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Proteomics of stress response in *Bifidobacterium*

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1. ABSTRACT

Bifidobacteria are commensal microorganisms of the human gastrointestinal tract which are largely being used in functional foods. Some strains are considered as probiotics since they beneficially affect the composition and the metabolic activity of intestinal microbiota, as well as the health status of the host. The lack of genetic tools has hindered the development of functional genomic studies in bifidobacteria, like the identification of molecular mechanisms underlying their survival under different environmental challenges. Some of these experimental obstacles have been successfully overcome with the use of proteomics, a set of techniques that, when applied to microorganisms, are directed to the identification of all the proteins produced by the cells under a given physiological condition. The aim of this review is to discuss and summarize some of the current knowledge of the stress tolerance in bifidobacteria, mainly identified by two-dimensional electrophoresis coupled to mass spectrometry, and compare the most recent proteomic results with the currently available transcriptomic studies. The input and advantages of novel high throughput proteomic techniques are considered as well.

2. INTRODUCTION

The gastrointestinal tract (GIT) of a healthy adult is a complex ecosystem which contains representative species of the three domains of life: bacteria, archaea and eukaryota. All these populations, which as a whole are known as GIT microbiota, display one of the highest cellular densities in nature: between 10^{12}-10^{14} CFUs per gram of intestinal content (1). In spite of such a large microbial population inhabiting our GIT, its biodiversity is rather low compared to other ecological niches; thus *Bacteroidetes* and *Firmicutes* divisions represent up to 99% of intestinal eubacteria, whereas archaebacteria are mainly represented by the species *Methanobrevibacter smithii* (2). Probiotics are microorganisms involved in the improvement or maintenance of GIT health by influencing its microbial activity, composition and ecology. Bifidobacteria are the most widely used probiotics in human functional foods together with lactobacilli, and they are believed to confer beneficial effects on the host such as treatment of diarrhoea, balancing of the intestinal microbiota, and alleviation of atopic eczema in infants, among many others, although the genetic basis of these promoting effects are still unknown (3). The genus

Bifidobacterium, strictly anaerobic and pleiomorphic rod-shaped bacteria belonging to the *Actinobacteria* class, is mainly constituted by species isolated from human or other animal GITs, representing in some cases up to 1-3% of all the ensemble of the human intestinal microorganisms in adults (4). Survival of bifidobacteria ingested with functional foods largely depends on their capacity of adaptation or resistance to the different adverse conditions of the GIT, mainly acid pH in the stomach and bile salts in the small intestine, which may significantly influence their success as probiotics.

Like other living organisms, bifidobacteria appear to exert strict regulation of their gene expression, which provides them with the right amounts of the different proteins that they need to adapt their metabolism to the physiological fluctuations of their habitat (5). In recent years, proteomic approaches have become very popular for the characterization of changes in protein synthesis after the exposure of a microorganism to a given stress factor. Proteomics has been defined as the "large-scale characterization of the entire protein complement of a cell line, tissue, or organism" and comprises an ensemble of different experimental techniques that include high-throughput protein identification and quantification, protein-protein interaction studies and characterization of protein function, locations and modifications (6). One of the most widely used proteomic techniques is two-dimensional electrophoresis (2DE) performed in immobilised pH gradients coupled to protein identification by mass spectrometry (MS). 2DE gathers two different techniques: isoelectrofocusing (IEF) or first dimension, and SDS-PAGE or second dimension, allowing high-throughput and accurate protein separation according to their isoelectric point and their molecular mass, respectively (6). 2DE has been the technique of choice for many scientists carrying out research in the field of stress response, due to its adequacy to quantify shifts in protein synthesis caused by environmental changes. Furthermore, 2DE is a reasonable technique to get a first impression on protein post-translational modifications, since proteins that show more than one position on the 2D gel are good candidates for post-translational modification reactions. However, the low resolution power of this technique for very small or very large proteins, as well as low-abundant proteins, proteins with extreme pI and *M*r values, or very hydrophobic proteins is well-known. Techniques based on liquid chromatography-mass spectrometry systems (LC-MS) or on the differential tagging of molecules under different environmental conditions using molecules like ICAT (Isotope-Coded Affinity Tags) or heavy isotopes of some amino acids seem to be able to solve such problems, and therefore its use is becoming popular among scientists (7).

The aim of this review is to compile the current knowledge of the characterization of the stress response and adaptation mechanisms of bifidobacteria to physiological conditions of the human GIT, as well as to other physico-chemical and environmental factors. Changes in protein synthesis and in metabolic pathways conditioning tolerance to such a factors, as well as common molecular responses to different stresses are also discussed.

3. THE PROTEOMES OF *BIFIDOBACTERIUM*

When the classic definition of proteome is applied to microbiology it can be stated as the "complete protein complement of a cell or subcellular fraction of a microorganism in a defined growth phase under concrete and precise physiological conditions" (8). As a response to internal or external stimuli, proteins can be easily modified, synthesized or degraded, accurately reflecting the environment in which they are studied. Furthermore, if all the possible factors affecting the proteome of a microorganism are considered, any genome can potentially originate an infinite set of proteomes. Although current proteomic techniques are not very efficient when studying proteins differing largely in relative abundance, they have been extensively used for the characterization of global changes in the microbial protein synthesis as a response to particular stimulus.

3.1. General features of *in silico Bifidobacterium* proteomes

Proteomics is becoming a very appropriate option to undertake functional studies in bifidobacteria in view of the scarcity of efficient transformation systems and genetic molecular tools for these microorganisms (9). Theoretically, the proteome of a bacterium can easily be obtained by plotting the isoelectric points (pI) against the molecular weights (MW) of all the predicted ORFs from a genome. Experimentally, this task is almost impossible due to both technical and physiological limitations. For that reason a theoretical proteome can be divided into subproteomes according to different criteria, which largely facilitates its study. The first criterion of choice is the pI; protein distribution along bacterial theoretical proteomes present two regions where the majority of proteins can be found, one in the acidic and another in the alkaline region. In this way the theoretical proteomes of *Bifidobacterium longum* biotype longum and *Bifidobacterium adolescentis*, until now the two publicly available genomes, agree with this statement and show two main protein peaks, the first between pIs 4 and 7 and the second between pIs 8-12. Another criterion is to establish cellular subproteomes, introducing a previous step of subcellular fractionation prior to protein extraction, thereby considering the cytoplasmic and membrane proteomes, and the secretome (cell-wall and extracellular proteins). From a bioinformatic analysis of *Bifidobacterium* genomes it can be inferred that cytoplasmic proteins are about two thirds of the total protein number and they are mainly distributed along an acid pI gradient. Thus, analysis by 2DE using immobilized pH gradients between 4 and 7 is suitable for a large percentage of bifidobacterial soluble proteins. On the other hand, membrane proteins are mainly alkaline, whereas secreted proteins are homogeneously distributed along a pH range between 4 and 12 (Figure 1 and Table 1).

