Stress Response in Bifidobacteria

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REVIEW

Stress Response in Bifidobacteria

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SUMMARY Bifidobacteria naturally inhabit diverse environments, including the gastrointestinal tracts of humans and animals. Members of the genus are of considerable scientific interest due to their beneficial effects on health and, hence, their potential to be used as probiotics. By definition, probiotic cells need to be viable despite being exposed to several stressors in the course of their production, storage, and administration. Examples of common stressors encountered by probiotic bifidobacteria include oxygen, acid, and bile salts. As bifidobacteria are highly heterogenous in terms of their tolerance to these stressors, poor stability and/or robustness can hamper the industrial-scale production and commercialization of many strains. Therefore, interest in the stress physiology of bifidobacteria has intensified in recent decades, and many studies have been established to obtain insights into the molecular mechanisms underlying their stability and robustness. By complementing traditional methodologies, omics technologies have opened new avenues for enhancing the understanding of the defense mechanisms of bifidobacteria against stress. In this review, we summarize and evaluate the current knowledge on the multilayered responses of bifidobacteria to stressors, including the most recent insights and hypotheses. We address the prevailing stressors that may affect the cell viability during production and use as probiotics. Besides phenotypic effects, molecular mechanisms that have been found to underlie the stress response are described. We further discuss strategies that can be applied to improve the stability of probiotic bifidobacteria and highlight knowledge gaps that should be addressed in future studies.

KEYWORDS DNA repair system, SOS response, acid stress, bifidobacterium, bile stress, heat stress, osmotic stress, oxidative stress, protein quality control

INTRODUCTION

Bifidobacteria are high-G+C-content, Gram-positive prokaryotes of the phylum *Actinobacteria*. The presence of bifidobacteria in the gastrointestinal tracts of humans and animals is associated with good health and a strong immune function of the host. Due to the well-documented health-promoting effects of bifidobacteria, several *Bifidobacterium* strains are commercially used as probiotics in food, including dairy products, and pharmaceutical products, including strains of the species *Bifidobacterium adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, and *B. longum* (1–3). According to the definition by the World Health Organization, probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (4). This definition underlines the fact that probiotic products must contain a sufficient number of viable microorganisms to provide a health-promoting effect. Although survival through the gastrointestinal tract is not an explicit requirement for probiotics by definition (5), it can be intuitively considered an important criterion for the probiotics to exert their beneficial effects (6). The effectiveness of a probiotic product is strain specific and depends on factors such as the product matrix and the intended health benefit (7). Therefore, the minimum effective dosage of probiotic products cannot be generalized, and the recommended effective dosage varies significantly among individual probiotic products (10^9 to 4×10^{10} colony forming units (CFU)/day) (7).

Throughout their life span, industrially used probiotic strains pass through several stages, comprising production (fermentation and downstream processing), formulation (e.g., as foods), storage, and administration (Fig. 1). In each stage, they are subjected to various stressors (2, 8–10). Depending on the stage, different groups of stressors prevail that can affect the survival of the cells and, thus, can challenge the delivery of sufficient numbers of viable microorganisms in the probiotic product (Fig. 1) (2, 8–10).

In recent years, evidence has been collected that not all health benefits of probiotics are strictly linked to viability and that nonviable microorganisms and/or cell components, so-called postbiotics (or paraprobiotics), may also confer some health-promoting effects (11, 12). The health effects of postbiotics are not as extensively studied as those of probiotics, but previous studies have shown that the functionality and effectiveness of a probiotic and its nonviable counterpart can differ (13–17). Moreover, the procedure used for inactivation of the microorganism seems to determine the health benefit of the postbiotic (18). Consequently, probiotic microorganisms that lose their viability during the shelf life of a probiotic product cannot simply be considered postbiotic without further proof of their health benefit (5). Thus, the delivery of viable cells in probiotic products is indispensable and it is important to differentiate between the concept of postbiotic and probiotics.

Since probiotic strains must retain their viability and functionality, robustness and stability are important criteria to be considered when selecting strains for industrial applications. Stability can be defined as the ability of a strain to remain viable under given environmental conditions during storage. Robustness is the ability of a strain to sustain its functionality despite being exposed to perturbations (19), which is closely related to the stress tolerance of a strain. Whether a cell survives and remains functional when exposed to stress depends on its ability to sense and efficiently respond to the perturbation. A stress response can comprise both physiological and metabolic changes. On the other hand, some strains might already be equipped with enhanced tolerance to a stressor before the induction of any phenotypic changes due to constitutively active mechanisms that allow them to cope with the stressor.

The stability and robustness of probiotic strains might be determined by intrinsic strain properties, including genetic and metabolic characteristics, but may also be affected by the conditions chosen for the production process, formulation, packaging and storage of the probiotic product. The low stability and robustness of some *Bifidobacterium* strains

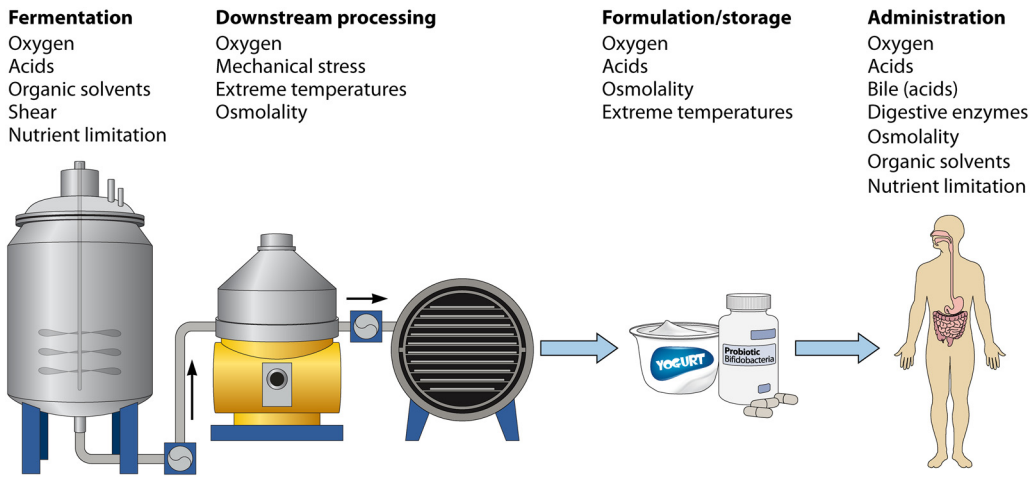

FIG 1 Common stresses that may affect the viability of industrially used probiotic strains throughout their life span, from fermentation until administration. After fermentation at high cell density, the cell broth is concentrated by centrifugation or membrane filtration. Subsequently, the cells are subjected to different long-term preservation treatments, such as freeze drying, that should guarantee that the cells remain viable throughout storage until administration. Probiotic bifidobacteria are employed as food supplements as well as pharmaceutical products. In each stage of their life span, different groups of stressors prevail.

hamper their industrial production and commercialization. Therefore, established production methods may need to be modified to enhance the stability of less stable strains. Other strains, such as *B. animalis* subsp. *lactis* BB-12, are relatively stable (6) in terms of consistent biomass yields during production, maintained viability during downstream processing, and a long shelf life.

To design high-performance production processes leading to robust and stable products, sufficient knowledge of the sensitivity of *Bifidobacterium* strains to various stressors is of great importance. Insights into the stress physiology of bifidobacteria can form the basis for the rational selection of technologically well-suited probiotic strains and allow knowledge-driven optimization of production processes of strains with promising health benefits (20). Moreover, understanding the metabolic and physiological changes induced by stress exposure is important to guarantee the delivery of probiotic strains with desired functionality (21, 22). Multiple putative links between stress responses, mainly to gastrointestinal tract stressors, and their effect on *in vivo* survival and the probiotic functionality can be suggested based on *in vitro* studies. For example, bile exposure has been shown to increase exopolysaccharide (EPS) production in *B. animalis* IPLA 4549 (23) and EPS production in *B. breve* UCC2003 has been shown to promote persistence of the strain in the mouse gut and reduce pathogen infection of the host (24). The potential effects of stress response and stress adaptation on probiotic functionality have been the topic of previous reviews (10, 22). However, due to a lack of conclusive *in vivo* evidence, these effects must still be considered putative (22).

Over the last decades, the stress physiology of bifidobacteria has been increasingly studied in order to gain insights into the molecular mechanisms underlying stability and robustness of members of the genus. Different strategies have been applied (Fig. 2). Besides studying the response of single strains to individual stressors, several studies have compared strains that differ in their stability and robustness. Some studies have applied adaptive laboratory evolution (ALE) or genetic engineering to modify the stability of *Bifidobacterium* strains, before comparing the mutants with the parental (reference) strain (Fig. 2). Sublethal stress treatments, so-called metabolic conditioning, have been applied to investigate how the exposure to individual stressors affects the strain's stress resistance and physiology. In addition, *Bifidobacterium* strains with intrinsic resistance above average have been compared with reference strains with lower tolerance to environmental stressors (Fig. 2). Storage (short or long term) and stress treatments have

