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REVIEW

How do bifidobacteria counteract environmental challenges? Mechanisms involved and physiological consequences

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Abstract An effective response to stress is of paramount importance for probiotic bifidobacteria administered in foods, since it determines their performance as beneficial microorganisms. Firstly, bifidobacteria have to be resistant to the stress sources typical in manufacturing, including heating, exposure to low water activities, osmotic shock and presence of oxygen. Secondly, and once they are orally ingested, bifidobacteria have to overcome physiological barriers in order to arrive in the large intestine biologically active. These barriers are mainly the acid pH in the stomach and the presence of high bile salt concentrations in the small intestine. In addition, the large intestine is, in terms of microbial amounts, a densely populated environment in which there is an extreme variability in carbon source availability. For this reason, bifidobacteria harbours a wide molecular machinery allowing the degradation of a wide variety of otherwise non-digestible sugars. In this review, the molecular mechanisms allowing this bacterial group to favourably react to the presence of different stress sources are presented and discussed.

Keywords Bifidobacteria · Stress · Technological processes · Gastrointestinal tract

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Introduction

The human gastrointestinal tract (GIT) harbours a microbial community of great richness and complexity. The total number of intestinal microorganisms represents more than 10 times that of eucaryotic cells in the human body. This complex community constitutes the so-called *intestinal microbiota*. Different bacterial groups and levels are found throughout the gut, corresponding with the different ecological niches present from mouth to colon. In the sparsely populated small intestine, genera such as *Lactobacillus* and *Bacteroides* are considered predominant; while in the heavily populated large bowel, the most frequently found microorganisms include *Bacteroides*, *Clostridium*, *Fusobacterium*, *Ruminococcus*, *Eubacterium* and *Bifidobacterium*. Most people are aware of the presence on the microbiota of potentially detrimental microbes causing health problems (e.g. diarrhoea), but do not know that there are also bacteria naturally present as part of the intestinal microbiota beneficially affecting health. Such beneficial microbes constitute the basis for the use of probiotics and have often been isolated to be included in probiotic products used to improve host health. Indeed, numerous health-promoting properties have been attributed to certain members of the intestinal microbiota, such as bifidobacteria, and these microorganisms are extensively used as probiotics by the food industry.

Probiotics are defined as *live microorganisms which when administered in adequate amounts confer a health benefit on the host* [19]. This definition underlines the importance of having a sufficient number of viable microorganisms throughout the entire shelf-life of the product in which they are included. Nevertheless, several reports have indicated a relatively poor survival of probiotic strains during most of the technological processes used

by the food industry [11, 24, 30]. This has limited the use of probiotics in most food product categories. Therefore, strains with increased stress tolerance would open new opportunities for the development of novel products. Moreover, in addition to technological stress, once they are ingested, probiotics must overcome biological barriers present in the GIT to reach their place of action and exert their beneficial effects. These barriers include digestive enzymes, acidic pH and bile. Candidate probiotic strains should thus possess traits such as resistance to gastric acidity and bile toxicity. Given the gut origin of most probiotic microorganisms (e.g. bifidobacteria), like other members of the gut microbiota, they must have developed adaptive processes to tolerate these harsh conditions found in the gut.

Several strategies are under investigation to improve probiotic survival, mainly by using pre-exposure to sublethal stresses or selection of derivative strains by stress adaptation. In 1994, it was shown that exposure to sublethal temperatures increases the stress tolerance of *Lactobacillus bulgaricus* [90]. More recent studies confirm this result, indicating that a stress pre-treatment may increase the survival of probiotics under stress conditions [4, 5, 68]. It is also possible to select stress-tolerant derivatives from originally sensitive strains adaptation [13, 44, 54]. One of the first indications of the selection of derivative *Lactobacillus* strains with increased thermal-tolerance was reported in the 1950s [51], and there is also recent evidence that selected strains of probiotics can adapt to other industrial stresses [20]. Nevertheless, care should be taken as stress adaptation may alter other probiotic functions. Therefore, understanding the molecular basis of the stress response is a key element not only for the further development of strains with increased stress tolerance but also to know the nature of the modifications in the cell physiology, in order to avoid undesirable changes in the strain properties.

In recent years, different authors have studied the molecular response of probiotic bacteria to stress and some genes have been identified [16, 94]. Proteomic approaches have also been used to this end [5, 57, 72, 73]. This manuscript aims to review the characteristics of the response of bifidobacteria to both technological and gastrointestinal stresses, as well as the different options to improve stress tolerance (Fig. 1).