3.2. The establishment of reference maps

Combination of all the possible subproteomes of a bacterium is a good approach to obtain its complete proteome. 2DE complemented with identification of proteins by matrix-assisted laser desorption/ionization–time-of-flight/mass spectroscopy (MALDI-TOF/MS) or

Table 1. Basic features of the proteomes of *B. longum* biotype longum NCC2705 and *B. adolescentis* ATCC 15703 compared to the other gram positive bacteria *Bacillus subtilis* subsp. *subtilis* 168 and *Lactococcus lactis* subsp. *lactis* IL1403

	B. adolescentis	**B. longum**	**B. subtilis**	**L. lactis**
Total proteins	1631	1727	4105	2321
Acid proteins	1115	1220	2486	1358
Basic proteins	516	507	1619	963
Cytoplasmic proteins	1089	1160	2788	1674
Membrane proteins	336	340	825	411
Secreted proteins	206	227	492	236
COG category	*B. adolescentis*	*B. longum*	*B. subtilis*	*L. lactis*
Translation	-	102	135	116
RNA processing and modification	-	1	-	-
Transcription	-	122	285	135
Replication, recombination and repair	-	97	136	168
Cell cycle control	-	19	32	15
Defense mechanisms	-	41	52	39
Signal transduction mechanisms	-	57	156	47
Cell wall/membrane biogenesis	-	65	190	99
Cell motility		3	55	7
Intracellular trafficking and secretion	-	19	42	23
Chaperones	-	46	97	41
Energy production and conversion	-	46	179	70
Carbohydrate transport and metabolism	-	137	206	115
Amino acid transport and metabolism	-	150	293	162
Nucleotide transport and metabolism	-	58	75	68
Coenzyme transport and metabolism	-	46	114	64
Lipid transport and metabolism	-	33	114	58
Inorganic ion transport and metabolism	-	58	171	89
Secondary metabolites metabolism	-	8	100	28
General function prediction only	-	169	389	228
Function unknown	-	75	249	138
Not in COGs	-	518	1425	788

The protein classification of the four bacteria was carried out according to the Cluster of Orthologous Genes (92)

more sophisticated MS techniques like electrospray ionization (ESI)-MS/MS are widely established for generating proteome maps. However, this technology currently fails to visualize certain proteins, mainly the extracytoplasmic proteome, high and low molecular mass proteins and low abundant proteins (10). For these reasons narrow pI gels, gradient gels or other chromatographic pre-fractionation techniques have been used in an effort to include such proteins into the proteome maps (11).

The establishment of a reference map represents the starting point for a deeper proteomic analysis, since it provides an overview of the synthesis and distribution of proteins in the microorganism, and to which further changes in protein production subsequent to different physiological conditions should be referred. In comparison to other Gram positive bacteria, few reference maps have been established for bifidobacteria. More specifically, the reference maps for *B. longum* biotype longum NCC2705, *B. longum* biotype longum NCIMB8809, *B. longum* biotype infantis BI07, *Bifidobacterium animalis* subsp. *lactis* Bb-12, *B. animalis* subsp. *lactis* IPLA 4549 and *B. animalis* subsp. *lactis* 4549dOx have been already established using 2DE coupled to different MS techniques (10, 12-16). Unfortunately, these approaches lack information on hydrophobic, basic, and surface proteins due to the technical limitations mentioned above. Some of these disadvantages have been overcome in another study, in which a reference map of *B. longum* biotype infantis has been obtained using a multi-dimensional chromatography system coupled to MS identification (MudPIT). This allowed the identification of 136 different proteins with pIs ranging between 4 and 12 (17). However, quantitative proteomic analyses could not be performed using that approach, thus limiting its applicability for the study of stress response. Another alternative is subcellular fractionation prior to 2DE, an easy-to-perform step to enrich proteomes for low abundant proteins that otherwise are difficult to detect if analysed together with cytoplasmic proteins. Examples of this strategy are the map of secreted proteins described for the strain *B. longum* NCIMB 8809, or the proteome of the cell wall associated proteins of the strain *B. animalis* subsp. *lactis* BI07 (18, 19). The number of proteins detected with this approach is much smaller compared to 2DE analysis of cytosolic proteins, 17 spots for the *B. longum* secretome and around 40 spots in the cell wall proteome of *B. animalis* subsp. *lactis* BI07.

4. QUANTITATIVE PROTEOMICS OF THE BIFIDOBACTERIAL RESPONSE TO GIT STIMULI

In spite of the hegemony of 2DE for the proteomic study of the stress response, this method is heavily biased towards proteins produced at high concentrations. Different staining methods, such as fluorescent dye labelling (DIGE), have been developed to improve the sensitivity of protein detection and quantification, yet proteins expressed at low concentrations may pass unnoticed (20, 21). Nowadays, non-gel-based techniques relying on the separation of peptides, rather than proteins, are emerging as powerful tools, in association with the use of cellular solubilizing reagents, for the characterization and complete profiling of the proteome of a bacterium. After protease digestion, the complex peptide