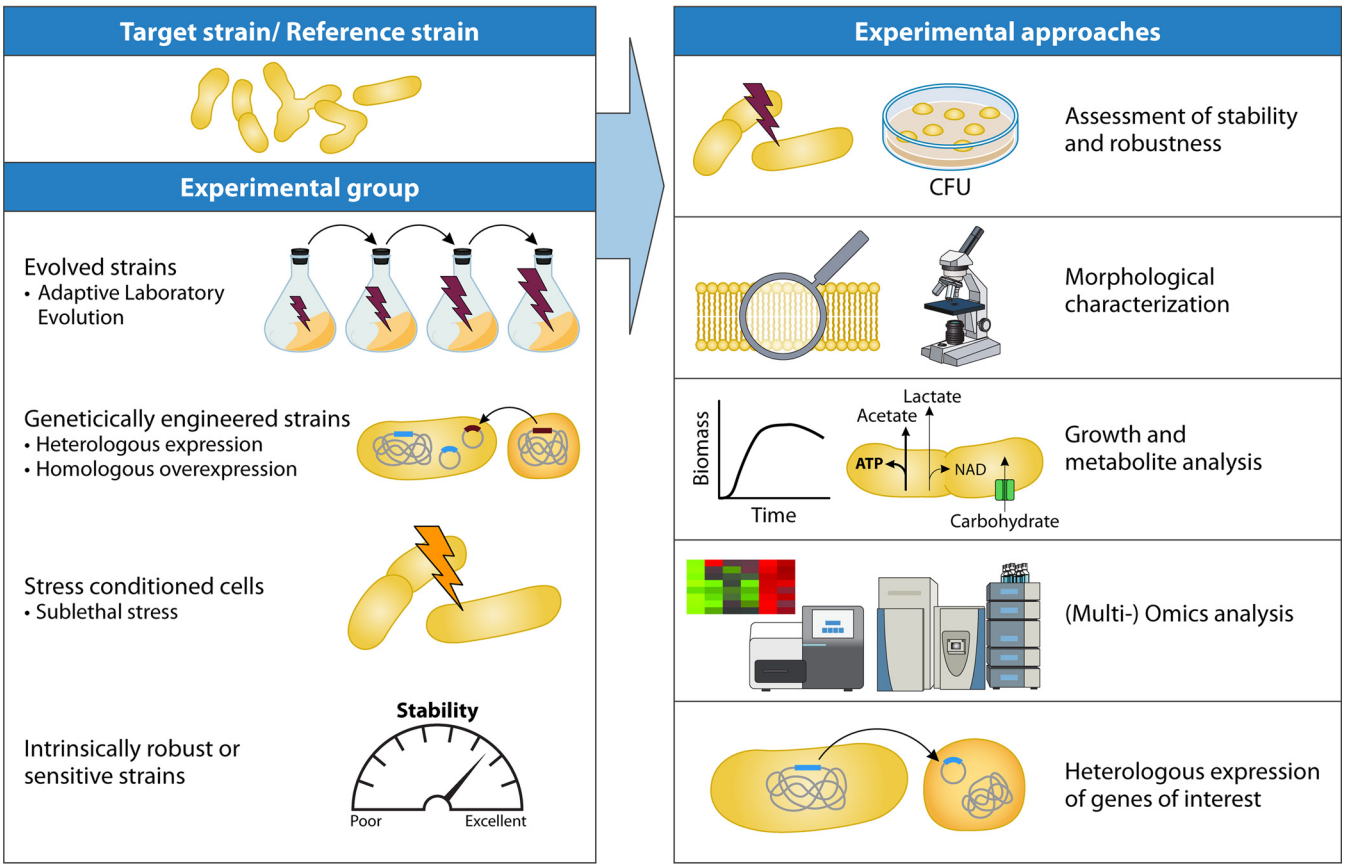

FIG 2 Experimental approaches employed to investigate the molecular response of bifidobacteria toward various stressors and the molecular basis underlying stress tolerance. Studies have investigated the stress tolerance and response of single target strains and have compared strains with different stability and robustness using various methods. Assorted study designs and strain selection criteria have been used.

been applied to assess the stability and robustness of the studied strains as well as to study the effect on the phenotype of the strains. Strains have commonly been analyzed in terms of their morphology, growth and metabolite profiles. Moreover, genes hypothesized to be linked to stability and robustness of *Bifidobacterium* strains have been expressed in heterologous hosts to elucidate their function. Furthermore, the application of omics technologies, in particular genomics, transcriptomics, and proteomics, has opened new avenues over the last decade to expand knowledge on responses of *Bifidobacterium* strains to various stressors (Fig. 2).

Although not all of the studied *Bifidobacterium* strains are currently applied as probiotics, some of them might become applied in the future, as already suggested for species isolated from bees (25). Moreover, the mechanisms underlying the stress response might be conserved across species, so that knowledge gained from these studies can contribute to our general understanding of probiotic species.

The first reviews on the molecular mechanisms of stress physiology of bifidobacteria were published around 10 years ago (21, 26, 27). Since then, new insights have emerged, not least due to the rise of omics technologies. More recent reviews have focused on the relationship between stress exposure and probiotic functional properties of bifidobacteria and lactobacilli (10) as well as on mechanisms of adaptation to stress exposure of propionibacteria, lactobacilli, and bifidobacteria (28). In this review, we provide a comprehensive overview of the phenotypic effects and underlying molecular mechanisms of the stress response specifically in bifidobacteria, including the latest developments in the field. We highlight knowledge gaps concerning bifidobacterial stress physiology and discuss strategies to improve the stability of probiotic bifidobacteria.

HEAT STRESS

The heat stress response in bifidobacteria comprises several universal stress response elements of bifidobacteria and is, therefore, discussed first. Although bifidobacteria are not exposed to temperature stress in their natural intestinal environment, industrially relevant strains can be subjected to heat stress during downstream processing and preservation (2, 8). Heat stress in bacteria is generally attributed to the denaturation and aggregation of proteins, the destabilization of macromolecules, and changes in membrane fluidity (29).

Phenotypic Effects of Heat Stress

Bifidobacteria are mesophilic, with optimal growth temperature between 37°C and 41°C (30, 31). In contrast to human isolates of the genus, most animal isolates can grow at temperatures up to about 45°C (32). Exceptions are *B. mongoliense*, *B. tibiigranuli*, and *B. aquikefiri*, which prefer lower temperatures, between 25°C and 30°C (31, 33, 34), and *B. thermacidophilum*, which has a maximum growth temperature of 49.5°C (35). *Bifidobacterium* strains show reduced growth rates when exposed to temperatures above their optimum, whereas their viability seems not to be significantly affected at temperatures at which growth is already reduced (36, 37). The ability to survive heat stress was found to vary among *Bifidobacterium* species (38). While members of some species exhibited poor survival when exposed to 52°C, strains of other species, including *B. animalis* subsp. *lactis* BB-12 and closely related strains, showed high survival rates when exposed to 57°C or even 60°C for 5 min (38).

Little is known about the physiological and metabolic stress response in bifidobacteria to heat, such as the effect of heat stress on the carbon flux through the central metabolism, to compensate for stress-induced perturbations in the requirements for energy, reducing power and biomass precursors. All bifidobacteria dissimilate carbon compounds anaerobically through a unique heterofermentative pathway, the bifid shunt, which relies on the key enzyme xylulose 5-phosphate/fructose 6-phosphate phosphoketolase (Xfp; EC 4.1.2.22) (see Fig. S1 in the supplemental material) (39, 40). Theoretically, 3 mol of acetate and 2 mol of lactate are produced per 2 mol of glucose consumed (41). However, besides acetate and lactate, bifidobacteria are capable of producing formate, ethanol and succinate as fermentation end products (42–48). In general, changes in the carbon flux distribution affect the final ATP yield on consumed substrate as well as the redox balance. A shift of the metabolic flow toward acetate production allows the formation of additional ATP, while formation of lactate and ethanol adds to the regeneration of NAD^+ (47–49). Increased ethanol production at the expense of lactate increases the ATP yield (43) by regenerating an additional NAD^+, thereby making carbon available for ATP formation via acetate production. Thus, studying the effect of a stressor, such as heat, on the fermentation end product profile of a strain may provide insights on how the stress affects energy supply and redox balancing. However, how heat stress affects the fermentation end product profile of bifidobacteria remains to be investigated.

To date, only one study has described how heat stress can affect the morphology of bifidobacteria. In strains of the species *B. pseudolongum* subsp. *globosum*, the production of most cell wall proteins increased with increasing temperature (50). In addition, the hydrophobicity of the studied strains changed depending on the temperature, with higher hydrophobicity at both low and high growth temperatures than at moderate temperatures (50).

Molecular Mechanisms of Heat Stress Response

Transcriptional and translational response. The exposure to severe heat stress was found to cause significant modifications of gene expression in *B. breve* UCC2003 and *B. longum* subsp. *longum* NCC2705 at the transcriptional level (36, 51) as well as in *B. longum* 3A and *B. animalis* subsp. *lactis* BB-12 at the translational level (37, 52). For example, 46% of the genes were differentially expressed in *B. longum* NCC2705 when shifting cells from 37°C to 50°C (36). In particular, annotations to several heat shock proteins (Hsps) were found to be overrepresented in the proteome or transcriptome in these strains

upon heat stress (36, 37, 51, 52). In addition, a transcriptome analysis of *B. longum* NCC2705 revealed the downregulation of many genes related to the translational machinery, cell division, and chromosome partitioning machineries upon severe heat treatment at 50°C, indicating a slowdown of the general metabolic activities upon heat stress (36). Moreover, a heat-resistant (59°C) derivative of *B. longum* NCC2705 showed lower constitutive production of proteins associated with the central carbon metabolism than the parental strain, including the key enzyme Xfp (53). In *B. longum* NCC2705, the expression of a gene encoding the transfer-messenger RNA (tmRNA; SsrA) binding protein SmpB was induced upon severe heat stress (36). SmpB is essential for the *trans*-translation machinery, which allows the release of stalled ribosomes that can be formed due to various reasons such as a low tRNA level (36, 54). In order to rescue ribosomes, a complex of tmRNA, SmpB, and elongation factor Tu (EF-Tu) interacts with the stalled ribosome, allowing the degradation of the nascent polypeptide and mRNA and the release of the ribosomes (54). Taken together, reduced metabolic activity and simultaneous activation of protective functions appear to be a survival strategy of bifidobacteria in response to heat stress (36).

Interestingly, in *B. animalis* subsp. *lactis* BB-12 heat stress resulted in an increased abundance of thioredoxin peroxidase (EC 1.11.1.24), which is known to detoxify hydrogen peroxide (H_2O_2) under oxidative stress in bacteria (52). Both before and after the heat shock, a heat-tolerant derivative had higher expression of thioredoxin peroxidase than the wild type, on the translational level (52). This response might be attributed to the fact that heat stress can promote oxidative stress under aerobic conditions (55). Alternatively, the upregulation of the thioredoxin peroxidase in *B. animalis* subsp. *lactis* BB-12 might be explained by leaky regulation or indicate that the induced thioredoxin peroxidase is part of the general stress response in bifidobacteria.

Molecular players of the protein quality control system. The heat stress response in bifidobacteria is characterized by induction of a protein quality control (PQC) system consisting of several transcriptional regulators and a variety of heat shock proteins that act as molecular chaperones and proteases. Despite their denomination, heat shock proteins are involved in maintaining protein homeostasis not only upon heat stress but also upon exposure to other stressors in bacteria (56), which is further discussed below.

Heat shock proteins are classified into families according to their molecular mass. Bifidobacterial genomes encode representatives of the Hsp100 (ClpC, ClpB, and ClpX), Hsp70 (DnaK), Hsp60 (GroEL), Hsp40 (DnaJ and DnaJ2), and small Hsp (Hsp20) families, cochaperones (GrpE and GroES), and Hsp100-associated proteases (ClpP); the names in parentheses represent the members of each family whose genes can be found in bifidobacterial genomes (56). Genes coding for proteins of the Hsp33 and Hsp90 families appear to be absent in bifidobacteria (56, 57).

The regulation of heat shock proteins in bifidobacteria was found to partially overlap the regulon of the so-called SOS response system (51), which is induced upon DNA damage and which is well studied in *Escherichia coli* (58, 59).

The majority of information on the genomic organization and regulation of molecular actors involved in the heat stress response in bifidobacteria derives from studies by Zomer et al. and Ventura et al., using *B. breve* UCC2003 as a representative strain (51, 57). Based on multiple studies on the heat stress-induced regulons in *B. breve* UCC2003, applying genetic and transcriptional analyses, a model for the stress gene regulatory network describing the genomic setup and regulation of the PQC system and DNA repair system (SOS response system) in bifidobacteria has been proposed (51). The original model further includes hypothetical interactions based on knowledge from other high-G+C Gram-positive bacteria, such as *Streptomyces coelicolor* (51). Here, we focus on the part of the model which is based on *Bifidobacterium*-specific data (Fig. 3).