Response to technological stresses

The preparation of probiotic formulas in large-scale for oral delivery, either in pharmaceutical preparations or in food carriers, involves the utilization of several technologies that could affect the viability and functionality of these bacteria. For the generation of a functional probiotic ingredient, dried formats such as freeze-drying and spray-drying are very attractive [48]. However, these technologies expose the bacterial cultures to extreme conditions mainly dealing with temperature and presence of oxygen [10, 88]. Additionally, when probiotics are added as ingredients into food formulations, the type of food matrix and presence of other live microorganisms, as well as the processing technologies and food storage conditions, must be carefully taken into account. Traditionally, fermented dairy products are considered the best carriers for probiotic delivery [75], but other food matrices are also suitable approaches to keep probiotic functionality such as vegetable and cereal beverages among others [59, 62]. Conventional food processing uses heat treatments to preserve the safety and quality of raw materials. But emerging strategies (high pressure, pulsed electric fields, ultrasound, etc.) are under consideration to be implemented in the processing of functional foods, which could present new challenges for probiotic microorganisms [33]. Thus, current food processing submits bacteria to heat, mechanical and aeration stresses, as well as to an acidic environment resulting from the metabolic activity of other microorganisms or the food matrix composition. Some of these challenges are still present during the shelf-life of functional foods, which are stored under refrigerated conditions; therefore, cold shock is an additional stress.

From a technological point of view, bifidobacteria are not robust microorganisms. They are strict anaerobes and only a few species, such as *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium boum*, *Bifidobacterium thermophilum, Bifidobacterium dentium* and *Bifidobacterium psychraerophilum,* can tolerate a microaerophilic environment [32, 41, 87, 101]. In general, their optimal growth temperature ranges between 36–38°C and 41–43°C for human and animal origin strains, respectively, although *B. thermacidophilum* is able to grow at 49°C and *B. psycraerophilum* at 4°C. The optimal growth pH is around neutrality (6.5–7.0), but *B. animalis* and *B. thermacidophilum* survive well at pH 3.5–4.0 [18, 46, 87]. From these values, it is clear that a tight range of oxygen, temperature and acidic conditions is compatible with bifidobacterial life. However, some bifidobacteria are able to survive under more extreme conditions (e.g. *B. animalis* subsp. *lactis* strains), while others display a low tolerance to stress (e.g. some *Bifidobacterium bifidum* and *Bifidobacterium longum* strains), although the physiology beyond their adaptation is far from being understood. The mechanisms most extensively studied in response to technological stress of bifidobacteria are those related to heat, oxygen and acid stress. The last one will be reviewed in Sect. "Response to gastrointestinal stress factors", since it is also one of the main barriers that these microorganisms must overcome to survive the gastric transit.