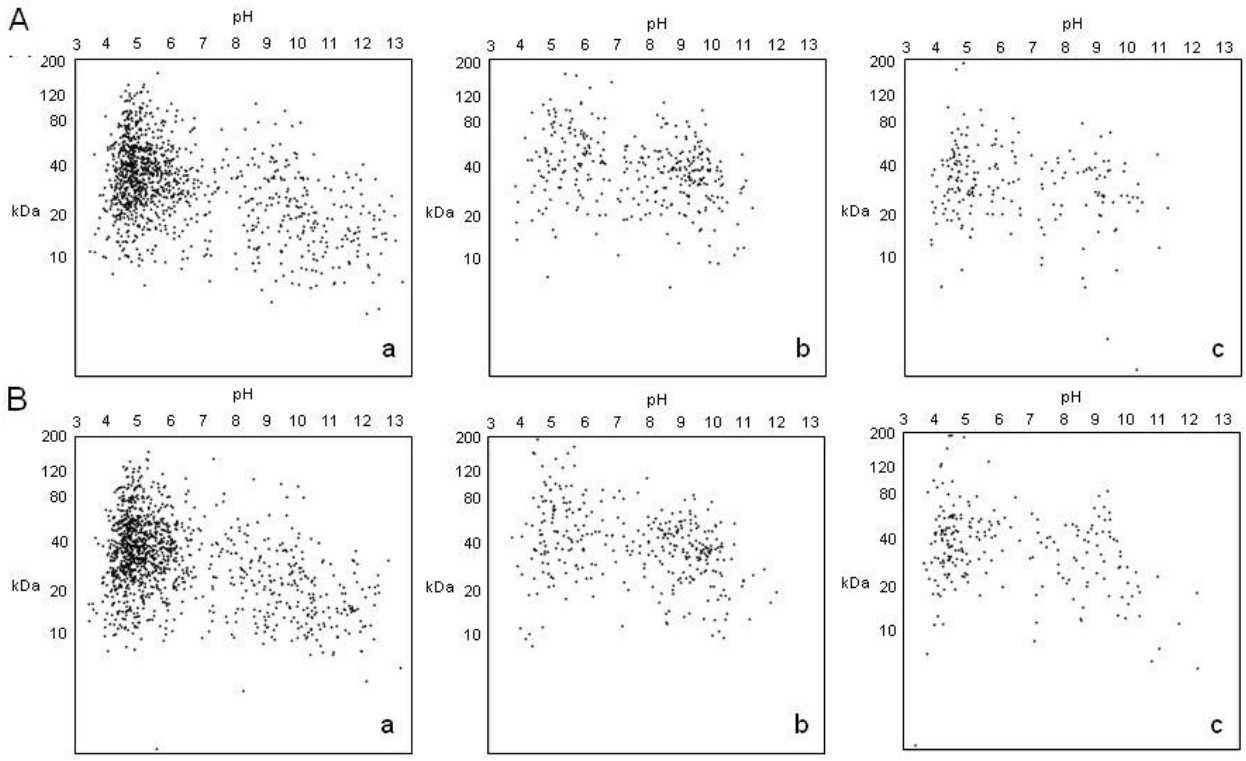

Figure 1. Theoretical proteomes obtained from the genomic sequences of the bifidobacteria *B. adolescentis* ATCC 15703 (A) and *B. longum* NCC2705 (B) using the software JVirGel 2.0 (91). Cytoplasmic (a), membrane (b) and secreted proteins (c) are represented.

mixtures can be fractionated by multidimensional LC and then analysed online by ESI-MS or offline by MALDI-TOF-TOF-MS, the peptides being identified with high confidence. With these techniques even low-abundant proteins become accessible and high proteome coverages can be accomplished (22). Such systems allow some of the experimental difficulties of 2DE analysis to be overcome, but their capacity for quantification of differences in the protein synthesis, as well as the capacity to correlate changes observed at the peptide level with individual protein isoforms, is still very limited (22).

With the aim of developing reliable methods to differentially quantify proteomes, the combination of different labelling techniques with chromatographic separations coupled to MS has been proven to be adequate. Isotope Coded Affinity Tagging (ICAT) and the use of amino reactive isobaric tagging reagents (iTRAQ) seem to be very useful in microbiology (23-26). Recently, a technique called SILAC (stable isotope labelling of amino acids in cell culture) has been successfully applied on *B. longum* NCIMB 8809 cultures (27). This technique is dependent on the efficient incorporation to the cell of a heavy isotope of an abundant and well represented amino acid, i.e. leucine. SILAC uses MS analysis to compare the peptides originated by in-gel protein digestion of the cellular extracts of one bacterium grown in two different conditions and allows the identification of proteins regardless their pI, molecular mass or subcellular localizations. Another useful technique is the surface enhanced laser desorption/ionization-time of flight (SELDI-TOF) coupled to an MS detector, which offers some advantages over 2DE, such as a higher throughput capacity in the low molecular mass range (< 20 kDa) and a more precise level of sensitivity, normally at the level of fM. In spite of this, SELDI-TOF counts with the disadvantage of being unable to directly identify proteins (28).

In the description of the tolerance to stress in the following paragraphs we will differentiate between two different specific processes, i.e. response and adaptation. Response is a transient process at DNA expression and protein synthesis levels, in which reversible alterations are involved due to the physiological shift introduced in the environment, disappearing when the specific pressure is released. Adaptation would involve a process in which bacteria can acquire mutations at specific sites as a reaction to environmental conditions (adaptative mutations), leading to stable stress-resistance phenotypes that can be transmitted to the next bacterial generations (12, 13, 16).

4.1. Acid adaptation and response

Bifidobacteria are often included in fermented dairy products, therefore these microorganisms usually cope with acidic stress right from the moment in which functional foods are produced. After being ingested, bifidobacteria again experience severe acidic conditions

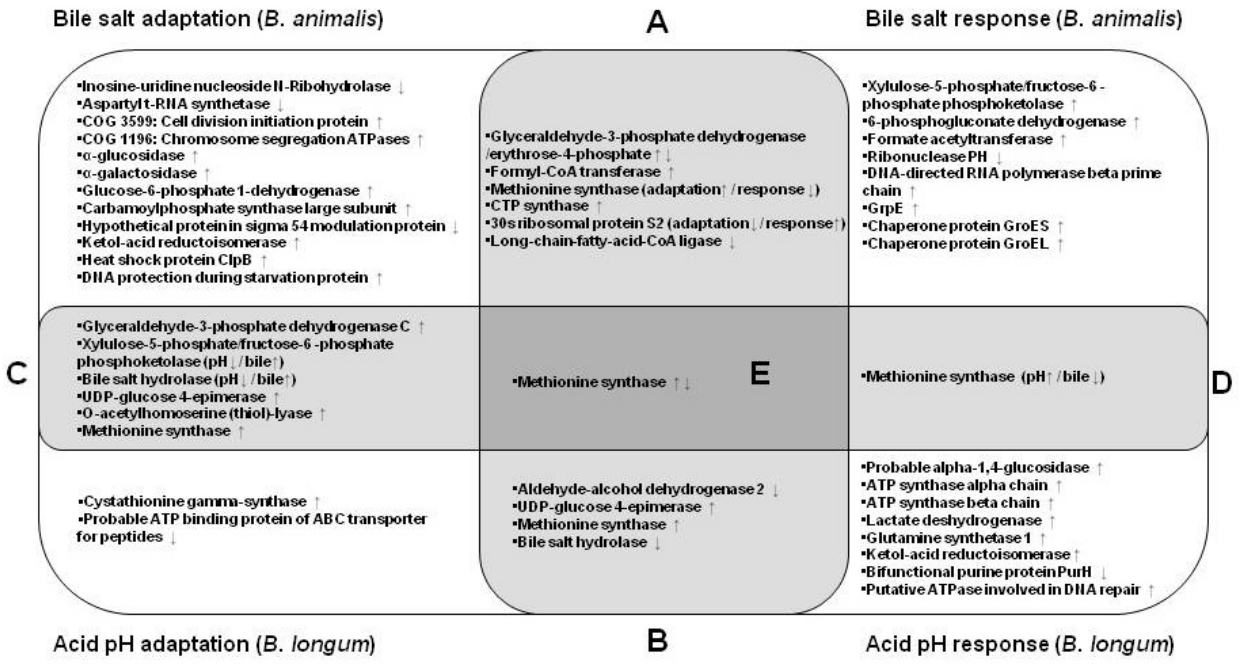

Figure 2. Main proteins involved in the response and adaptation to acid pH and bile salts in the genus *Bifidobacterium*. Protein induction or repression is indicated with arrows, and set intersections represent proteins shared by the processes of bile adaptation and response in *B. animalis (*A), acid adaptation and response in *B. longum* (B), acid and bile adaptation (C), acid and bile response (D), and acid and bile response and adaptation (E).

when they arrive in the human stomach (pH ≈ 2). In this way, good tolerance to acid pH is required for a probiotic strain in order to exert its beneficial effects on the host (29). Moreover, isolation of strains showing high resistance to acid pH is a suitable feature for industry as long as they also show good tolerance to other stresses like bile salts or to technological processes (30, 31).