(i) Hsp100. Hsp100 chaperones are ATP-dependent proteins that can be categorized into two subgroups. While the ClpB/Hsp104 subgroup presumably mediates protein disaggregation and refolding in collaboration with the Hsp70/DnaK chaperone system, the subgroup of ClpA, ClpC, ClpE, ClpX, and ClpY can form Clp holoenzyme complexes

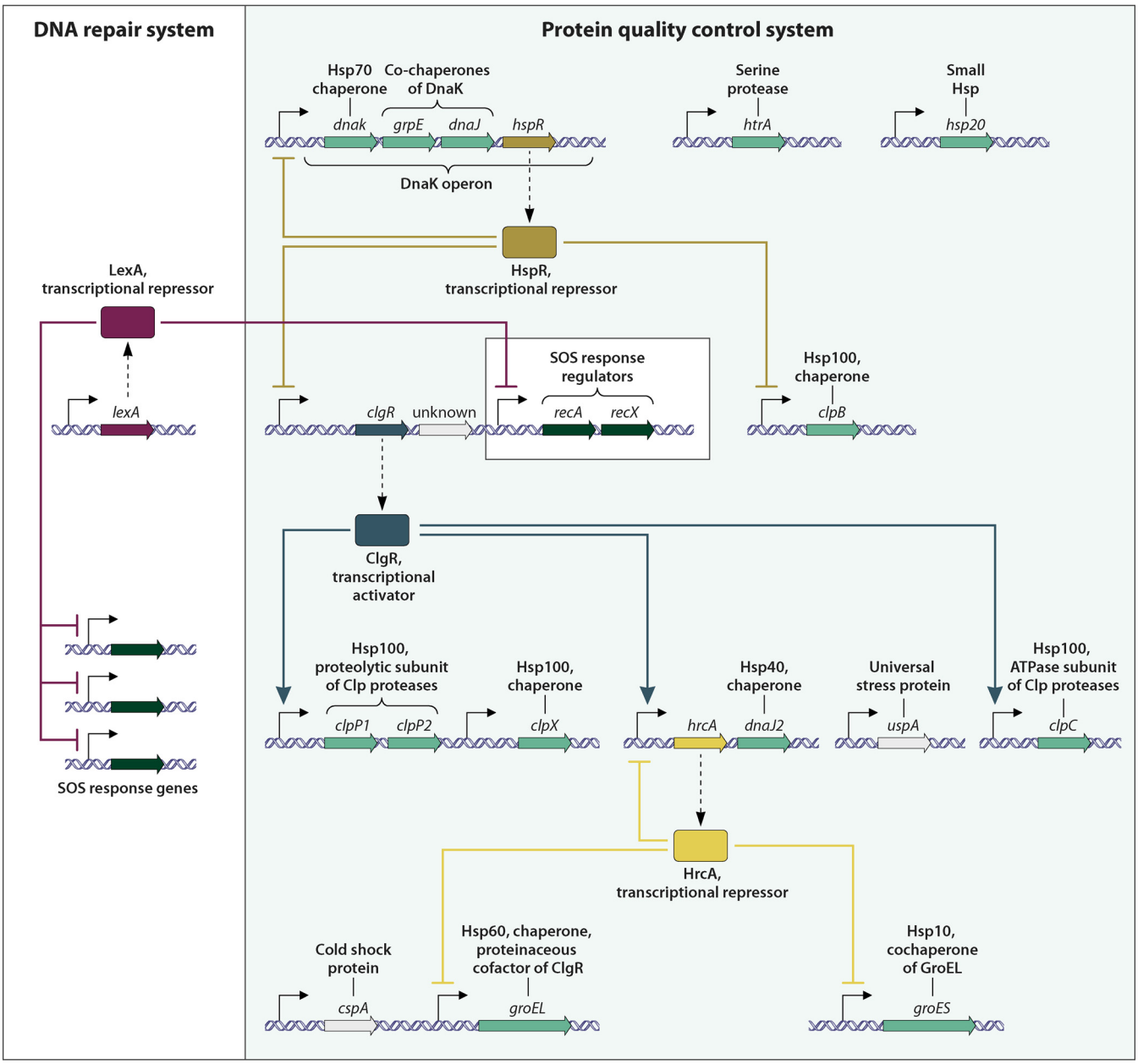

FIG 3 Proposed regulatory network of the stress-induced protein quality control and DNA repair system in bifidobacteria, mainly based on studies of the heat stress response in *B. breve* UCC2003 (51). Lines ending in bars indicate repression; lines ending in arrowheads indicate activation.

with ClpP or ClpQ proteases for ATP-dependent proteolysis of misfolded proteins (56). Genes encoding the Hsp100 proteins ClpB and ClpC have been identified and characterized in *B. breve* UCC2003 (60, 61). Whereas other high-G+C Gram-positive bacteria possess multiple *clpC* homologs (62), studied bifidobacterial genomes revealed the presence of only a single *clpC* copy, located next to a homolog of the *uspA* gene, encoding a universal stress protein UspA (61). The biochemical function of universal stress proteins in bacteria remains still to be explained (63). The expression of *clpB* was found to be induced upon severe heat stress (61), whereas *clpC* may be induced upon moderate heat stress in *B. breve* UCC2003, but conflicting results have been obtained (51, 60). The sequential order of the *clpC* gene and *uspA* gene seems highly conserved among investigated *Bifidobacterium* strains; however, in *B. breve* UCC2003, the *uspA* gene does not belong to the *clpC* gene locus and was not induced by heat stress (61). Moderate heat stress may further induce the *clpP* operon in *B. breve* UCC2003, which

comprises genes for the ClpP proteases ClpP1 and ClpP2 (EC 3.4.21.92) that are transcribed bicistronically in the strain (64). Also for the induction of the *clpP* operon, conflicting results have been obtained (51, 64), which may be due to different cultivation conditions used across experiments (51). A *clpX* gene, located directly downstream of the *clpP1* and *clpP2* genes, does not belong the *clpP* operon and is not induced in response to heat shock in *B. breve* UCC2003 (64).

(ii) Hsp70 and Hsp40. The Hsp70 chaperone DnaK complex, which displays folding, refolding, protein transport, and quality control activity in *E. coli* (56), has been reported to be induced in strains of *B. adolescentis*, *B. breve*, and *B. longum* upon heat stress (36, 37, 65). The *dnaK* operon in *B. breve* UCC2003 comprises the genes *dnaK*, *grpE*, *dnaJ1*, and *hspR* and is transcribed as a single polycistronic mRNA (57, 66). DnaJ1 (Hsp40) and GrpE function as cochaperones of DnaK, and HspR (heat shock protein repressor) represents a common transcriptional repressor of chaperones in *Actinobacteria* (57).

(iii) Hsp60 and Hsp10. The expression of GroEL (Hsp60) and its cochaperone GroES (Hsp10), known to promote protein folding (67), is induced in multiple *Bifidobacterium* strains in response to heat stress (36, 37, 52, 68). Moreover, a slightly higher constitutive level of GroEL and GroES at the translational level has been detected in a heat (70°C)-tolerant derivative of *B. animalis* subsp. *lactis* BB-12 (69) and was proposed as one factor contributing to the mutant's increased heat tolerance (52). The opposite has been described for a heat-resistant (59°C) derivative of *B. longum* NCC2705 (53), suggesting a noncompelling link between the level of GroEL and GroES and the heat tolerance of a *Bifidobacterium* strain.

While commonly organized in a monocistronic operon in other bacteria, the genes encoding GroEL and GroES were found to be located in different loci in studied bifidobacterial genomes (68). In addition, bifidobacteria appear to possess only a single copy of the *groEL* gene (68), while some other high-G+C Gram-positive bacteria contain multiple copies (70). Although the significance of this genomic arrangement remains to be elucidated, phylogenic analysis indicates that acquisition of the *groES* gene through horizontal gene transfer or an incomplete duplication of the GroELS operon followed by deletion of the original *groEL* gene can provide potential explanations (68). Genomic analysis of *Bifidobacterium* strains revealed that *groEL* is located directly downstream of a gene predicted to encode a cold shock protein, CspA (68). Initial experiments indicated cotranscription of *groEL* and *cspA* in *B. breve* UCC2003 (68); however, an active promoter upstream of the *groEL* gene was identified later, suggesting monocistronic transcription of the genes (51). Cold shock proteins have been proposed to function as RNA chaperones under cold stress or other stresses in bacteria by preventing the formation of secondary structures in RNA (71); however, their function in bifidobacteria remains unknown.

(iv) sHsps. Small heat shock proteins (sHsps) are ATP-independent molecular chaperones whose size usually ranges from 15 to 30 kDa (72) and which have various functions in different organisms, e.g., being involved in biofilm formation and cell protection during dormancy, besides having chaperone activity (73). Previous analysis of bifidobacterial genomes revealed that a subgroup of *Bifidobacterium* strains possesses a gene encoding a small heat shock protein of around 20 kDa, Hsp20 (27, 74). The presence of Hsp20 was suggested to be exclusive to *Bifidobacterium* isolates from the human intestine (74). Phylogenetic analysis of the protein sequence of Hsp20 further suggested that the *hsp20* gene might have been acquired by horizontal gene transfer from *Firmicutes* (74). The *hsp20* gene was found to be among the most strongly induced genes in *B. breve* UCC2003 and *B. longum* NCC2705 in response to severe heat stress, as well as to osmotic, oxidative, and starvation stress (51, 74, 75). In addition, the tolerance of *B. longum* NCC2705 to heat, salt, bile salt, low-pH, and cold stress was enhanced by homologous overexpression of *hsp20* (76). Taken together, these findings suggest that Hsp20 plays a substantial role in the response to multiple stressors and could therefore function as a biomarker for stress in bifidobacteria that possess the *hsp20* gene, as was also suggested in reference 75.

(v) HtrA. Transcriptomic and proteomic analysis suggested that the serine protease HtrA (EC 3.4.21.107) might contribute to degradation of misfolded proteins under severe heat stress in *B. longum* strains (36, 37, 77).

Transcriptional regulation of the protein quality control system. Based on studies of *B. breve* UCC2003, the PQC system in bifidobacteria appears to be under the complex control of two stress-related transcriptional repressors, the heat shock protein repressor (HspR) and the heat regulation at CIRCE (HrcA) regulator, as well as one transcriptional activator, the Clp gene regulator (ClgR) (Fig. 3) (51).