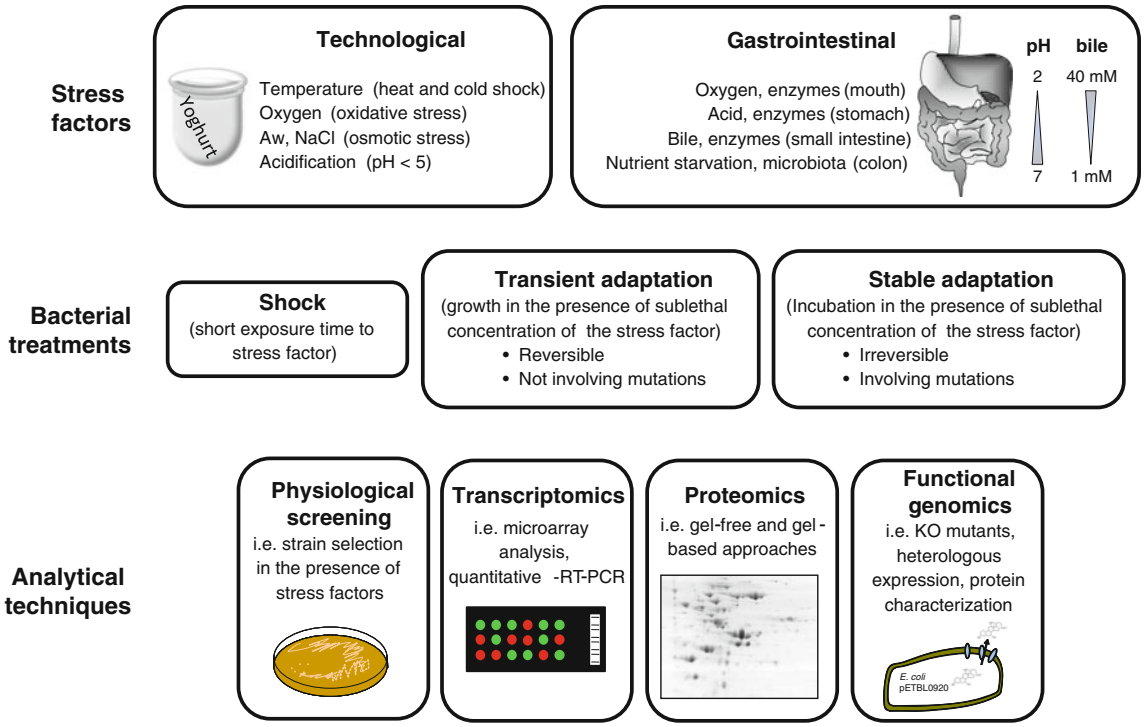

Fig. 1 Schematic representation summarizing the different stress sources, stress treatments and the techniques employed for the study of their effects on bifidobacteria. *Upper panel*, stress sources can be classified into two groups, technological or gastrointestinal. *Middle panel*, relating to the duration of the treatment, a short exposure, cell growth for a given time, or long-time exposure to the stress may lead to a stable (or transient) phenotype of resistance. *Lower panel*, different strategies used to study stress responses in *Bifidobacterium* strains

Oxygen

Oxidative damage is related to the production of reactive oxygen species. From the results of scarce studies dealing with the oxygen detoxifying mechanisms in bifidobacteria, it seems that oxygen tolerance could be related to the presence of some enzymes and also to modifications in morphology and cell surface components. The anti-oxidative enzymes catalase and superoxide dismutase seem not to be involved in the aerotolerance of *Bifidobacterium*. However, the detoxification of hydrogen peroxide by means of NADH oxidative enzymes might play a key role improving bifidobacteria survival under oxygen [86]. Besides, in presence of oxygen, *B. longum* increases its lag growth phase and morphological changes became apparent (long size and rough surface) associated with changes in the fatty acid profile (shortened chains) and induction of the synthesis of some proteins (Osp proteins) [1]. Variations in surface hydrophobicity, in relation to a reduction in the carbohydrate levels and an increase in protein content detected by FT-IR spectroscopy, were reported as well in *B. animalis* subsp. *lactis* Bb12 in presence of oxygen [84].

Heat

Heat shock (HS) is the most studied technological stress in the *Bifidobacterium* genus. In response to this challenge, several HS proteins, such as chaperons, proteins related to DNA and RNA synthesis and cell division, are increased, this being a mechanism conserved in several species such as *B. longum, Bifidobacterium adolescentis* and *Bifidobacterium breve*. This is the case of the chaperon DnaK which is induced at transcriptional level by increasing temperatures [82]. Most investigations into HS response and adaptation have been performed in *B. longum* NCC2705 and *B. breve* UCC2003, taking advantage of the knowledge of its genome sequence and using advanced molecular tools. The global transcriptomic analysis of *B. longum* NCC2705 exposed to HS treatment (50°C for 3, 7 and 12 min) showed an alteration of expression in 46% of the genes [60]. Induction of chaperon systems (DnaK, GroEL and HtrA) was previously reported in this species by proteomic approaches [80]. Additionally, strain NCC2705 also responded to HS by inducing the *smpB* gene, coding the tmRNA-associated small protein B. Thus,

the trans-translation machinery is up-regulated with a high constitutive expression of tmRNA gene [60]. For this strain, couples of wild (parental) and derivative (heat adapted) strains could be compared. The HS-derivative NCC2912 was obtained from *B. longum* NCC2705, and the proteomic analysis of both strains showed quantitative differences in the relative abundance of 19 common proteins. These proteins were involved in glucose metabolism and protein synthesis, and some HS proteins, previously identified by other authors, were also detected [28, 80]. The induced HS proteins were chaperons and proteases, mainly ClpC, ClpA/B, GrpE, DnaK and fk506-binding protein [28]. Regarding DnaK chaperon, it has recently been shown that its up-regulation by HS is due to mutations in *hspR* gene, coding for the negative regulator of *dnaK* operon. The mutations affect its capability for binding to the *dnaK* promoter, thus rending an overexpression of DnaK [8]. The differences between these two wild-type and HS-derivative *B. longum* proteomes and transcriptomics reveal a HS-resistance pattern that could reflect the metabolic adaptation to heat stress.

Regarding the HS response of *B. breve*, it is clear that chaperones play a predominant role. Ventura and co-workers demonstrated that the molecular response to heat takes place, in the strain UCC2003, with an over-expression of two groups of chaperones. The first group, composed of the proteins GroEL, GroES, ClpC and ClpPs, is involved in the response to moderate heat shock, whereas the second (DnaK, GrpE, DnaJ and ClpB) is associated with more severe increases in the processing temperatures [94]. In addition, the gene *hsp*20, coding for a member of the small heat-shock protein family, has been shown to be highly over-expressed after heat shock [95].