It has been suggested that the acid tolerance of bifidobacteria is linked to the activity of the membrane H^+-F_1F_0-ATPase, an enzyme responsible for the maintenance of the cellular pH homeostasis in anaerobic microorganisms (32, 33). Also, transcriptomic studies from Ventura and co-workers demonstrated that the operon coding for the enzyme complex F_1F_0-ATPase in *B. animalis* is induced in acidic pH conditions (34). Some proteomic data on acid tolerance of *B. longum* biotype longum were obtained by Sanchez and co-workers, by using an acid-resistant mutant isolated from the originally acid pH-susceptible strain *B. longum* biotype longum NCIMB8809 (13). The parent and acid adapted strains of *B. longum* revealed an over-expression of the soluble subunits of the enzyme, AtpA and AtpD, at acidic conditions but not at neutral pH, which points to the pH inducible nature of this enzyme (35). Similar results of F_1-F_0-ATPase induction at low pH were obtained for *B. animalis* subsp. *lactis* by western blot analysis of cytoplasmic membranes (36). Additionally, measurement of the intracellular pH showed that the lower the external pH, the lower the intracellular pH of both original and acid adapted *B. longum* strains, the acid-adapted strain being able to maintain its intracellular pH closest to appropriate physiological values. These findings agree with previous results where the tolerance mechanism to acid pH was shown to be associated with the F_1-F_0-ATPase in bifidobacteria by non-proteomic approaches (32, 33).

Growth kinetics revealed that both *B. longum* strains, acid-susceptible and acid-adapted, had a longer lag phase at acid pH when compared to neutral conditions. This indicates that cellular metabolism and protein pool is reorganized allowing growth when the pH of the media was fixed to 4.8. Analysis of the protein extracts by 2DE showed that metabolic reorganization affected notably the synthesis of enzymes involved in the formation of fructose-6-phosphate from other carbon sources (Figure 2) (13). In addition, the carbon balance of the glucolytic pathway was higher at acid pH in the adapted strain, suggesting an optimization of the bifid shunt, probably directed towards an increase in the ATP formation. This ATP surplus could be directed to the maintenance of the proton gradient across the cytoplasmic membrane by means of proton-extruding mechanisms like the F_1-F_0 ATPase.

Acid pH also strongly influences amino acid metabolism in *B. longum.* On the one hand, enzymes involved in the biosynthesis of certain intermediates of the synthesis of the sulphured amino acids are over-expressed, although it remains unclear whether these changes on the sulphur metabolism could have an effect on acid pH tolerance, or otherwise they are just a side effect of pleiotropic mutations. On the other hand, growth at acid pH