(i) HspR. The transcriptional repressor HspR is known to bind to the so-called HspR-associated inverted repeat (HAIR) sequences in promoter regions of the targeted genes (78). In *B. breve* UCC2003, HAIR-like motifs were found in the promoter region of multiple genes (60); however, binding of HspR could be experimentally validated only for the operators of the *dnaK* operon, the *clpB* gene, and the *clgR* gene (Fig. 3) (51). Based on alignments of the HAIR motifs in the promoter region of these gene loci, the consensus sequence of the bifidobacterial HAIR motif was suggested to be AAAsTTGAGysw-N_6-CTCAAsTTTT (the number of Ns was incorrectly given as five in the original publication; nucleotide codes follow the IUPAC nomenclature [79]). The adenine- and tyrosine-rich extensions of the motif appeared to be crucial for the binding efficiency of HspR (51). A physiological and genomic study of heat-resistant (59°C or 62°C) derivatives of *B. longum* NCC2705 provided further evidence for the transcriptional regulation of the *dnaK* operon and the *clpB* gene by HspR. A point mutation in the DNA-binding domain of the *hspR* gene was found to be responsible for constitutive upregulation of the *dnaK* operon and the *clpB* gene in the heat-tolerant mutants, presumably by impairing the binding of the repressor to the promoter regions and thus diminishing its negative regulation (77). A shorter HAIR motif (TGAG-N_9-CTCA) was identified in the promoter region of additional genes in bifidobacterial genomes, including genes predicted to encode the nucleoside triphosphate pyrophosphohydrolase MutT, a methyltransferase, and a 4-α-glucanotransferase (EC 2.4.1.25) (60), which might participate in the oxidative stress response in bifidobacteria (80, 81). However, binding of HspR to this less-extended HAIR motif has not been experimentally validated and appears unlikely, considering that the motif lacks the adenine- and tyrosine-rich extensions that have been found to be important for HspR binding.

(ii) HrcA. The second transcriptional repressor of the PQC system in bifidobacteria, HrcA, binds to promoter regions at inverted repeat sequences designated controlling inverted repeat of chaperone expression (CIRCE) (51, 82). In *B. breve* UCC2003, CIRCE-like motifs (TTAGCACTC-N_9-GAGTGTAA) have been detected in the promoter region of the *hrcA* gene, *groEL* gene and *groES* gene (Fig. 3), suggesting a transcriptional coregulation of GroEL and GroES and a negative feedback regulation of HrcA expression (51). While a single CIRCE-like motif was found in the promoter region of the *groES* and *hrcA* gene, two CIRCE-like motifs were detected in the promoter region of the *groEL* gene (51). In all genome sequences of *Bifidobacterium* strains available at the time of the study, the *hrcA* gene was located upstream of a second *dnaJ2* gene, which was found to be a characteristic genetic organization of the two genes in *Actinobacteria* (83). In *B. breve* UCC2003, the two genes formed a bicistronic operon (51, 83).

(iii) ClgR. ClgR is the only transcriptional activator shown to participate in the PQC system of bifidobacteria (51). In *B. breve* UCC2003, purified ClgR was found to bind in the promoter regions of the *clpC* gene and of the *clpP* operon in the presence of a proteinaceous cofactor, later suggested to correspond to the chaperone GroEL (51, 61, 64). In contrast to ClgR homologs encoded by other high-G+C Gram-positive bacteria, the amino acid sequence of ClgR in bifidobacteria possesses an N-terminal extension that might prevent its binding activity in the absence of chaperones such as GroEL (51). In DNA-binding assays with a N-terminally truncated ClgR, the activator bound upstream of the *hrcA* gene, besides binding the promoter regions of the *clpC* gene and of the *clpP* operon (Fig. 3) (51). This indicates that ClgR causes the repression of the HrcA regulon, including HrcA, DnaJ2, GroEL, and GroES, by activating the expression of

the *hrcA* gene. Based on comparative sequence analysis of the identified ClgR motifs in *B. breve* UCC2003 and the results from DNA-binding assays, ClgR binds an imperfect repeat TNCGCT-N_3-GGCGNAA in bifidobacteria (51).

In *B. breve* UCC2003, the level of heat stress was found to determine which molecular players of the PQC system are induced at the transcriptional level (51, 57). Slightly inconsistent results have been obtained, depending on the cultivation method applied as well as the method used for the quantification of transcripts (51). The proposed differential induction system in response to moderate and severe heat stress in *B. breve* UCC2003 correlates fairly well with induction patterns of heat shock proteins and regulators in other *Bifidobacterium* strains at the transcriptional and translational level. It should be noted, however, that the comparison is hampered by the different durations of heat stress treatments applied across the studies as well as variations in the methods of quantifying gene expression.

The HspR regulon, presumably comprising the *dnaK* operon, the *clpB* gene, and the *clgR* gene as well as the *hsp*20 gene, exhibited strong induction upon exposure to severe heat stress (47°C or 50°C) in *B. breve* UCC2003 (up to 416-fold after 1 h) and *B. longum* NCC2705 (up to 16-fold after 12 min at 50°C) (36, 51). The regulon was also overexpressed to a lesser extent upon moderate heat stress (42°C or 44°C) in *B. breve* UCC2003 (up to 27-fold after 1 h) (84). In line with this observation, expression of DnaK was induced in *B. longum* 3A (above 70-fold after 30 min at 47°C) at the translational level and in *B. longum* NCC481 and *B. adolescentis* NCC251 at the transcriptional level upon moderate to severe heat stress (37, 65). However, none of the other members of the HspR regulon were found to be overproduced in *B. longum* 3A upon severe heat stress, which might be attributed to the incomplete identification of all upregulated proteins (37).

The ClgR regulon, presumably including the *clpP* operon and the *clpC* gene, showed high constitutive expression in *B. breve* UCC2003 and was downregulated upon severe heat stress in *B. breve* UCC2003 (to −4-fold after 1 h) and not induced in *B. longum* NCC2705 (36, 51, 84). These results conflict with the high expression level of the activator ClgR when both strains are exposed to severe heat stress (36, 51). Under moderate heat stress, the ClgR regulon might be slightly induced in *B. breve* UCC2003; however, as mentioned before, conflicting results have been obtained (51, 61).

The expression of the *groEL*, *groES*, and *hrcA* genes, which are all potential members of the HrcA regulon, was induced upon severe heat stress in *B. breve* UCC2003 (up to 11-fold after 1 h at 47°C) and *B. longum* NCC2705 (up to 4-fold after 12 min), as well as upon moderate heat stress in *B. breve* UCC2003 (up to 4-fold after 1 h), but to a significantly lower extent than the members of the HspR regulon (36, 51, 84). Despite its minor induction, the *groEL* gene became the most transcribed gene at 44°C and 47°C in the strain, because of its very high constitutive expression level in *B. breve* UCC2003 (51). The gene encoding DnaJ2, an additional candidate HrcA-regulated protein, was not significantly induced upon elevated heat stress in *B. longum* NCC2705 and was downregulated in *B. breve* UCC2003 (to −6-fold after 1 h) (36, 51, 84). Consistent with the upregulation of the corresponding genes in *B. breve* UCC2003 and *B. longum* NCC2705, GroEL and GroES were overproduced in *B. animalis* subsp. *lactis* BB-12 (up to 2- and 4-fold after 2 h) and GroEL in *B. longum* 3A (between 5- and 10-fold after 30 min) upon severe heat stress (37, 52). Production of HrcA was not found to be induced upon severe heat stress in either of the strains (37, 52); however, again, the possibility that the protein was simply not identified in the studies cannot be excluded.

(iv) Alternative sigma factors. Housekeeping sigma factors 70 (σ^{70}, RpoD) enable specific binding of the RNA polymerase to the promoter regions of genes that are required for growth of bacteria (85). In *B. breve* UCC2003, a homolog of RpoD (HrdB) was found to be upregulated upon severe heat shock (51), whereas the expression of σ^{70} in *B. longum* NCC2705 was not significantly upregulated upon exposure to 50°C (36).

In addition to the housekeeping sigma factor, bacteria possess alternative sigma factors that respond to specific stimuli and control the transcription of genes in response to

the change (86). *B. longum* NCC2705, *B. longum* subsp. *longum* BBMN68, and *B. breve* UCC2003 and closely related strains were reported to possess one or two genes coding for an extracytoplasmic function (ECF) RNA polymerase sigma factor RpoE (σ^E) (36, 57, 75, 87). Most alternative sigma factors are known to be cotranscribed with a single or multiple negative regulators that inhibit binding of the sigma factor to the RNA polymerase until being released upon an environmental stimulus (86). The gene located downstream of σ^E in the genome of *B. longum* NCC2705 was proposed to encode a negative effector of the ECF-type sigma factor (36, 75). Interestingly, the transcription of the putative negative effector was found to be upregulated upon exposure to heat stress in *B. longum* NCC2705, while the expression of σ^E was not significantly affected, suggesting independent regulations of the genes or varying mRNA stability (36, 75). The consequences of the described expression pattern of σ^E and its negative effector for the corresponding protein levels upon heat stress remain to be elucidated. However, it can be assumed that an upregulation of the negative effector decreases the amount of free σ^E and thereby slows the transcription of the genes connected to the ECF sigma factor. To fully understand the consequences of the upregulation of the negative effector upon heat stress, it would be necessary to identify the genes whose transcription is controlled by σ^E. As no consensus binding motifs were identified in the promoter region of the *hsp20* gene in bifidobacteria, it has been suggested that the expression of Hsp20 might be regulated by an alternative sigma factor (51, 74). However, the *hsp20* gene was found to be highly upregulated upon heat stress in *B. longum* NCC270; thus, the gene is likely not regulated by σ^E, assuming that the gene downstream of σ^E encodes its negative effector.

Molecular players of the DNA repair system and their regulation. Besides genes involved in the PQC system, genes of the SOS response are induced upon heat stress in *Bifidobacterium* strains. The SOS response in bacteria is an inducible DNA repair system controlled by the repressor LexA (EC 3.4.21.88) and inducer RecA (59). In the presence of single-stranded DNA, the transcriptional repressor LexA is cleaved by RecA, which activates the expression of genes involved in the SOS response (59). Members of the RecA-LexA-dependent SOS response system were upregulated at the transcriptional level in *B. longum* NCC2705 and *B. breve* UCC2003 upon heat stress at 50°C and 44°C to 50°C, respectively (36, 51). The set and the level of induction of SOS response genes varied between the two strains and were further shown to depend on the level of heat stress in *B. breve* UCC2003 (36, 51). Besides RecA and LexA, RecX (negative regulator of RecA), RecN (a recombination protein), RuvA (Holliday junction ATP-dependent DNA helicase), and additional potential members of the DNA repair system (including an ImpB/MucB/SamB family protein and DNA-cytosine methyltransferase [Dcm], referred to as modification methylase) were induced in one or both strains upon heat stress (36, 51). The functions here (indicated in parentheses) were inferred from the role of the corresponding protein homologs in *E. coli* (59, 88).

In *B. breve* UCC2003, the genes coding for RecA and RecX are located directly downstream of a gene locus encoding the transcriptional repressor ClgR and a hypothetical protein (Fig. 3) (51). Experimental evidence was collected for the existence of a transcriptional unit comprising the whole set of genes from *clgR* to the *recX* gene. The upregulation of the SOS response in *B. breve* UCC2003 overlapped with the upregulation of the HspR regulon. HspR might thus control the SOS response in *B. breve* UCC2003 by expression of RecA. A potential binding motif of LexA was detected directly upstream of RecA, indicating that an alternative transcriptional unit exists that comprises only the *recA* and *recX* genes (51). LexA binding motifs were further identified upstream of the *lexA* gene, indicating a negative feedback loop for its regulation, as well as upstream of additional genes involved in DNA repair, including *impB*, *ruvA*, and *dcm*, which is in line with the finding that their expression is co-upregulated (51). The LexA binding motif was also identified upstream of a gene encoding MutY (A/G-specific adenine DNA glycosylase), which was not significantly induced by heat stress in *B. breve* UCC2003 (51). Moreover, no LexA binding motif was found upstream of the

recN gene, which still was found to be upregulated in *B. longum* NCC2705 upon heat stress (36). These findings indicate that additional, yet-unidentified regulators may be active in the control of the DNA repair system.