Microbial competition

Another technological challenge that needs to be addressed is the simultaneous presence of two or more microorganisms in the food matrix. The competition for nutrients and the production of antimicrobial metabolites, mainly organic acids, could affect the microbial growth and functionality. In this way, Ruiz and co-workers [66] observed by means of two-dimensional electrophoresis (2DE) that 16 proteins changed their expression in *B. longum* and *B. breve* when grown simultaneously in compartmentalized co-culture in comparison with monocultures. These included ribosomal proteins and those involved in carbohydrate metabolism, gene regulation, cell envelope biogenesis and transport. Thus, it is clear that the physiology of probiotics could be affected when they are added in combination with other probiotic or starter cultures.

Response to gastrointestinal stress factors

Bifidobacteria display a set of mechanisms in order to overcome the adverse situations encountered through the human GIT and transiently colonize this competitive environment. This section gives an overview on how these microorganisms surmount these difficulties, the processes involved in its response and tolerance to those factors, and the most recent insights into the field. The main molecular mechanisms and gene/protein players are summarized in the Table 1, and some of them are represented in Fig. 2.

Acid pH

Orally administered bifidobacteria firstly experience severe acidic conditions in the stomach, where the pH is close to 2, and this strongly compromises bacteria viability. In general, bifidobacteria tolerance to acid is reduced with the exception of *B. animalis* [34, 45]. Isolation of strains with good acid tolerance is desirable, since it is usually related to cross-resistance to some other stress factors, either those characteristic of the intestinal environment or typical of technological processes [13, 79].

A common bacterial response to acidic conditions is the so-called acid tolerance response (ATR), in which acid adaptation is achieved through an assemblage of inducible molecular mechanisms [14, 15, 70]. ATR is induced in bifidobacteria after the exposure of cells to sublethal acidic conditions, enhancing its survival to more severe exposures [47]. pH resistant bifidobacteria strains with stable phenotype have also been isolated after long exposure to acid pH [13]. This stable phenotype was related to changes in the surface properties of the strains, such as better adherence to mucin or pathogen displacement [13].

Up to date, only one comparative study, in which an acid pH resistant *B. longum* strain and its parental (acid pH sensitive) strain were compared, has been performed [72]. In this work, the two main cytoplasmic subunits of the F_0F_1-ATPase (*atpA* and *atpD*) were pointed out to be overrepresented on 2DE patterns after acid exposure, providing a key role of this enzyme in the *Bifidobacterium* ATR. The activity of this enzyme was also higher in membranes of *B. animalis* subsp. *lactis* grown under acidic conditions [74]. This multimeric enzyme is responsible for the active extrusion of protons that acidify the bacterial cytoplasm under acidic conditions and is directly related to the acid resistance of a given strain [46]. F_0F_1-ATPase contains eight subunits coded in an operon that is organized in two groups, each of them coding for each of the subunits (F_0, membrane subunit and F_1, cytoplasmic subunit). Ventura and co-workers established that the F_0F_1-ATPase operon is transcribed in two different mRNAs, the

Table 1 Main strategies of response of bifidobacteria to different stress factors

Stress source	Strategy of response	Molecular mechanisms/players
Heat shock	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, ClgR
Acid pH	Proton extrusion	F_1F_0-ATPase
	Cytoplasm buffering/ammonia production	Branched-chain amino acid production
		Glutamine synthetase
	Unknown	CysD, MetE
Bile salts	Bile salt/acid detoxification	Multidrug transporters and bile efflux pumps (BetA, Ctr)
	Bile salt deconjugation (unclear)	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, GroEL, DnaK
	Degradation of misfolded proteins	Proteases: ClpC
Antimicrobials	Cytoplasm detoxification	Efflux pumps
Carbon source fluctuations	Ability of degrading a high range of carbohydrates	Extracellular oligosaccharide-binding proteins, glycosydases and transcriptional regulators