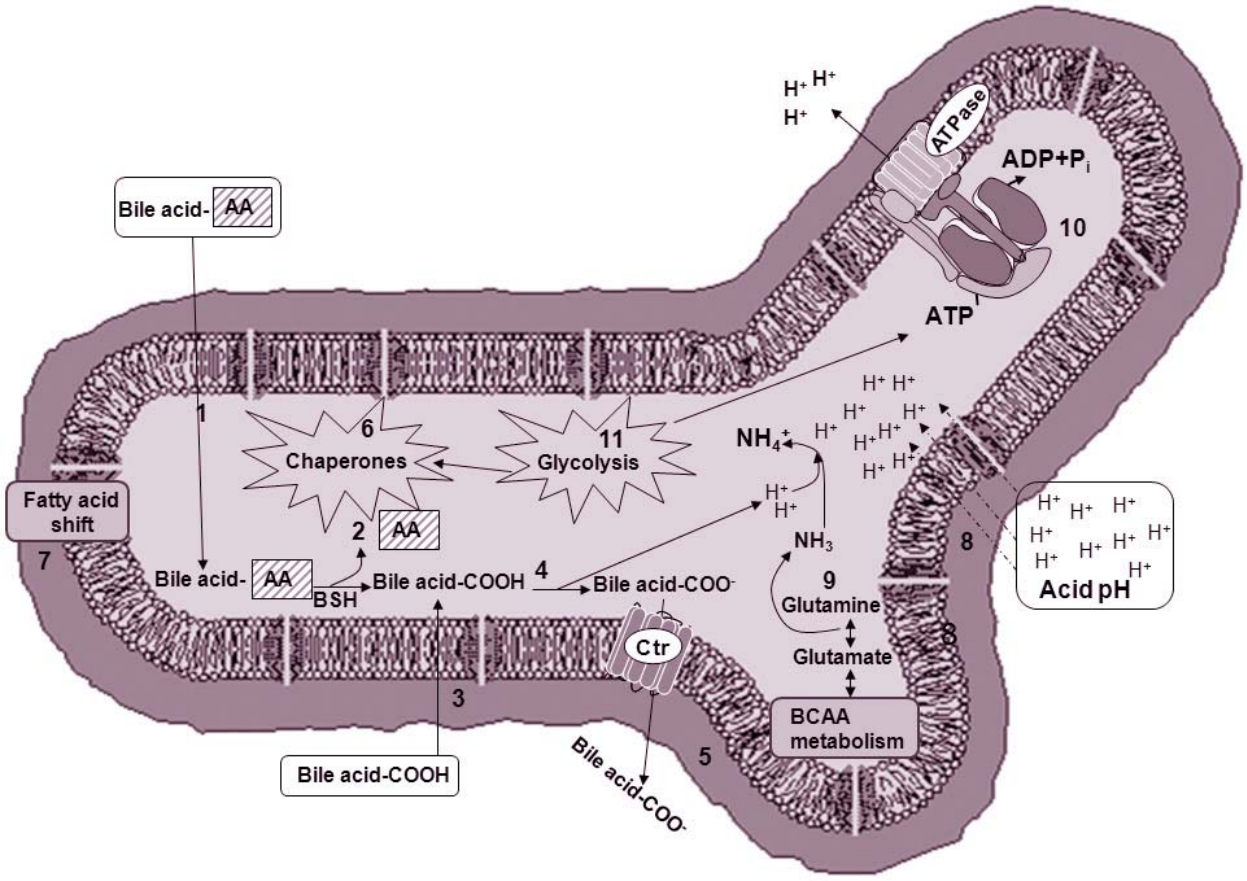

Figure 3. Schematic representation of the main physiological mechanisms involved in the processes of acid pH and bile salt tolerance in bifidobacteria. Conjugated bile acids/salts diffuse to the bifidobacterial cytoplasm (1), being cleaved by the bile salt hydrolase (BSH; bile adaptation and acid adaptation protein) and rendering one amino acid (glycine or taurine) and one deconjugated bile acid moiety (2). The last can also enter the cytoplasm by passive diffusion (3), being deprotonated at the slightly acid pH of the cytoplasm (4). Ionized bile acids are non-permeable, and must be excreted by the action of certain transporters, such as the cholate transporter Ctr of *B. longum* (5) (93). In addition, the process of tolerance to bile salts is associated with an increase in the synthesis of molecular chaperones (ClpB, GrpE, GroES, GroEL, DnaK and others) (6) and a decrease in the synthesis of long-chain-fatty-acid CoA ligase (bile response and adaptation protein) together with a shift in the fatty acid composition (7). On the other hand, acid pH exposure or bile acid deprotonation cause cytoplasm acidification (8), which is counteracted with mechanisms such as ammonia production from glutamine deamination (9) or proton pumping by the F_1F_0-ATPase (acid response and bile adaptation protein) (10). The amounts of ATP needed for feeding these mechanisms are provided through the bifid shunt (11).

was shown to cause an over-expression of enzymes involved in the biosynthesis of branched-chain amino acids, as well as an increase in the production of glutamine synthetase. Furthermore, high levels of ammonium and valine were measured as a response to acid pH. Thus, the mechanism of response to acid pH in *B. longum* seems to be in accordance with the hypothesis suggested by Len and colleagues, where the increase on branched-chain amino acid levels and ammonia production are coupled via the enzyme glutamine synthetase in *Streptococcus* mutants (37). In this process, one molecule of ammonia is released which, in acidic conditions, could gain a proton acting thus as a cytoplasmic buffer.

Finally, a decrease in the production of bile salt hydrolase (BSH) was detected in response to acidic conditions. The relevance of this enzyme for the physiology of bifidobacteria in the intestine still remains unclear (38). Surprisingly, no increase of any general stress proteins was detected as a response to acidic pH in *B. longum*, except for the higher content of the chaperone GroES in the acid-resistant strain *B. longum* biotype longum 8809dpH. Even a decrease of GroES was detected for the non-adapted *B. longum* strain. This fact could be related to some studies conducted in *Propionibacterium freudenreichii*, where the increase of certain chaperones was only detected after prolonged acid adaptation (39). The different effects of acidic conditions on *B. longum*, detected by 2DE and corroborated using physiological and biochemical experiments, are summarised in Figure 3.

4.2. Bile adaptation and response

Tolerance to bile salts is a crucial property for the persistence of intestinal microbiota in the human GIT,

this being an important factor for the survival of the bacteria ingested with food. With regards to the genus *Bifidobacterium*, tolerance to bile salts is dependent on the species, but usually deconjugated bile salts are more toxic than their corresponding taurine or glycine conjugates (40). The enzyme responsible for bile salt deconjugation is BSH, but to date there is no solid evidence of a relationship between this enzyme and the resistance level to bile salts (41).

Only a small number of proteomic studies on the influence of bile on the physiology of *Bifidobacterium* species have been performed. *B. animalis* subsp. *lactis* NCC362 grown at different bile salt concentrations was used for the identification of a stress-related fingerprint by MALDI-TOF/MS (42). This approach allowed the identification of up to five ions (with molecular masses ranging from 11.5 to 16.5 kDa) statistically associated with the presence of bile salts, although no information at the identification level was provided. Savijoki and co-workers reported changes in 3 proteins of the strain *B. longum* 3A after 20 minutes in the presence of bile; this rather low response was attributed to the interference between bile salts and the radioactive amino acid used in the study, which could result in low protein labelling efficiency (43). By using a 2DE approach it was shown that bile salts produced changes in the synthesis of a large number of proteins, indicating that such compounds affect the global metabolism of bifidobacteria (12, 16). Those observations are in agreement with a recent transcriptomic study of the strain *B. animalis* subsp. *lactis* Bb-12, in which the presence of bile revealed changes in the expression of 209 genes (86 over-expressed and 123 under-expressed), which represents 12.9% of their genes being affected by the presence of bile salts in the growth medium (15).

2DE analysis of cytoplasmic extracts of *B. longum* and *B. animalis* subsp. *lactis* revealed different strategies as a response to bile salts depending on the species (12, 16). While *B. longum* showed an over-expression of most of the enzymes of the glycolitic pathway, *B. animalis* subsp. *lactis* displayed an increment of the synthesis of enzymes involved in the formation of fructose-6-phosphate from other sugars, with Xylulose-5-phosphate/fructose-6-phosphate phosphoketolase and Glyceraldehyde 3-phosphate dehydrogenase C as the sole enzymes of the bifid shunt showing changes in their expression. Moreover, the bile salt adapted strain *B. animalis* subsp. *lactis* 4549dOx showed an over–expression of α-glucosidase, α-galactosidase and other enzymes involved in the formation of fructose-6-phosphate from more complex carbon sources. This data points to an increase in glucose degradation to lactate in *B. longum*, but not in *B. animalis*, as affected by the presence of bile. Experimental measurement of the glucose consumption by both species in the presence of bile salts corroborated that, in fact, it was higher in *B. longum* and lower in *B. animalis*. Furthermore, the *B. animalis* bile-adapted strain was able to grow more efficiently than its counterpart in the presence of maltose as compared to glucose as the sole carbon source, indicating a shift in carbohydrate preferences of this species promoted by bile (44).

The modification of the synthesis of enzymes involved in carbohydrate metabolism suggests an influence of bile salts in the production of energy and redox equivalents, as described before for other intestinal Gram positive species (45). As deduced from the proteome maps in the absence/presence of bile salts, energy production could be increased in *B. longum* by the over-expression of glucolytic enzymes, whereas in *B. animalis* subsp. *lactis* this could be achieved by an increase in the acetic acid formation through the bifid shunt that, theoretically, could allow bifidobacteria to synthesize more ATP by phosphorylation at the substrate level. Experimentally, it has been shown that intracellular ATP concentration decreases as the bile salt concentration in the growth medium increases, this inverse correlation being attenuated in the bile-resistant strain *B. animalis* subsp. *lactis* 4549dOx (36). This suggests that the response to bile salts is ATP dependent, energetic expenses being lower in strains adapted to those compounds.