The model for the bifidobacterial stress gene regulatory network appeared to be valid for all characterized *Bifidobacterium* strains at the time when it was established in *B. breve* UCC2003, based on a high genetic conservation of the regulons and binding sites across strains (51, 61, 64, 66, 68, 83). In a recent genomic study, we reconfirmed that genes associated with PQC and DNA repair are highly conserved among 171 genome sequenced *Bifidobacterium* strains (20). However, the presence of binding sites of the transcriptional regulators (51) was found to vary for strains of different phylogenetic groups (20). Hence, the general validity of the proposed regulatory network should be further validated for other members of the genus. A lack of physiological data regarding the heat stress response in *Bifidobacterium* strains other than *B. breve* UCC2003 and *B. longum* NCC2705, as well as limited proteomic data, which can deliver a direct measure of changes in protein quantities, limit the current knowledge on the regulation of the PQC and DNA repair system in bifidobacteria. In addition, the specificity and functions of the individual proteins in the network remain to be investigated in bifidobacteria, using classical molecular biology approaches that focus on their complex interplay, which is not grasped in omics studies.

COLD STRESS

During freezing and when used as ingredients in refrigerated functional foods, probiotic bifidobacteria are subjected to cold stress (Fig. 1), which can influence their viability. The minimum growth temperature of most bifidobacteria lies between 25°C and 28°C (31), with the exception of a few animal isolates such as *B. bombi* and *B. psychraerophilum*, which can still grow at 10°C and 4°C, respectively (89, 90). The analysis of the cell membrane fatty acid profile of *B. bombi* BluCl/TP^T revealed a high content of unsaturated fatty acids (90), potentially allowing maintenance of membrane fluidity at low temperatures (91). The cold stress response in bifidobacteria is almost uncharted, and the genetic and metabolic bases of their cold stress responses remain to be studied in depth. However, as mentioned above, the *groEL* gene in *B. breve* UCC2003 and some other *Bifidobacterium* strains was found to lie directly downstream of a *cspA* gene, encoding a cold shock protein (Fig. 3) (68). The CspA protein sequence of *B. breve* UCC2003 shows high similarity to several cold shock proteins from other high-G+C bacteria and *E. coli* and contains a consensus cold shock domain, which is highly conserved among CspA homologs (68). As mentioned above, CspA may function as RNA chaperone (71). The *cspA* gene in *B. breve* UCC2003 was found to be significantly expressed upon severe heat stress (47°C and 50°C) but not in response to other tested stress treatments (moderate heat, osmotic, and solvent stress) (51). The effect of cold stress on *cspA* expression has not been evaluated yet.

OXIDATIVE STRESS

Throughout their life span, probiotic bifidobacteria can be exposed to molecular oxygen (O_2) during fermentation, and in particular during downstream processing and storage (Fig. 1) (2, 8). While O_2 itself is not toxic for bacteria, cellular damage is commonly attributed to reactive oxygen species (ROS) that are formed from O_2 under aerobic conditions. The predominant ROS include the superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO^{\cdot}$), which are formed by one-, two- and three-electron reductions of molecular O_2, respectively (Fig. 4). These redox reactions can be of an enzymatic or chemical nature. For example, H_2O_2 and $O_2^{\cdot -}$ are produced when O_2 oxidizes a redox enzyme that facilitates electron transfer to other substrates (92). H_2O_2 formation further results from spontaneous or enzymatic breakdown of $O_2^{\cdot -}$ (93), while $HO^{\cdot}$ can be formed from H_2O_2 through the oxidation of Fe^{2+} to Fe^{3+} in the Fenton reaction (92) (Fig. 4). The toxicity of ROS is linked to oxidation of biomolecules, which can cause cell death.

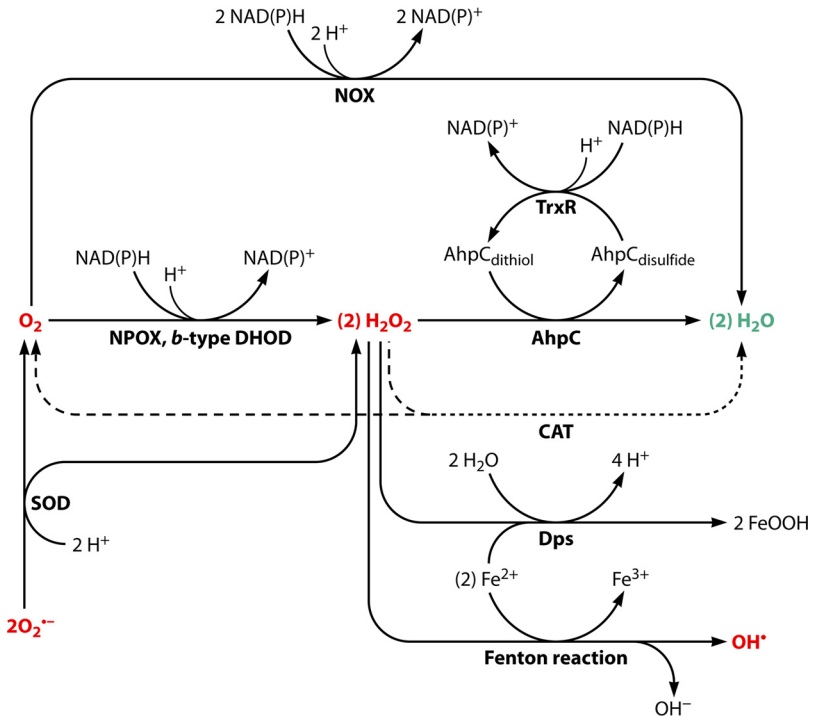

FIG 4 Overview of O_2-scavenging and ROS detoxification enzymes in bifidobacteria. NPOX, H_2O_2-forming NADP oxidase; DHOD, dihydroorotate dehydrogenase with H_2O_2-forming activity; NOX, H_2O-forming NAD(P)H oxidase; AhpC, alkyl hydroperoxide reductase subunit C; TrxR, thioredoxin reductase; Dps, DNA-binding protein from starved cells; SOD, superoxide dismutase; CAT, catalase. The prevalence of genes encoding these enzymes varies across *Bifidobacterium* strains.

Phenotypic Effects of Oxidative Stress

Bifidobacteria are generally classified as anaerobes, but their sensitivity to aerobic conditions varies significantly among species (38, 94–97) and among strains of the same species (98). Recently, it was suggested to categorize bifidobacteria into four groups according to their sensitivity to O_2: O_2 hypersensitive, O_2 sensitive, O_2 tolerant, and O_2 hypertolerant (99). While O_2-hypersensitive species (*B. longum* subsp. *infantis* and *B. adolescentis*) and O_2-sensitive species (*B. bifidum*, *B. breve*, *B. animalis* subsp. *animalis*, and *B. longum* subsp. *longum*) are inhibited by O_2 concentrations below and around 5%, respectively, members of the O_2-tolerant group (*B. minimum*, *B. asteroides*, *B. indicum*, and *B. animalis* subsp. *lactis*) can tolerate oxygen concentrations up to around 10% to 15%, and the O_2-hypertolerant group (*B. boum* and *B. thermophilum*) can grow in the presence of 20% O_2 (41, 99). The use of a standardized nomenclature for the classification of O_2 tolerance of bifidobacteria among studies would facilitate the comparison of results from different studies. However, as this classification was introduced only recently, older publications do not follow this notation.

Oxidative damage is one of the prevailing factors that affect the survival of probiotic bifidobacteria (100). Aerobic bacteria harbor efficient systems to detoxify ROS, comprising enzymes such as catalase (EC 1.11.1.6), superoxide dismutase (SOD; EC 1.15.1.1) and NADH peroxidase (EC 1.11.1.1). In contrast, most *Bifidobacterium* strains appear to lack a sufficient ROS detoxification system, resulting in O_2 sensitivity. For example, accumulated H_2O_2 has been found to severely affect the central carbon metabolism of bifidobacteria by specifically inhibiting the key enzyme Xfp (97).

Molecular Mechanisms of Oxidative Stress Response

Carbon metabolism. During fermentation, the presence of O_2 reduces the growth rate and the final biomass yield of bifidobacteria (100, 101). Moreover, oxidative stress was found to induce changes in the carbon flux through the bifid shunt and consequently

affect the profile of fermentation end products. Previous studies showed that some *Bifidobacterium* strains, including the O_2-tolerant strain *B. animalis* subsp. *lactis* IPLA 4549, produce less lactate and more acetate under oxidative stress (100, 102). This change in the end product formation is thought to result from the competition between lactate dehydrogenase (EC 1.1.1.27) and NADH-dependent O_2-scavenging and ROS detoxification enzymes. Under aerobic conditions, NAD^+ is partially regenerated by the latter group of enzymes, and less NAD^+ needs to be regenerated by lactate formation, which spares pyruvate for acetate formation along with ATP production (100, 102). In line with this, *B. animalis* subsp. *lactis* IPLA 4549 has a higher intracellular ATP content under aerobic conditions (100). The additional ATP generated from the breakdown of pyruvate to acetate might provide extra energy for repair processes that counteract O_2 damage (100). In contrast to these findings, a decrease of the acetate/lactate ratio has been reported for the O_2-tolerant strains *B. longum* subsp. *infantis* ATCC 17930 (103) and *B. animalis* subsp. *animalis* DSM 20140 (41) in the presence of O_2. The relative decrease of acetate formation under aerobic conditions might be due to high O_2 sensitivity of pyruvate formate lyase (EC 2.3.1.54), which catalyzes the conversion of pyruvate and coenzyme A (CoA) to formate and acetyl-CoA, the latter being the precursor of acetate (41).

Transcriptional and translational response. Oxidative stress was found to modify the gene expression in *Bifidobacterium* strains to different extents. For example, no changes in gene expression have been observed for *B. animalis* subsp. *lactis* DSM 1040 after exposure to sublethal H_2O_2, whereas the gene expression of *B. animalis* subsp. *lactis* BL-04 showed significant changes under the same conditions (81). Moreover, changes in gene expression were found to depend on the time of exposure to the stressors. For example, enzymes involved in oligosaccharide utilization were upregulated in *B. animalis* subsp. *lactis* BL-04 after 5 min but not after 20 min exposure to 1.25 mM H_2O_2 (81). Interestingly, in *B. longum* BBMN68, genes linked to disaccharide and oligosaccharide transport and metabolism were downregulated after exposure to 3% O_2 for 0.5 h and 1 h (80).

The application of omics technologies has not only provided further evidence on previous observations but also provided new insights to be further investigated in future studies. Even though initial hypotheses might have been proposed on the differential expression of some proteins in response to oxidative stress, the biochemical and physiological functions of many of those proteins remain to be elucidated. An overview of genes and proteins that have been found to be differentially expressed in *Bifidobacterium* strains in response to oxidative stress is provided below.