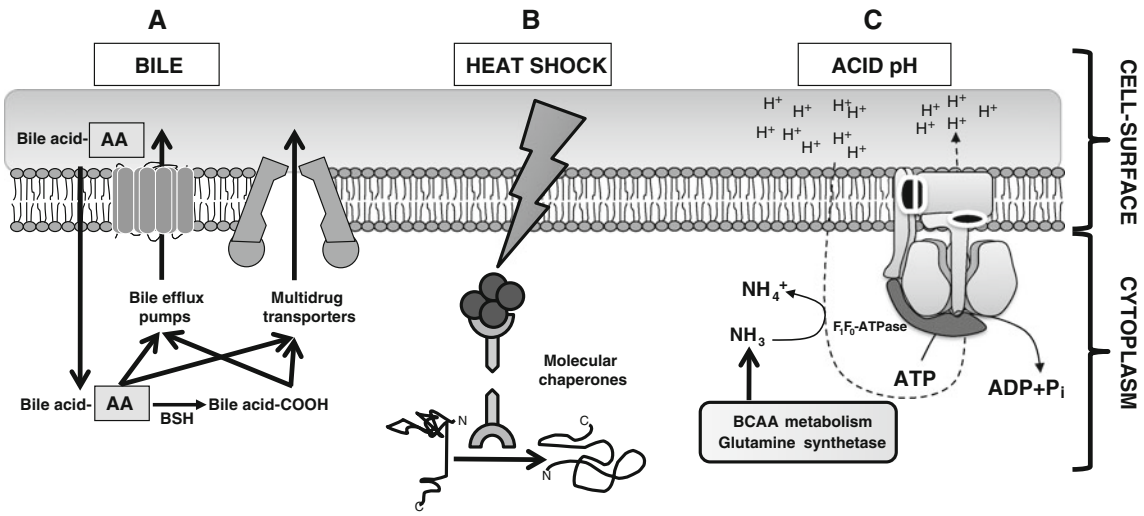

Fig. 2 Main molecular mechanisms involved in the response of bifodobacteria to different stresses. **a** Bile is detoxified from the cytoplasm by the activity of bile efflux pumps and/or multidrug transporters. Conjugated bile acids are deconjugated by the bile salt hydrolase (BSH), although the relationship of this enzyme with the resistance to bile is unclear. **b** Finally, 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

production of both of them being induced under acidic conditions [93].

ATR in *B. longum* has been associated with deep reorganizations affecting the level of glycolytic enzymes involved in the metabolism of complex carbohydrates. In addition, the branched-chain amino acid metabolism was also connected to ATR. Specifically, a higher concentration of glutamine synthetase and enzymes involved in branched amino acid synthesis was detected under acidic conditions in *B. longum*. This proteomic data, together with some

physiological results [72], are in agreement with the hypothesis formulated by Len and co-workers [39], where the increase in branched-chain amino acids and ammonia production are coupled through the enzyme glutamine synthetase. The ammonia released would capture protons, acting as a cytoplasmic buffer [92]. Finally, ATR in *B. longum* also involved stable changes in the production of enzymes implied in sulphur amino acid metabolism [72] although, to date, it still remains unclear whether those changes are associated to the ATR.

Bile salts

Bile is the second biological barrier that probiotic bifidobacteria have to overcome after being ingested. Bile acids and salts are the main components of the bile flow [7], determining its antimicrobial and detergent-like properties. Bile acids are weak organic acids that can passively enter the bifidobacteria cytoplasm [37]. This passive accumulation of deconjugated bile acids has a deep impact on the metabolic processes and homeostasis of the cell, causing leakage of ions and other cellular components, and leading finally to cell death. In particular, bile acids are more toxic than conjugated bile salts (taurine or glycine conjugates), since the latter need specific transporters to be taken up by bacteria [54]. Bile tolerance is very dependent on the species within the genus *Bifidobacterium*. However, bifidobacteria can develop stable bile resistance phenotypes as a response to bile exposure, regardless of its intrinsic tolerance [77], and often this process involves the appearance of cross-resistance to other stress factors [74]. Additionally, adaptation to bile has been related to changes in carbohydrate metabolic profiles [44, 54, 64], changes in lipid and protein composition of membranes [44, 67], in the ability to compete with enteropathogens [26, 27] and in the antibiotic resistance patterns, among others [34, 52].

The structure and composition of the bacterial surface have a profound influence on bile tolerance. More concisely, lipid composition, membrane protein profiles and cell wall functionality play key roles in the response to bile in the genus *Bifidobacterium* [22, 44, 67]. Production of extracellular polysaccharides (exopolysaccharides; EPSs) is also a mechanism mediating protection against bile in this genus [3, 63], and a direct correlation has recently been established between EPS production and bile tolerance in *Bifidobacterium* spp. [3]. Furthermore, bile decreases the concentration of several enzymes involved in fatty acid and phospholipid synthesis in *B. animalis* subsp. *lactis* [21, 73], those observations being in accordance with previous findings related to the induction of changes in membrane composition of bifidobacteria [36, 37, 67]. These changes in the lipid composition of the membranes are thought to reduce the bile salt diffusion rate to the cytoplasm, thus contributing to bile tolerance. In the case of surface-associated proteins, such as adhesins, some of them are overproduced as a response to bile, and they may enhance the colonization of the human gut, although this statement is merely speculative [9, 35, 65].