Oxidative damage is a well-known deleterious effect of bile salts in cells (46). Almost all living cells have developed systems able to counteract oxidative damage (47, 48). 2DE analysis of the bile-resistant strain *B. animalis* subsp. *lactis* IPLA4549dOx revealed that adaptation to bile salts induced the constitutive over-expression of several enzymes involved directly or indirectly in redox reactions, notably those that catalyse the last two steps of methionine biosynthesis, whose sulphur group is susceptible to oxidisation. Changes in the redox balance of *B. animalis* bile-susceptible and *B. animalis* bile-adapted strains were confirmed by fluorescence spectroscopy (16). One of the outcomes of this change in the redox balance could be the presence of isoforms revealed by 2DE for certain proteins, i.e. Glyceraldehyde 3-phosphate dehydrogenase C. Different reduction states of this enzyme were shown to have a regulatory role in the glycolytic pathway of *Lactococcus lactis* (49).

Bile also modifies chaperon levels in bifidobacteria, which are a group of proteins that, among other functions, conduct the correct folding of proteins, thus helping to acquire their appropriate three-dimensional structure (50). Under certain environmental conditions, as in the presence of bile salts, there is an acceleration of the processes of protein misfolding and aggregation, which negatively affect cell viability. It is known that the synthesis of the chaperone DnaK is induced after heat shock in the species *B. adolescentis*, *Bifidobacterium breve,* and *B. longum* and, in addition, genetic analysis of the *gro*EL, *gro*ES*, clp*B loci and the *dna*K operon revealed an overexpression in *B. breve* UCC2003 after heat shock, osmotic stress or both (51-54). 2DE analysis of *Bifidobacterium* proteins revealed an increase in the chaperone synthesis as a response to, or adaptation to, bile salts (12, 16). However, in the presence of bile, the resistant strain *B. animalis* subsp. *lactis* 4549dOx displayed a lower number of over-expressed chaperones than the non-resistant strain *B. animalis* subsp. *lactis* IPLA4549, maybe as a direct consequence of its previous adaptation to those compounds. On the other hand, proteomic comparison of both strains, original and resistant, grown in the absence of

bile salts showed that the chaperones HtrA and ClpB (both with protease activity) were constitutively over-expressed in the bile-adapted strain, which could represent the maintenance of molecular mechanisms directed to fight against the harmful effects of bile salts even if such compounds are absent. The number of over-expressed chaperones detected in the strain *B. longum* NCIMB 8809 when grown in bile was markedly lower than in *B. animalis*. Savijoki and colleagues showed that bile salts induce moderate changes in the chaperone syntesis when compared to those caused by heat shock in the strain *B. longum* 3A (43). This fact could be related to the lower bile salt tolerance of *B. longum* with respect to *B. animalis*.

Finally, significant changes in several metabolic processes were demonstrated with proteomic techniques. Remarkably, it is known that bile induces changes in the lipidic composition of bifidobacterial membranes (45). *B. animalis* subsp. *lactis* showed a decrease in the production of long-chain-fatty-acid-CoA ligase as a response to, or adaptation to, bile salts, suggesting a modification of the fatty acid synthesis and hence variations on the phospholipid ratios of their membranes (16). Changes in the membrane fatty acid composition could favour tolerance to bile salts, decreasing the bile salt diffusion rate to the cytoplasm. Experimental evidence obtained by measuring the lateral mobility of lipids in the *B. animalis* membranes treated with bile indeed support this hypothesis (55). Relating to this, a transcriptomic analysis conducted by Garrigues and co-workers showed an inhibition in the expression of a whole cluster of enzymes involved in fatty acid biosynthesis in the strain *B. animalis* subsp. *lactis* Bb-12 after growing in 0.1% bile salts (15). Also related to the bifidobacterial surface and shape was one of the more notorious consequences of bile salt adaptation in bifidobacteria: a decrease in cell size (56). In our proteomic studies two proteins (cell division initiation protein and chromosome segregation ATPase), directly related to the cell division process were detected at higher amounts in the bile salt adapted strain *B. animalis*. Another enzyme whose synthesis and activity increases in *B. animalis* as a consequence of adaptation to bile was BSH (16, 57). However, no changes were found for this enzyme in both *B. longum* and *B. animalis* as a response to bile salts. Thus, an enhanced BSH activity could play a role in the bile salt adaptation phenomenon, although the expression of this enzyme seems to be constitutive in *Bifidobacterium* in contraposition to some lactobacilli, where it can be regulated by bile salts (45, 58).

4.3. Host-induced proteome changes

Currently, innumerable open questions remain in the microbiologist's pipeline regarding the dynamic interplay among bacteria and between host and commensal gut bacteria. Furthermore, it is unquestionable that *in vitro* laboratory conditions cannot completely mimic the events that occur in the human intestinal system. In this respect, the current -omics revolution is helping to unravel these events, by allowing a comprehensive understanding of the biological processes responsible for the cross-talk in our GIT environment. The limitations of using an *in vitro* model as a surrogate for the rapidly changing *in vivo* environment are being highlighted during recent years mainly by transcriptomic studies. In this respect, using murine models host–*E. coli* cross-talk not predicted by previous *in vitro* analyses was shown, with unusual patterns of stress response genes, a response to host induced metal ion limitation, and a failure to achieve stationary phase (59). Also, whole genome transcriptional profiling of *B. longum* and *Bacteroides thetaiotaomicron* in germ-free mice revealed that the presence of *B. longum* elicits an expansion in the diversity of polysaccharides targeted for degradation by *B. thetaiotaomicron*, and induces host genes involved in innate immunity (60). However, the information obtained from proteomic studies is scarcer, mainly due to the technical difficulties of isolating enough of the bacterial proteins for a quantitative analysis. This could be solved by specific immunological bacterial separation (61). Recently, the host-induced proteome of *B. longum* NCC2705 in a rabbit model was investigated. Yuan and co-workers implanted *B. longum* cultures contained in dialysis tubes in a rabbit intestine, and the obtained proteomes were compared with the proteome of the bacteria incubated *in vitro* (62). Several proteins were up and down regulated more than 3-fold, including key stress proteins, metabolism-related proteins, and proteins related to translation. Some of them reflected the adaptation of *B. longum* NCC2705 to the intestine, such as the elongation factor Tu, an adhesin-like factor, or BSH, which might play an important role in the defence of *B. longum* against the action of bile salts. The most relevant finding of that work was that several proteins exhibited clear post-translational modifications. Among them, phosphoglycerate kinase (Pgk) and autoinducer-2 production protein LuxS were identified for the first time as bifidobacterial phosphoproteins *in vivo*. Thus, it seems that the determination of the phosphoproteome of *B. longum* will be crucial for understanding the metabolic processes inherent in these bacteria and its quorum sensing mechanisms.