Prevalence and function of O_2-scavenging and ROS detoxification enzymes. As some O_2-scavenging enzymes and ROS detoxification enzymes produce H_2O_2 while others decompose it, the interplay between the individual enzymes is crucial to sufficiently decrease O_2 stress (Fig. 4). In bifidobacteria, a strong production of H_2O_2 or its weak decomposition under aerobic conditions is thought to determine the O_2 sensitivity of some strains (94, 96, 97, 104, 105). In line with this, accumulation of H_2O_2 has been detected in O_2-senstive *Bifidobacterium* strains (strains of *B. adolescentis*, *B. bifidum*, and *B. longum*), whereas strains with higher O_2 tolerance (strains of *B. boum* and *B. thermophilum*) seem to accumulate less or no H_2O_2 during O_2 exposure (94, 96).

(i) NAD(P)H oxidase. There are two types of NAD(P)H oxidases: those that catalyze the two-electron reductions of O_2, resulting in the formation of the detrimental H_2O_2 (EC 1.6.3.1), and those that catalyze four-electron reduction, resulting in the formation of H_2O (EC 1.6.3.2), thereby contributing to the detoxification of O_2 (Fig. 4). The formation of H_2O_2 in O_2-sensitive *Bifidobacterium* strains is thought to be mainly associated with NAD(P)H oxidases catalyzing the two-electron reduction of O_2 (94, 96, 97). H_2O_2 accumulation and NAD(P)H oxidase activity were reported to be induced in the presence of O_2 and to increase with rising O_2 concentrations (94, 96, 102).

Two enzymes with H_2O_2-forming NAD(P)H oxidase (Fig. 4) activity have been purified and characterized from *Bifidobacterium* strains, including a *b*-type dihydroorotate dehydrogenase (DHODb) from the O_2-sensitive strain *B. bifidum* ATCC 29521 (106), and

a NADPH oxidase (NPOX) from the O_2-hypersensitive strain *B. longum* subsp. *infantis* ATCC 15697, showing homology to proteins in the nitroreductase family (105). Both the *b*-type DHOD and NPOX showed high level of similarity with the corresponding genes from other *Bifidobacterium* strains (105, 106). DHOD, which is composed of a PyrK and a PyrDb subunit, is traditionally known to oxidize dihydroorotate to orotate in pyrimidine biosynthesis. However, the *b*-type DHOD purified from *B. bifidum* was proposed to additionally contribute to H_2O_2 formation at O_2 concentration above 10% in N_2, using NADH as electron donor (106). The activity of the H_2O_2-forming NPOX in *B. longum* subsp. *infantis* ATCC 15697 was shown to be determinative for the strain's growth inhibition at moderate O_2 concentration around 5% as well as to contribute to H_2O_2 formation in 10% and 20% O_2 atmosphere (105). Knocking out the NPOX gene (*npoxA*) in *B. longum* subsp. *infantis* ATCC 15697 improved its growth in the presence of 5% O_2 but did not eliminate the strain's sensitivity to O_2 concentrations above 10%, at which residual H_2O_2 accumulation in the medium of the mutant could be detected (105). These results suggested the presence of a second H_2O_2-forming NADH oxidase in the strain (105). The *b*-type DHOD protein of *B. longum* subsp. *infantis* ATCC 15697, which we found shares 78% sequence identity with the *b*-type DHOD protein of *B. bifidum* ATCC 29521, was suggested to be a potential candidate (105). The role of NPOX was corroborated by heterologous expression of the *npoxA* gene in the O_2-tolerant strain *B. minimum* DSM 20102^T, which diminished growth of the mutant at O_2 concentrations above 10%, accompanied by H_2O_2 formation (105).

A recent study on the transcriptional stress response in the O_2-sensitive strain *B. longum* BBMN68 to a low O_2 concentration of 3% in the headspace (i.e., a growth-inhibiting but not lethal O_2 concentration) showed upregulation of genes annotated as nitroreductase (*nfnB2*) and DHOD (*pyrD2*) (80). We found that the upregulated BBMN68 nitroreductase displays 99.6% protein sequence identity with NPOX characterized from *B. longum* subsp. *infantis* ATCC 15697 (105). In contrast, the induced DHOD shares only 27% similarity with the PyrDb subunit of *B. bifidum* ATCC 29521 and is annotated as a class 2 quinone-dependent DHOD (106). In line with the hypothesis that the *b*-type DHOD contributes to H_2O_2 formation only at elevated O_2 levels, the NADH-dependent *b*-type DHOD of *B. longum* BBMN68, which has 79% sequence identity with the *b*-type DHOD of *B. bifidum* ATCC 29521, was not upregulated in 3% O_2–97% N_2. The upregulated quinone-dependent DHOD might participate in the oxidative stress response under microaerobic conditions. The H_2O_2-forming NAD(P)H activity in *B. longum* 6001 had previously been found to be enhanced in the presence of the growth factor 2-amino-3-carboxy-1,4-naphthoquinone (ACNQ), suggesting that the quinone serves as an electron mediator from NAD(P)H to O_2 in the given reaction (107). In *B. longum* 6001, the presence of ACNQ appeared to reduce the overall oxidative stress in the strain by simultaneously enhancing the detoxification of H_2O_2 by a quinone-dependent NADH peroxidase (107).

No H_2O-forming NAD(P)H oxidase (NOX) has yet been characterized in O_2-tolerant bifidobacteria. Nevertheless, the increased expression and activity of the NADH oxidase in the O_2-tolerant *B. animalis* subsp. *lactis* IPLA 4549 under aerobic conditions were linked to its potential role in detoxification of O_2 to H_2O (100). Moreover, in two O_2-hypertolerant *Bifidobacterium* strains, H_2O-forming NAD(P)H oxidase activity was reported to exceed H_2O_2-forming activity under acidic conditions (96). However, instead of being solely linked to a high H_2O-forming NAD(P)H activity, high O_2 tolerance of *Bifidobacterium* strains might also be due to sufficient peroxidase activity, detoxifying the formed H_2O_2, as discussed below.

(ii) NAD(P)H peroxidase. Several studies report a positive correlation between the O_2 tolerance of *Bifidobacterium* strains and their ability to decompose H_2O_2 under aerobic conditions (94, 100, 104). Initially, the decomposition of H_2O_2 by *Bifidobacterium* strains was thought to be linked to the activity of NAD(P)H peroxidase (EC 1.11.1.1/2), which reduces H_2O_2 to H_2O (94, 102, 104). However, in the last decade, genomics and proteomics analyses revealed that bifidobacteria lack a gene for NAD(P)H peroxidase and that H_2O_2 decomposition may instead be associated with the activity of an alkyl

hydroperoxide reductase subunit C (AhpC) and a thioredoxin reductase-like protein encoded in their genomes (75, 108, 109). Commonly, alkyl hydroperoxide reductases are thiol-specific antioxidant proteins that consist of the subunits AhpC (EC. 1.11.1.15) and AhpF (EC 1.6.99.3). The AhpC subunit possesses peroxidase activity, while AhpF functions as a flavoprotein disulfide reductase that regenerates reduced AhpC (110). A thioredoxin reductase-like protein (TrxR; EC 1.8.1.9; encoded by *trxB*) possessing the N-terminal domain of AhpF is considered to replace the function of AhpF (Fig. 4) (75, 111, 112). The *trxB* and *ahpC* genes are tandemly located in the genome of *B. longum* NCC2705 and *B. bifidum* ATCC 29521 (75, 112). Previous studies confirmed upregulation of AhpC, also referred to as peroxiredoxin or thioredoxin peroxidase, at the transcriptional and translational level in strains of *B. longum*, *B. bifidum*, and *B. animalis* when exposed to oxidative stress in the form of H_2O_2 or O_2 (75, 80, 81, 100, 101, 105, 112–114). Likewise, the expression of the *trxB* gene was strongly induced as part of the oxidative stress response (75, 80, 81, 100, 112–114). The assumption that AhpC and TrxR cooperate was reinforced by the observation that homologous overexpression of AhpC in *B. longum* NCC2705, which resulted in increased O_2 tolerance and endogenous H_2O_2 detoxification activity, also caused an upregulation of *trxB* expression (111). In addition, TrxR, purified from a NAD(P)H oxidase-active fraction of the O_2-sensitive *B. bifidum* ATCC 29521, which contributed to H_2O_2 formation in the absence of AhpC, degraded H_2O_2 when interacting with recombinant AhpC under aerobic conditions (112). In *B. longum* subsp. *infantis* ATCC 15697, the expression of AhpC appeared to be induced prior to the expression of the H_2O_2-forming NADPH oxidase NPOX upon exposure to O_2, demonstrating a timely activation of the defense to avoid H_2O_2 accumulation (105). Taken together, these observations indicate that O_2 sensitivity of *Bifidobacterium* strains appears to originate from the accumulation of H_2O_2 due to activity of H_2O_2-forming NADH oxidases as well as insufficient H_2O_2 detoxification by the AhpC-TrxR system (112).

A new member of the thiol-specific antioxidant protein (TSA)/AhpC family, a bacterioferritin comigratory protein (BCP; EC 1.11.1.24), might contribute to thioredoxin-dependent H_2O_2 peroxidase activity in *Bifidobacterium* strains. BCP was upregulated at the transcriptional level in the H_2O_2-tolerant strain *B. animalis* subsp. *lactis* 01 upon oxidative stress (113). In *E. coli* and *Helicobacter pylori*, the enzyme was found to reduce linoleic acid hydroperoxide rather than H_2O_2 (115, 116). The substrate specificity of BCP in bifidobacteria is unknown; however, *B. longum* strains have been shown to inhibit linoleic acid peroxidation (117).

In *B. bifidum* ATCC 29521 and *B. longum* NCC2705, a second gene encoding a thioredoxin reductase was identified. However, it lacks the N-terminal domain of AhpF (112) and was not upregulated at the transcriptional level upon oxidative stress in *B. longum* NCC2705 (75).

(iii) Oxygen-independent coproporphyrinogen III oxidase. A gene predicted to encode oxygen-independent coproporphyrinogen III oxidase (HemN; EC 1.3.98.3), known as a key enzyme in heme biosynthesis, was induced in the O_2-tolerant strain *B. animalis* subsp. *lactis* IPLA 4549 when cultivated under aerobic growth conditions (100). In addition, homologous overexpression of AhpC in *B. longum* NCC2705 resulted in increased expression of the HemN-encoding gene, suggesting feedback control by AhpC (111). The function of HemN in the oxidative stress response in bifidobacteria remains elusive, in particular as it is annotated to be oxygen independent and thus seems not to contribute to O_2 scavenging. Moreover, the HemN-encoding gene in bifidobacteria was found to share high sequence similarity with that for a putative heme chaperone, HemW, of *Lactococcus lactis* that lacks HemN activity (20, 118).