The cell membrane is one of the main targets of bile, and cells can be detoxified from bile salts by the action of efflux pumps. In the particular case of bifidobacteria, two bile efflux pumps have been described up to date, Ctr and BetA, being directly related to bile resistance through a bile extrusion activity [25, 58]. Interestingly, the gene coding for BetA has been shown to be under the control of a bile-inducible promoter, guaranteeing in this way its expression in the human intestine, where bile salts are present.

Remarkably, particular responses to bile seem to be species dependent [71, 73, 77]. While most of the enzymes of the glycolysis were overrepresented upon bile exposure in *B. longum*, xylulose-5-phosphate/fructose-6-phosphate phosphoketolase and glyceraldehyde-3-phosphate dehydrogenase were the only enzymes of this pathway overrepresented in *B. animalis.* This observation was correlated to glucose consumption, increased in *B. longum* but not in *B. animalis,* in response to bile.

Oxidative damage is another harmful effect of bile salts on the cells [55]. Among the mechanisms employed by bifidobacteria in order to deal with such damage, induction of proteins involved in SOS response, including a thioredoxin-dependent thiol peroxidase, is noticeable [73]. Adaptation to bile salts in *B. animalis* was also related to changes in methionine biosynthesis enzymes, thus suggesting methionine metabolism (whose sulphur group is susceptible to oxidization) and regulation of redox state of the cells are crucial for regulation of oxidative stress [29, 77].

Other groups of proteins whose production were affected by the presence of bile salts were chaperones and proteases, overproduced in order to conduct proper folding of proteins, and to promote a quicker recycling of misfolded and aggregated proteins [31]. In this regard, a complete set of chaperones has been revealed to be overexpressed in *Bifidobacterium* in response to bile exposure or adaptation, among which are ClpB, GrpE, HtrA, GroEL, GroES and DnaK [71, 73, 80].

Finally, it is noteworthy to highlight that bile adaptation in *B. animalis* was related to a constitutive increase in the production and activity of the enzyme bile salt hydrolase (BSH) [53, 73]. BSH is responsible for bile salt deconjugation, but its role in the response to bile is, to date, unclear [23]. However, this observation suggests some role of BSH in bile adaptation, although its expression does not seem to be regulated by bile. On the contrary, in vivo assays have demonstrated an intracellular accumulation of BSH in *B. longum* in the GIT of rabbits, suggesting that intestinal factors different from bile could trigger BSH expression [104].

Carbon source availability

Fluctuation in carbon source availability in the gut is an important factor conditioning the balance of microbial groups within the human intestinal microbiota. Bifidobacteria are, in this regard, very well adapted to these fluctuations, displaying a high metabolic versatility allowing utilization of different carbon sources. In silico analysis of the available *Bifidobacterium* genomes has revealed the presence of an extensive battery of genes potentially involved in carbohydrate transport and metabolism, including a broad arsenal of glycosidases. The simultaneous bioactivity of these enzymes allows bifidobacteria to metabolize from galactomannan-rich plant gums to human milk oligosaccharides (HMOs) and glycans from mucin [6, 83, 91]. Remarkably, *B. longum* subsp. *infantis* genome contains the largest amount of genes coding for predicted extracellular oligosaccharide-binding proteins (21 copies) versus, for instance, the 10 or 11 present in the genomes of *B. longum* subsp. *longum* or *B. adolescentis*, respectively. In this context, there is evidence for genetic duplication in the origin of some carbohydrate utilization clusters. This fact reflects the strong selective pressure to which *B. longum* genomes are exposed, in order to obtain a high carbohydrate catabolic diversity and thus increase their fitness in the GIT environment [83].

Overall, the broad diversity of carbohydrate metabolism activities coded in *Bifidobacterium* genomes agrees with the high carbohydrate transport and metabolism genes overrepresentation in human gut microbiomes [38, 40], which reflects the importance of those gene categories in adaptation to the intestinal environment [43]. All this catabolic machinery, allowing bifidobacteria to quickly adapt to the wide substrate fluctuations occurring at the intestinal level, seem to be orchestrated by the action of the large percentage of transcriptional repressors encoded in their genomes [6, 81, 83, 91].

