bearson *et al.,* 1997) or "exposure to pH values below the growth range" (Jan *et al.,* 2000). The understanding of acid stress response or acid tolerance and adaptation is expected to contribute to enhancement of probiotic survival in

the GI tract. Furthermore, this understanding is important during fermentation since *Lb. casei/paracasei* growth on lactose is always accompanied by lactic acid accumulation.

In order to evaluate the survival of lactobacilli under acidic conditions, Corcoran *et al.* (2005) compared the survival of five *Lactobacillus* strains in simulated gastric juice, pH 2.0, for 90 min. *Lb. rhamnosus* GG had the highest survival rate over the 90 min of exposure to simulated gastric juice (pH 2.0), while the poorest survivor was *Lb. paracasei* NFBC 338, whose viabilty declined to undetectable levels after only 30 min of exposure. However, with the adjustment of the pH of simulated gastric juice, a protective effect did occur for *Lb. paracasei* NFBC 338. These data indicated that glucose provides ATP to F0F1-ATPase via glycolysis, enabling proton exclusion and thereby enhancing survival during gastric transit, therefore the survival of these probiotic lactobacilli in acidic condition was enhanced in the presence of metabolized sugars. The F0F1-ATPase has been found to be an important complex in the survival of *Lb. casei* in acidic environments. In addition, higher activity has been observed in *Lb. casei* than in *Actinomyces viscosus* (Bender and Marquis, 1987), which may have resulted in greater potential for survival of this strain.

Broadbent *et al.* (2010) optimized conditions for the acid tolerance response (ATR) in *Lb. casei* ATCC 334 and then analyzed its effects on membrane lipid composition and global gene expression. The membrane lipid composition of acid-adapted cells showed a dramatic increase in the ratio of saturated to unsaturated membrane FAs and cyclopropane FA content. Comparisons between the transcriptome of cells grown at pH 6.0 (control) to that of acid-adapted (5 or 20 min

at pH 4.5) or acid-adapted and then acid-challenged (20 min at pH 4.5 and then 10 min at pH 2.0) cells showed differential expression of numerous genes in acid-treated versus control cells. Overall, functional predictions for these genes indicated that acid adaption invoked a stringent-type response that was accompanied by other functions which likely helped these cells resist acid damage, including malolactic fermentation and intracellular accumulation of histidine which were important for enhanced acidurance. Validation of microarray data was provided by follow-up experiments that showed that *Lb. casei* survival at pH 2.5 was improved at least 100-fold by chemical induction of the stringent response or by the addition of 30 mM malate or 30 mM histidine to the acid challenge medium.

Results of Wu *et al.* (2009) showed differentially expressed proteins between the exponential and stationary phase of *Lb. casei* among which 70% up-shifted in the stationary phase, which was confronting a gradually acidic condition, including proteins involved in energy metabolisms. Wu *et al.* (2011) reported that multiple metabolic pathways were involved in the response of *Lb. casei* during acid stress, particularly in relation to carbohydrate metabolism. This confirmed that glycolytic enzymes were involved in the generation of sufficient energy for the cell during growth under acidic conditions.

Hosseini Nezhad *et al*. (2010) studied the mechanisms of growth under acidic conditions and the impact of low pH on the relative level of protein expression of a typical strain of *Lb. casei*. Late log-phase cells cultured at pH 4.0 showed obvious changes in Gram staining properties while transmission electron microscopy analysis revealed evidence of structural distortions of the cell

surface relative to the controls cultured at pH 6.5. When comparing cytosolic or whole cell preparations on SDS-PAGE, few changes in protein profiles were observed under the two growth conditions. However, analysis of surface protein extracted by 5M LiCl demonstrated changes in the proportions of proteins present in the molecular weight range of 10 to 80 kDa, with some proteins more dominant at pH 6.5 and other at pH 4. The results suggested that surface proteins of this strain are associated with growth and survival at low pH. The function of these proteins was subject to further investigation (Hosseini Nezhad *et al.*, 2012). In this research, change in relative abundance of the cell surface proteins of *Lb. casei* was investigated in response to acidic conditions, using 2-DE, Western blot analyses and MALDI-TOF-TOF mass spectrometry. Results showed that many enzymes involved in glycolysis were up-regulated on the cell membrane fraction following growth at low pH, including enolase, lactate dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase. Several of these proteins were also related to adhesion and generalized stress responses. It was demonstrated that growth of *Lb. casei* under acidic conditions caused molecular changes in the cell surface to develop an adaptive strategy to enable growth at low pH. Given that these proteins may be important for probiotic function, this work suggests that preconditioning cells prior to application may be important for survival, competiveness and functionality of probiotic strains.

Conclusion

This review was intended to present a comprehensive overview of the stress response in *Lb. casei/paracasei* strains of industrially important and probiotic LAB. The environmental conditions in the stomach and in GI track are challenging to probiotic organisms because of the

presence of bile salts, acids and enzymes. NSLAB including *Lb. casei* group are also exposed to a variety of stresses, mainly heat or cold shock, salt stress, acid stress and starvation, during the manufacturing of cheeses and other dairy products, which limit growth and influence the physiology and biochemistry of cells. Given the importance of these strains in industrial applications as well as their health promoting effects, rapid advances have been made in characterizing stress responses of *Lb. casei* group in recent years, although a great deal still remains to be revealed. Future research will need to differentiate between the influence of specific stressors and their combined impact on cell growth and survival, particularly differentiating between changes due to growth stage and the impact of specific stressors, given that most studies to date have not controlled variables associated with measuring stress responses against the changing physiology of cells that occurs during the movement from exponential to stationary phase of growth.

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