(iv) Class I pyridine nucleotide-disulfide oxidoreductase. A gene predicted to encode a class I pyridine nucleotide-disulfide oxidoreductase (PNDR) was upregulated in *B. longum* NCC2705 upon exposure to 1.25 mM H_2O_2 at the transcriptional level (75) as well as in *B. longum* BBMN68 at the transcriptional and translational level after short-term (0.5 h and 1 h) exposure to 3% O_2 (80, 101). PNDR is a heterogenous protein

family with different functional activities (119). The subfamily class I includes mercuric reductase, higher eukaryotes thioredoxin reductases, trypanothione reductase, lipoamide dehydrogenase, and glutathione reductase (119), but the function of the bifidobacterial enzyme has not been biochemically proven.

(v) DNA-binding protein from starved cells. In the O_2-sensitive *B. longum* BBMN68, a gene coding for the DNA-binding protein from starved cells (Dps; EC 1.16.3.1), which is also referred to as DNA-binding ferritin-like protein, was found to be upregulated in mid-exponential growth phase cells in response to short-term (1 h) oxidative stress (3% O_2) at the transcriptional and translational level (101). Dps protects prokaryotic cells from oxidative damage through (i) binding genomic DNA and thereby shielding it from ROS and (ii) oxidizing Fe^{2+} using H_2O_2 as electron donor and thereby simultaneously detoxifying H_2O_2 and sequestering Fe^{2+} that might otherwise react in the Fenton reaction (Fig. 4) (120). Dps might further have a protective effect against other stresses than O_2, e.g., acid and base stress (121). The Dps from *B. longum* BBMN68 was found to have a protective function against oxidative stress *in vitro* and *in vivo* when expressed heterologously in *E. coli* (101). Moreover, its expression was proposed to be activated by RecA (101), suggesting a link between the SOS response and Dps expression in bifidobacteria.

(vi) Methionine sulfoxide reductase. Genome sequencing of the H_2O_2-tolerant *B. animalis* subsp. *lactis* 01 and of *B. longum* NCC2705 revealed the presence of a gene encoding a peptide methionine sulfoxide reductase MsrAB (EC 1.8.4.1.2) (108, 113). MsrAB protects enzymes from oxidative damage by reducing methionine sulfoxide back to methionine and thereby indirectly scavenges ROS (122). It was found to be induced by oxidative stress in *B. animalis* subsp. *lactis* 01 at the transcriptional level (113).

Recently, we confirmed that most of the described O_2-scavenging and ROS detoxification enzymes are present in the genomes of strains of multiple *Bifidobacterium* species (20). However, strains of *B. angulatum* and some strains of *B. adolescentis* lack multiple genes encoding ROS detoxification enzymes, including AhpC, TrxR, BCP, Dps, and MsrAB, which might explain the exceptionally high O_2 sensitivity of these strains (20, 94, 104, 123).

(vii) Superoxide dismutase. Superoxide dismutase (SOD) catalyzes the dismutation of $O_2^{\cdot-}$ to O_2 and H_2O_2 (Fig. 4). SOD activity has been detected in several strains of the genus, including strains of the species *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, and *B. longum* (94, 102, 104). Most of the studied *Bifidobacterium* strains showed low SOD activity (94, 104), and no correlation between the O_2 sensitivity and the SOD activity of the strains was found (94, 102, 104). Particularly high SOD activity was reported for the O_2-sensitive strain *B. adolescentis* ATCC 15703 (104).

Despite SOD activity being detected in multiple strains, most *Bifidobacterium* strains lack SOD-homologous genes (75, 124). In exception, genome sequencing of *B. xylocopae* XV2, *B. aquikefiri* CCUG 67145^T, *B. tibiigranuli* TMW 2.2057^T, and *B. asteroides* PRL2011 revealed the presence of a gene encoding SOD (125–127).

Based on the absence of SOD-encoding genes in most *Bifidobacterium* strains, it was suggested that the detoxification of $O_2^{\cdot-}$ in these strains might be purely of nonenzymatic nature, via scavenging of $O_2^{\cdot-}$ by manganese and other bivalent metal ions taken up from the medium (75, 128). The uptake of manganese by bifidobacteria under aerobic conditions was proposed to be realized by a P-type ATPase or the divalent metal cation transporter MntH, which were induced in *B. longum* NCC2705 and *B. animalis* subsp. *lactis* 01 upon exposure to H_2O_2 (75, 113). However, this hypothesis can be challenged. First, it might be questionable why exposure to H_2O_2 should promote SOD activity by manganese uptake, since H_2O_2 is not known as a precursor for $O_2^{\cdot-}$, while H_2O_2 is a product of the SOD reaction. Second, even though manganese accumulation had been correlated with SOD activity in lactobacilli that lack a SOD-encoding gene (129), a more recent study of *E. coli* suggested that the protective role of manganese against oxidative stress is not linked to redox activity of the ion but is instead due to its ability to replace ferrous iron in the active sites of mononuclear metalloenzymes, which are susceptible to the Fenton reaction (130). In line with the suggested protective

effect of manganese against oxidative stress, an elevated manganese concentration in the growth medium of the O_2-hypersensitive *B. longum* BBMN68 was found to improve the strain's O_2 tolerance (80). As *B. longum* BBMN68 is known to be O_2 hypersensitive and, thus, probably lacks a sufficient detoxification system for H_2O_2, increased nonenzymatic dismutation of $O_2^{\cdot-}$ by manganese would probably not improve the strain's intrinsic O_2 tolerance because of the associated accumulation of H_2O_2. Therefore, also in *Bifidobacterium* strains, the protective effect of manganese might rather be linked to its function in the stabilization of metalloenzymes. SOD activity in strains lacking a SOD-homologous gene might originate from the rather rapid spontaneous destruction of $O_2^{\cdot-}$ (93).

Taken together, these results suggest that enzymatic SOD activity is a rare characteristic in bifidobacteria and that nonenzymatic SOD activity might contribute to O_2 sensitivity rather than to O_2 tolerance.

(viii) Catalase. Catalase activity, responsible for the decomposition of H_2O_2 to H_2O and O_2 (Fig. 4), was found to be absent or very low in strains of the species *B. adolescentis*, *B*, *longum*, *B. animalis*, *B. breve*, and *B. bifidum* (104). All *Bifidobacterium* strains that were found to harbor significant catalase activity are isolates from the digestive tract of bees (99, 131). This is considered to be an environment with a higher O_2 concentration than the digestive tracts of mammals, suggesting that the presence of a catalase homolog gene in the genome of these *Bifidobacterium* species is a result of evolutionary adaptation to the more aerobic growth environment (99). The O_2-inducible heme catalase of the O_2-tolerant *B. asteroides* JCM 8230^T is the only catalase characterized to date (131). Interestingly, the bee isolates *B. xylocopae* subsp. nov. XV2 and *B. aemilianum* subsp. nov. XV10 showed no catalase activity and different O_2 tolerances, even though both strains possess a catalase gene (126). This means that the mere presence of a catalase gene in the genome of a *Bifidobacterium* strain is not indicative of its catalase activity.

Combined heterogenous expression of catalase and SOD in *B. longum* NCC2705 and heterologous expression of catalase in *B. longum* 105-A enhanced the strains' survival under oxidative conditions significantly (132, 133). Addition of catalase to the cultures of O_2-sensitive *Bifidobacterium* strains, such as *B. longum* JCM1217^T and *B. bifidum* JCM1255^T (= ATCC 29521), improved growth under aerobic conditions but could not recover growth completely (96). In keeping with this, in *B. longum* 105-A, the addition of catalase to the medium resulted in a weaker protection than the heterologous expression of catalase, most likely due to its inability to sequester H_2O_2 intracellularly (132). Overall, these results again indicate that H_2O_2 detoxification is determinative for the aerotolerance of bifidobacteria.

(ix) Pyruvate oxidase. The O_2-tolerant water kefir isolates *B. aquikefiri* CCUG 67145^T and *B. tibiigranuli* TMW 2.2057^T possess additional genes which might contribute to their aerotolerance and which are unique within the genus, including a gene encoding pyruvate oxidase (EC 1.2.3.3) (127). Pyruvate oxidase uses O_2 to convert pyruvate into acetyl phosphate, H_2O_2, and CO_2. A gene encoding pyruvate oxidase is also encoded in the genome of the bee isolate *B. asteroides* PRL2011 (125). As noted in the previous paragraph, the pyruvate oxidase must be accompanied by H_2O_2 detoxification in order to contribute to aerotolerance.

(x) Electron transport chain. Genomic analysis of the aerotolerant honeybee isolate *B. asteroides* PRL2011 revealed that this strain might even harbor the capability of aerobic respiration, based on genes encoding enzymes for an electron transport chain in its genome (125). Comparative genomic analysis of strains of other species, mainly insect isolates, also revealed the presence of an electron transport chain (complex I to complex IV). This group includes strains of the species *B. actinocoloniiforme*, *B. bohemicum*, *B. bombi*, *B. coryneforme*, *B. indicum*, *B. mongoliense*, and *B. subtile* (124, 125). In contrast to other strains from *Bifidobacterium* species tested, *B. asteroides* PRL2011 was found to consume small amounts of oxygen (125). Based on global gene expression analysis, the expression levels of genes encoding the electron transport chain components appear to be similar under anaerobic and aerobic conditions; however, higher ATPase

activity was detected under aerobic conditions (125). A complete respiratory chain would provide a direct O_2-scavenging activity, provided that ROS do not leak from the system.

(xi) Glutathione system. The glutathione system, which contributes to the maintenance of redox homeostasis in Gram-positive bacteria (134), appears to be incomplete in bifidobacteria based on the absence of genes coding for glutathione peroxidase (EC 1.11.1.9) and glutathione reductase (EC 1.8.1.7) in their genomes (80, 132). While glutathione peroxidase uses reduced glutathione to quench oxidative radicals, the oxidized glutathione is regenerated by the glutathione reductase. In contrast, both *B. aquikefiri* CCUG 67145^T and *B. tibiigranuli* TMW 2.2057^T harbor a gene for glutathione peroxidase and genes for the heterodimeric ATP-binding cassette type transporter CydDC (127), which is known to contribute to glutathione uptake in prokaryotes, besides being involved in the assembly of cytochromes (134).

(xii) Hypothetical protein involved in H_2O_2 resistance. Acquisition of H_2O_2 resistance by *B. longum* NCC2705 was accompanied by increased constitutive expression of a protein with unknown function (135), whose role in H_2O_2 resistance was confirmed by heterologous expression of the encoding gene (BL_1404) in *B. thermophilum* (136). BL_1404 appears to encode a membrane protein; however, its exact function remains unknown (135).

Synthesis and integrity of DNA and protein. Besides the upregulation of O_2-scavenging and ROS detoxification enzymes, the defense mechanism of bifidobacteria against oxidative stress includes the induction of enzymes involved in safeguarding synthesis and integrity of DNA and protein. The effects of oxidative stress on the systems have not yet been examined as thoroughly as the effects of heat stress. The type of ROS used, as well as the duration and severity of the oxidative stress treatments, varied across studies, hampering the integration of individual results. Moreover, different results have been obtained for the same strain under comparable conditions (75, 114), and transcriptomic and proteomic analyses have sometimes indicated contradictory effects (80, 101).