Improving the robustness of bifidobacteria

Different *Bifidobacterium* strains may show big differences in their tolerance to technological and physiological stresses; therefore, the identification of strains showing greater tolerance among those presenting suitable probiotic properties is important for granting the efficacy of probiotic microorganisms included in food products. Several production and culture conditions, i.e. the presence of certain single sugars, can contribute to increase the tolerance of bifidobacteria to stress factors [53, 56]. Organic matrices, in some cases combined with prebiotics, have been employed in cell-entrapment techniques (e.g. microencapsulation) to improve the viability of bifidobacteria in foods or through the gastrointestinal transit [17, 50, 89]. The addition of protectors and supplements can be used with the same purpose [69, 78]. In this respect, milk, or milk-derived preparations, has been shown to act as effective protectors of bifidobacteria during simulated gastrointestinal digestions, thus increasing their survival in physiologically stressful conditions [3, 76]. Skimmed milk powder protects bifidobacteria during freeze-drying and spray-drying procedures used in the industry for preparing concentrated microbial cultures and facilitates the rehydration of the dried preparation [12, 49].

Apart from external factors helping bacteria to cope with unfavourable environmental conditions, the improvement of the stress tolerance of the microorganism itself constitutes one of the main targets of the current research in bifidobacteria. In this regard, stress adaptation and gene modification are important areas of research.

Variations in stress tolerance have been documented for bifidobacteria and stress-resistant mutants sometimes arise spontaneously [13, 42]. Exposure of bifidobacteria to sublethal stress factors is a relatively easy way to increase the appearance of such stress-resistant mutants. These derivatives usually present a stable stress-resistant phenotype, with changes affecting not only the targeted property but often also triggering other pleiotropic changes and presenting cross-resistance to other stresses and alteration of other physiological features [54, 77]. Functional properties should then be re-assessed in these new strains when they are intended for probiotic or technological use.

In general, the resistance of bifidobacteria to acidic pH is poor, with the exception of *B. animalis* [45, 61], which could partially explain the good survival rate of this species through the human GIT [2, 61]. However, it has been found that acquisition of resistance to bile implies a concomitant increase in resistance to low pH and vice versa, adaptation to low pH is associated with a bile resistance phenotype [72, 74]. The most relevant feature that links bile and acid adaptation seems to be the extrusion of protons from the cytoplasm through the activity of the membrane F_1F_0-ATPase [74]. In fact, the induction of the F_0F_1-ATPase seems to be very important for maintaining the viability of bifidobacteria under GIT conditions [74].

Regarding technological stresses, it is worth mentioning that heat-tolerant mutants of *B. longum* have been obtained by successive sublethal heat-shock treatments. The genetic bases of such adaptation have been characterized recently [8, 60, 80]), and a few *Bifidobacterium* derivative strains able to grow aerobically have also been reported [41, 103]. These adapted strains represent a promising option for the functional food industry.

Improvement of the stress tolerance can also be obtained by gene modification. This strategy was used to increase *B. breve* [85] tolerance to acidic and high osmolar

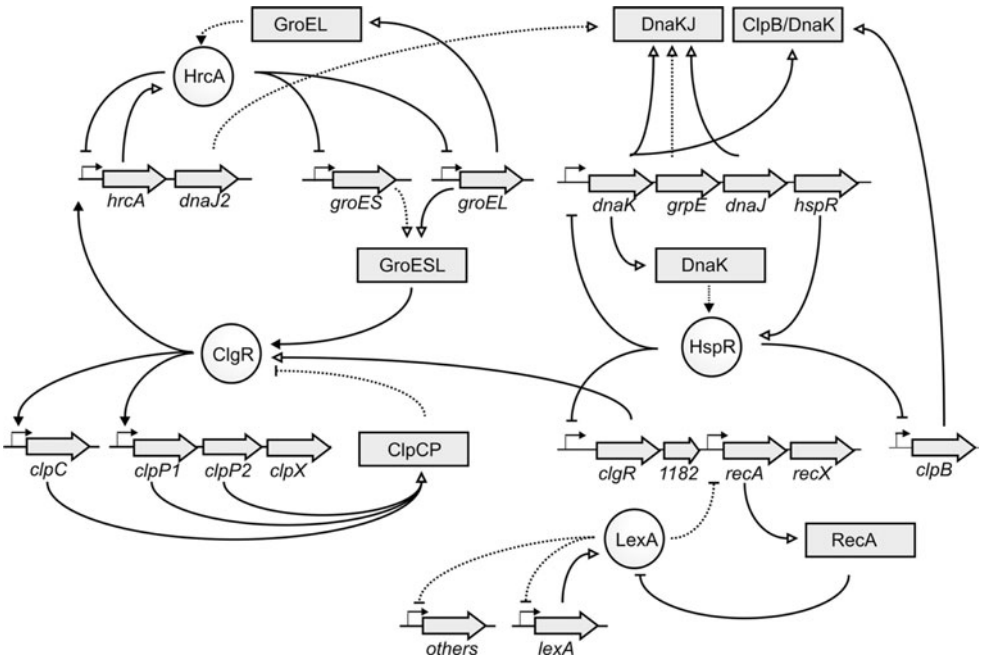

Fig. 3 Schematic representation of the stress gene regulatory network proposed for *B. breve* UCC2003. *Dotted lines* indicate the predicted interaction, and *closed lines* indicate a proven interaction. A *dash* at the end of a *line* indicates repression, while a *triangle* at the end of a *line* indicates activation. Connections between the various regulons occur at different levels, which is indicative of complex interactions. [Reproduced with ASM press permission, Copyright 2009; from [105]