4.4. Common responses to the gastrointestinal tract environment

In recent years it has been stated that the molecular mechanisms of different stress responses overlap. For example, *Bifidobacterium* strains most tolerant to oxidative stress and heat shock also showed maximum survival to technological processes like spray-drying or storage at 4ºC (63). It is also known that *B. breve* cell suspensions exposed to pH 5.2 for two hours are protected against subsequent lethal challenges of pH (pH 2-5), bile (0.2-1%) or hydrogen peroxide (100-1000 ppm) (64). In *Bifidobacterium* species, the acquisition of a stable bile resistance phenotype also led to a higher tetracycline resistance *in vitro* (65). Also, in *B. animalis* a previous and sublethal exposure to acid pH or bile protects against a lethal dose of the other stress (66). In light of the results of proteomic studies regarding the tolerance of *B. animalis* and *B. longum* to acid pH and bile salts, one of the main conclusions is that adaptation to one of the two stresses can confer protection against the other, even though both mechanisms seem to be apparently different, this cross resistance being strain dependent (13, 16, 36, 40).

Adaptation to low pH in *B. longum* caused the over-expression of enzymes related to the use of complex carbohydrates (13). Over-expression of enzymes involved in complex carbohydrate metabolism was also revealed in the process of tolerance to bile salts in *B. animalis* subsp. *lactis* (16). All together it suggests that the acquisition of acid pH or bile salt tolerance prepare the bacteria for a subsequent challenge in the GIT environment. This causes changes in the carbohydrate metabolism that, from a physiological point of view, could favour a more efficient use of the carbon sources available in the colon, increasing the synthesis of enzymes involved in the utilisation of sugars other than glucose, which is not abundant in the colon.

Considering the proteomic analysis of *B. animalis* subsp. *lactis* strains grown under different bile and acid pH conditions, and complementary physiological studies carried out, it can be deduced that there is a common link between the resistance to acid pH and bile salts in *B. animalis* subsp. *lactis*, the F_1-F_0-ATPase (16, 36). With our current data, the hypothesis can be summarized as follows: bile salts would enter bifidobacterial cells by passive diffusion, being hydrolysed inside the cell by the BSH. This reaction releases amino acids and deconjugated bile acid moieties, the latter being weak acids that, at the pH of the cytoplasm are quickly dissociated, generating a proton excess (67). A higher ATPase activity would therefore enable the bacteria to better survive bile challenging conditions (Figure 3). In a physiological context a higher ATPase activity as a response to the acid pH of the stomach would enhance tolerance to a subsequent bile salt exposure in the intestine by extruding part of the excess of protons generated as a consequence of bile salt deconjugation. Both acid pH and bile salts could act as physiological signals from which absence/presence cycles would indicate bifidobacteria that they are in the appropriate ecological niche, in the same way that occurs in enteric pathogens like *Campylobacter jejuni*, in which invasion-related proteins are synthesised as a response to the presence of bile salts (68).

An integrated analysis of all protein variations detected for the different bile and acid responses, and adaptation processes, for *B. animalis* and *B. longum* is shown in figure 2. It is notable that the methionine synthase was the only enzyme whose expression was modified under all the conditions tested. Methionine metabolism is crucial for the regulation of oxidative stress, and its variations could reflect modifications in the redox signalling in the bacterial cytoplasm, directed to maintain a viable physiological status (69).

5. ADAPTATION TO CARBON SOURCE FLUCTUATIONS

The human GIT is a highly competitive environment characterised by fluctuations in carbon source availability, usually the non-digestible components of the diet. Metabolic versatility allowing the utilisation of different carbon sources, mainly carbohydrates, is a suitable characteristic for a probiotic strain. As reported above, bifidobacteria contain in their proteomes an arsenal of proteins able to feed a wide range of carbohydrates into the fructose-6-phosphate phosphoketolase pathway, such enzymes being regulated by a high number of transcriptional repressors (5). Molecular mechanisms underlying the use of certain carbohydrates have been identified in some bifidobacteria. Proteomes of *B. longum* NCC2705 grown in the presence of glucose or fructose have recently been obtained. The analysis showed that 18 proteins displayed a variation of at least 3-fold in their level when the microorganism was grown in the presence of fructose with respect to the standard growth conditions in glucose (10). Among these proteins, a fructose ABC-transporter (BL0033/BL0034) and a sugar kinase of the PfkB family (BL1339) showed a clear up-regulation in the presence of fructose, this fact being additionally supported by RT-PCR analysis of the corresponding genes. With this approach some proteins responsible for the metabolic use of fructose were identified. Also, 5 isoforms of BL0033, the fructose-binding protein, differing in their charge were identified, and a potential role of post-translational modification of this protein in the regulation of fructose uptake was suggested. Remarkably, fructose uptake in *B. animalis* and *B. bifidum* is believed to be carried out via a fructose-6-phosphate-forming fructose-PTS system, indicating that different mechanisms are present among *Bifidobacterium* species to internalise this monosaccharide (70).

On the other hand, a proteomic approach using the technique SELDI-TOF MS allowed the analysis of proteins differentially expressed in *B. breve* DSM 20213 after being grown in the presence of glucose, galactose or lactose as the main carbon source (71). Several proteins showing changes more than 2-fold (up- or down-regulated) in their synthesis were detected, although due to limitations of the proteomic technique they could not be identified. Relating to this, it was also shown that *Bifidobacterium longum* NCC2705 displays a lactose-over-glucose preference, and a transcriptomic analysis of this bacterium was able to show that glucose utilization is impaired until the depletion of lactose. In the same way, glucose uptake experiments showed that glucose transport was repressed in the presence of lactose (72). This is a good example of the complementarity of transcriptomics and proteomics for the study of similar physiological processes, transcriptomics being advantageous for a global gene expression profile, and proteomics for identification of post-translational modifications.