conditions mimicking the gut environment by cloning the listerial betaine uptake system, BetL, into the probiotic strain *B. breve* UCC2003. A similar approach was followed to increase bile resistance in the same strain by heterologous expression of the *L. monocytogenes* bile resistance mechanism BilE [102]. An improvement of gastric transit and of intestinal persistence was obtained with both strains following oral administration to mice; additionally mice fed the recombinant strains showed reduced oral infections by *Listeria monocytogenes*. In spite of that the introduction of genes from pathogens into probiotic strains is unlikely to meet with immediate approval from regulatory food authorities.

Regulation of the stress response

The response of bifidobacteria to the different environmental challenges is a multifactorial process involving the action of several mechanisms and cellular pathways. In the general response to stress, chaperones hold a prevailing place. Chaperones are a subset of proteins involved in both the correct folding of proteins and in the enzymatic degradation of misfolded proteins, two common (and negative) effects promoted by stress. Firstly described as heat-shock proteins (HSPs), molecular chaperones include GroEL (HSP60), DnaK (HSP70), ClpB (HSP100), and proteases such as ClpC and ClpP, among others [94].

Regulation of stress response has been studied in detail in *B. breve* UCC2003, and it has been shown that different groups of chaperons are affected depending on the regulators involved. For instance, during HS the regulation of *clp*C and *clp*P genes is dependent on the product of the gene *clg*R, a transcriptional activator that requires the presence of the protein GroEL as cofactor [98, 100]. On the contrary, genes *gro*EL and *gro*ES are regulated by the repressor protein HrcA [96]. The other group of chaperones is basically regulated by the repressor HspR [97, 99]. This chaperone accumulation following HS has been confirmed by studies involving high-throughput techniques, such as DNA arrays or 2DE [60, 80].

Recently, the work by Zomer and colleagues has shed some light on how the response to different stresses may overlap. Indeed, the molecular regulation of chaperone induction appears to constitute a general molecular mechanism of stress response in *Bifidobacterium*, depending, in turn, on the accumulation of misfolded proteins [105]. In the genus *Bifidobacterium*, and in general in the *Actinobacteria* taxa, the genes coding for chaperones are arranged in operons, which are subjected to tight genetic regulation, in a sort of regulon. This is dependent on the existence of certain transcriptional regulators, mainly HrcA (heat regulation at CIRCE), HspR (heat-shock protein repressor) and ClgR (Clp gene regulator), each of those binding to more or less conserved inverted repeats present in the promoter regions of the above mentioned operons [105]. This regulatory network has been suggested as the paradigm for stress adaptation in bifidobacteria [105] and is represented schematically in Fig. 3. In this network, the different operons containing the different chaperones are regulated by the interaction at different levels of the transcriptional regulators ClgR, HrcA and HspR with chaperones (HrcA with free GroEL, ClgR with GroESL and

HspR with DnaK) and with the corresponding inverted repeats located in the different promoters. An in-depth explanation to all those processes can be found in Zomer and co-workers [105], and in Ventura and colleagues [94].

Perspectives and future challenges

The selection of bifidobacteria strains for being included in functional foods has traditionally been carried out mainly taking into account phenotypical aspects related to their appropriate technological performance, and their ability to survive the harsh conditions of the human GIT, without paying much attention to the mechanisms responsible for stress tolerance, which is largely unexplored at molecular level. However, during the last decade detailed physiological analyses, supported by functional genomics and proteomics data, shed some light on the molecular processes used by bifidobacteria to counteract environmental challenges, as well as in the mechanisms of stress sensing, gene regulation and adaptive behaviour of this bacterial group. Future and ongoing research in this field must take advantage of the enormous amount of data generated from bacterial genome projects and human microbiome projects. This will facilitate the understanding of the specific effects of probiotics, the processes involved in survival and the crosstalk mechanisms with the human host. Also, research on stress response might help to generate improved specific strain properties by building robust stress resistance phenotypes that prepare these microorganisms to cope with adverse conditions. Such data will also enable us to know which factors influence the performance of bifidobacteria, thus allowing a rational approach to strain improvement and, in turn, provide information about the functional properties of probiotics.

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