6. HEAT SHOCK

Stresses associated with the probiotic manufacturing process could affect and influence the survival and tolerance to the GIT conditions of probiotic bifidobacteria ingested with functional foods. Resistance to heat shock has been widely studied in probiotic bifidobacteria, since this is a suitable feature for industrial fermentations taking place at 42 ºC, such as the elaboration of some fermented milks. Growth temperature has been shown to have a strong influence on the synthesis of surface proteins in *Bifidobacterium globosum*, the presence

of some of them being linked to growth at 45°C and to an increase in cell hydrophobicity (73). Genome analysis showed that the strain *B. breve* UCC2003 possesses two groups of chaperones, one related to the response to moderate increases of temperature (*gro*EL, *gro*ES, *clp*C and *clp*P1P2) and the other involved in the response to more severe increases (*dna*K, *grp*E, *dna*J1 and *clp*B) (74). Regulation of both groups seems to be different. Regulation of *clp*C and *clp*P genes is dependent on the product of the gene *clg*R, a presumed transcriptional activator, which binds to the promoter of *clp*P1P2 and *clp*C, and may require the presence of the protein GroEL as cofactor (75, 76). On the other hand, *gro*EL and *gro*ES are probably regulated by the repressor protein HrcA (52). The second group of chaperones, in contrast, depends on the activity of the protein that acts as a repressor of the regulon, HspR (53, 77). Recently it has been shown that gene *hsp*20, a member of the small heat shock protein family, is highly over-expressed after heat shock (78). In *B. longum*, a global transcriptome analysis of its heat shock response revealed that the expression of 46% of its genes is altered upon exposure to 50°C, especially those related to the general metabolism and the classical heat shock stimulon (79). Also, the use of the radioactive amino acid (^{35}S)methionine in cell cultures in combination with 2DE allowed the labeling of proteins synthesized as a response to heat shock (43). This experimental approach has revealed that the response to heat shock is linked to the accumulation of the molecular chaperones GrpE, ClpA/B, HtrA, DnaK and GroEL in the cytoplasm, with HtrA undergoing proteolytic processing during heat stress. Relating to this, it has recently been suggested that chaperones accumulated under stress conditions could become susceptible to proteolysis following phosphorylation (80).

7. THE EFFECT OF ANTIMICROBIALS ON *BIFIDOBACTERIUM* PROTEOME

Many of the bacterial resistance mechanisms to antibiotics depend on the functionality of just one (or a few) proteins to be successful. However, there are still many unsolved questions regarding the mechanisms underlying antimicrobial activity as well as the mechanisms of resistance evolved by microorganisms against these molecules. In fact, using transcriptomic and proteomic techniques it has been shown that the robust metabolism of *Salmonella* severely limits the success of new antimicrobials (81). To unravel the molecular targets involved in antimicrobial resistance the proteomes of numerous bacteria have been studied (82-84). The molecular characterization of the resistance to antibiotics is also of great concern for probiotic bifidobacteria (85). 2DE analysis of a rifaximin spontaneous mutant of the strain *B. longum* biotype infantis BI07 has led to the identification of some proteins whose expression changed as a consequence of the acquired resistance phenotype to such antibiotic (14). It should be noted that, among principal categories of proteins affected, there was an increase of general stress proteins and some transcriptional regulators, which were linked to the activation of compensatory mechanisms to overcome the action of rifaximin, a powerful RNA polymerase inhibitor in bacteria (86). In this way, this study suggested the simultaneous use of the antibiotic rifaximin and probiotic bifidobacteria, without any risk of horizontal gene transfer of antibiotic resistance elements.

8. TOWARDS BIFIDOBACTERIAL SUBPROTEOMES

The aim of subcellular targeted proteomic studies is to provide relevant information to enhance the understanding of the different biological processes linked to the purified, or enriched, fractions for subsequent functional studies. For instance, the determination of the extracellular protein profiles or the profiling of the cell surface proteome of *Bifidobacterium*, alone or together with other bacteria or host cells, will be critical to understand its quorum sensing mechanism, and its cross-talk with the host. A recent work partially characterized the cell wall proteome of *B. animalis* subsp. *lactis* (19). Candela and coworkers identified 8 cell wall proteins able to bind plasminogen, and they postulated a role of some of them in bifidobacterial adhesion to the surface of the intestine. Interestingly, they reported that most of the plasminogen-binding proteins were highly conserved cytoplasmatic proteins that, when expressed on the bacterial cell wall, acquire a "moonlighting" function different from their well-known activity performed in the cytoplasm. None of these enzymes exhibited any detectable export and retention signal, and the mechanisms of secretion and cell anchoring remain to be determined. In another study, the preliminary identification of *B. longum* NCIMB8809 secretome was reported (18). Among the most abundant proteins, those involved in peptide intake were found. In fact, bifidobacteria are the main bacterial group in breast-fed infants, where milk peptides are an important part of nutritious substances (87). Regarding the proteins that could be involved in the interactions with other bacteria or with eukaryotic cells of the host mucosa, nine spots were identified in the culture supernatant of *B. longum* NCIMB 8809 which can be related to those processes (18). Some of them were identified as proteins with homology with TraG-like proteins, which play essential roles in DNA transfer during the process of bacterial conjugation in *Streptococcus* and whose function in bifidobacteria may be related to the interaction between cells (88). Another protein, BL1663, displays homology with the p60 invasion-associated protein of *L. monocytogenes*, a murein hydrolase involved in intracellular infection and motility (89). Further research is needed in order to elucidate the precise roles of TraG-like and p60 invasion-associated protein homologs on bifidobacterial ecology. Finally, for the analysis of the insoluble protein fraction, mainly the membrane subproteome, techniques based on improved solubilizing agents or isotope labelling are being used (27, 90). Current research is ongoing in our laboratory for the determination of the whole surface proteome of *B. longum*. Using SILAC methodology and subfractionation techniques we were able to identify more than 250 proteins in membrane and cell wall fractions of *B. longum* NCIMB8809, including a large percentage of the theoretical transporters of this species (Ruiz *et al.*, unpublished data). The influence of GIT conditions on the cell surface proteome of *B. longum* will

indeed provide relevant information about the transport processes of this bacterium.

9. PERSPECTIVE

The state-of-the-art of probiotic proteomics, although it can be considered nascent if compared with other bacterial groups such as pathogens, has already made a significant contribution to the understanding of the mechanisms of action of bifidobacteria. Till a few years ago, probiotics were selected according to their resistance phenotype against different conditions i.e. ability to tolerate bile salts, acid pH, survival through GIT or different technological conditions such as high salt concentrations, high and low temperatures and lyophylization processes. However, current international guidelines and standards have encouraged a change in this approach; nowadays, in order to propose specific health claims, the elucidation of the precise mechanisms by which probiotics influence human health will become necessary. Researchers are taking the necessary steps to identify *in vivo* probiotic biomarkers in animal models and, ultimately, in clinical trials. The lack of single-gene knockout analyses using mutants has burdened the progress of bifidobacterial functional genomics, but the current -omics era is somehow successfully mitigating those disadvantages. Proteomic approaches have enabled to elucidate some of the mechanisms responsible for the tolerance to gastrointestinal stress, to understand why the bifidobacterial stress responses overlap, and to generate ideas to improve specific strain properties by building robust stress resistance phenotypes that prepare these microorganims to survive under adverse conditions. The study of proteome cell fractions, or subproteomes, such as cell surface, secretome, or phosphoproteome, will be crucial to elucidate the mechanisms of communication among different *Bifidobacterium* strains, with other bacteria and with the host, but also to understand their own metabolic activities. Proteomics will also contribute to enable the selection of the most appropriate science-based probiotic strains for a particular condition/health benefit.

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