In *B. breve* UCC2003, exposure to 1.2 mM H_2O_2 for 1 h resulted mainly in the transcriptional upregulation of the LexA regulon (3.6- to 6.2-fold), comprising genes involved in the SOS response, and of *hsp20* (8.0-fold) (84). Transcription of the *hsp20* gene (*ibpA*) was also induced in exponentially growing cells of *B. longum* BBMN68 when subjected to 3% O_2 for 30 min and 60 min (6.8-fold) (80), as well as in *B. longum* NCC2705 upon exposure to 1.25 mM H_2O_2 (75). In contrast, no change in Hsp20 expression at the translational level was detected when mid-exponential-growth-phase cells or stationary-phase cells of *B. longum* BBMN68 had been exposed to 3% O_2 for 60 min (101). Moreover, Hsp20 expression was found to be downregulated when cells were harvested in the mid-exponential growth phase and exposed to 3% O_2 for 9 h (-2.5-fold) (101). Results from a transcriptomic analysis of the stress response in *B. longum* BBMN68 to 3% O_2 revealed a slight induction of additional members of the PQC regulon (80), in contrast to previous proteomic analysis that had not reported any significant induction of additional Hsps (101). However, MutT1, involved in the prevention of DNA mutations, was found to be induced at the transcriptional and the translational levels upon exposure to 3% O_2 for 1 h (101).

Across transcriptomic and proteomic studies, genes of ribonucleotide reductase class Ib (NrdHIEF) and class III (NrdDG) gene clusters were found to be induced in *B. animalis* and *B. longum* strains upon oxidative stress (75, 80, 81, 101, 113, 114). Ribonucleotide reductases, which catalyze the reduction of ribonucleotides to their corresponding deoxyribonucleotides, might be important for DNA replication and repair under stress conditions (80). In particular, the glutaredoxin-like protein NrdH was found to be upregulated (75, 80, 101, 113, 114). NrdH is known to function as an electron donor for ribonucleotide reductases. Due to the absence of glutathione reductase, which commonly regenerates glutaredoxin, thioredoxin reductase might reduce NrdH in bifidobacteria, as was described for *E. coli* (80, 137). In line with this hypothesis, induced transcription of thioredoxin reductase

as well as simultaneous induction of glutaredoxin and thioredoxin at the transcriptional and translational levels were suggested to improve the resistance of *B. bifidum* WBBI03 to oxidative stress, after being cocultivated with *Listeria monocytogenes* CMCC 54001 (138).

In *B. longum* BBMN68, additional genes that may play a role in translation, PQC, and DNA repair and replication were found to be overexpressed upon exposure to 3% O_2. For example, the ribosome-associated protein Y (also known as ribosome-associated inhibitor A [RaiA]), which was suggested to stabilize ribosomes against dissociation under environmental stress (139) and cause a reduction of the protein biosynthesis rate by blocking the ribosomal A site (140), was upregulated at the translational level (101). This might improve the fidelity of translation under oxidative stress (101). In addition, the transcription of genes involved in the biosynthesis of tetrahydrofolate was upregulated. Improved availability of tetrahydrofolate might contribute to efficient protein synthesis and repair as well as DNA repair (80). Genes involved in purine biosynthesis were transiently upregulated, while pyrimidine biosynthesis appeared downregulated at the transcriptional level (80). The exposure of *B. longum* BBMN68 to 3% O_2 further resulted in increased transcription of genes involved in the biosynthesis of branched-chain amino acids (BCAAs), which might be required for the synthesis of proteins involved in the oxidative stress response (80). In contrast, a gene encoding ketol-acid reductoisomerase (EC 1.1.1.86), which is part of the BCAA biosynthesis, was found to be downregulated in the O_2-tolerant *B. animalis* subsp. *lactis* IPLA 4549 under aerobic conditions (100). Genes of the SUF iron-sulfur (Fe-S) cluster assembly machinery were induced in *B. longum* BBMN68 upon oxidative stress (80). Fe-S is a prosthetic group of several proteins that have essential functions in a variety of cellular processes (141). As the biosynthesis of Fe-S clusters can be inhibited by O_2, increased expression of SUF proteins may be required to fulfill the demand of Fe-S clusters under oxidative stress (141).

Morphological and cell envelope adaptations. The effects of oxidative stress on the cell shape and cell wall properties have been studied in a group of *B. longum* strains that showed good survival in the presence of O_2 (95). In response to O_2, cells elongated and developed an irregular cell surface, most likely due to disturbed cell division (95). In addition, oxidative stress was reported to change the fatty acid profile of the cells in the form of an increased content of short-chain and cyclopropane fatty acids (95). As cyclopropane fatty acids are considered to be less susceptible to oxidation than unsaturated fatty acids, they might contribute to increased resistance of the cell membrane to O_2 damage (142). In alignment with the increased cyclopropane fatty acid content in other *B. longum* strains (95), the expression of cyclopropane-fatty-acyl-phospholipid synthase (EC 2.1.1.79) was upregulated in *B. longum* BBMN68 upon oxidative stress at the transcriptional level (80). Moreover, a higher concentration of $C_{19:0}$ cyclopropyl plasmalogen in the cell membrane of *B. animalis* subsp. *lactis* BL-04 than in the cell membrane of *B. animalis* subsp. *lactis* DSM 10140 was suspected to contribute to the higher intrinsic resistance to H_2O_2 of BL-04 than of DSM 10140 (143, 144), despite the small genetic differences between them (145). Plasmalogens, which are phospholipids that contain a vinyl-ether at the *sn-1* position, are considered to function as endogenous antioxidants (146). Moreover, the cell membrane of *B. animalis* subsp. *lactis* BL-04 showed a higher myristic acid ($C_{14:0}$) and palmitic acid ($C_{16:0}$) content as well as a lower oleic acid ($C_{18:1,n\text{-}9}$) content than *B. animalis* subsp. *lactis* DSM 10140 when grown in complex medium with 0.1% Tween 80 as an exogenous fatty source (81). Interestingly, when cultivated in the medium without an exogenous fatty acid source, *B. animalis* subsp. *lactis* DSM 10140 showed a fatty acid profile more similar to that of *B. animalis* subsp. *lactis* BL-04, accompanied by an improved resistance to lethal H_2O_2 concentration (81).

The function of plasmalogens as ROS scavengers has been suggested to result in a decreased content of plasmalogens under oxidative conditions (146). In line, the plasmalogen content of cell membranes of the *B. longum* strains E4, JI1, NCC2705, and D2957 decreased significantly when cultivated aerobically or exposed to oxidative

stress, while being originally high under anaerobic conditions (95, 114). Taken together, these results suggest that the fatty acid and lipid profile play an important role in the tolerance of *Bifidobacterium* strains to oxidative stress.

In addition to changes in the fatty acid and lipid profile, other morphological changes have been observed upon oxidative stress. Exposure of *B. longum* BBMN68 to aerobic conditions resulted in increased cell surface hydrophobicity and autoaggregation of the cells, which might limit penetration of O_2 into the cells (80). The authors linked these changed surface properties of the strain to the downregulation of genes involved in polysaccharide biosynthesis, such as rhamnosyltransferases, and suggested that a reduced number of polysaccharides surrounding the cell surface upon oxidative stress might expose protein receptors responsible for cell aggregation (80, 147).

ACID STRESS

Probiotic bifidobacteria are exposed to acidic conditions during their life span (Fig. 1). During fermentation, bifidobacteria produce organic acids, mainly acetic acid and lactic acid, as end products of carbon dissimilation (Fig. S1). pH is commonly kept near neutral during fermentation. However, when used as supplements in functional food matrixes, such as yogurt or fermented milk, probiotic bifidobacteria must cope with the acidic conditions in the products (10). Moreover, probiotic bifidobacteria must survive the low pH (around 2) in the stomach, caused by hydrochloric acid (10), as well as the organic acids produced in the gastrointestinal tract produced by the intestinal microbiota.

Organic and inorganic acids have deleterious effects on bacteria. The inhibition mechanism of weak organic acids is well studied. Under acidic extracellular conditions, weak organic acids are present in their undissociated form and can passively diffuse across the cell membrane (148). Inside the cytoplasm, the pH is close to neutral, and the weak organic acid dissociates and accumulates, as the anion can no longer passively cross the cell membrane. The released protons decrease the intracellular pH and challenge pH homeostasis (148). Several responses follow, with the common purpose to capture and export protons across the cell membrane (see below). A low intracellular pH can further have deleterious effects on the activity and integrity of biomolecules (149). At neutral pH, at which organic acids are mainly dissociated and cannot penetrate the cell, the inhibitory effect of organic acids on growth of bifidobacteria has been found to be due to osmotic pressure (150). Strong acids, such as hydrochloric acids, which are fully dissociated under a wide range of pH, cannot pass the cell membrane and instead exert their inhibitory effect by denaturing biomolecules on the cell surface (91).

Phenotypic Effects of Acid Stress

The common optimum pH for growth of bifidobacteria lies between pH 6.5 and 7.0 (31). Below pH 4.5 or above pH 8.0, bifidobacteria cannot grow (31), except for *B. thermacidophilum* strains and *B. dentium* strains, which are able to grow at pH 4.0 to 4.5 (35, 151). However, the ability of bifidobacteria to survive in low-pH environments varies greatly across *Bifidobacterium* strains, with exceptional resistance to low pH of strains belonging to the species *B. animalis* (98, 152, 153). For example, *B. animalis* subsp. *lactis* BB-12 showed good survival for 20 min at pH 2, whereas strains of the species *B. adolescentis* and *B. longum* did not survive at pH 3 or pH 4 for 10 min (152).

The acid resistance of bifidobacteria appears to depend on the fermentation conditions under which the cells are produced. A lower final culture pH was shown to improve the subsequent stress resistance of some *Bifidobacterium* strains, most likely due to successive adjustment of their metabolism and physiology to the acidification of the medium (154). A gradual adaptation to acidic conditions might be one reason why cells produced in non-pH-controlled fermentations that are harvested in the stationary growth phase appear to be more acid tolerant than exponentially growing cells (154). Thus, when studying the response of stationary growth phase cells to other stressors, such as oxygen or heat, it should be noted that the cells might have already responded to acid stress during their cultivation. Osmotic stress or nutrient starvation

can be additional stressors shaping the physiology of cells in the stationary phase, which can hamper the identification of molecular mechanisms that are specifically associated with other stressors.

The presence of fermentable carbon sources was reported to extend the survival of *Bifidobacterium* strains under acidic conditions, underlining the importance of energy delivery for the acid stress response in these strains (154, 155).

Exposure and adaptation to acid stress were found to affect the growth characteristics of *Bifidobacterium* strains. The acid-resistant (pH 4.0) derivative of *B. longum* biotype *longum* NCIMB 8809 and its parental strain showed a longer lag phase when growing under acidic conditions (pH 4.8) than under nonstressed conditions, indicating the need for the reorganization of their metabolism in response to the prevailing stress condition (156). Both strains showed a reduction of growth rate with increasing bile content; however, the acid derivative showed higher growth rates than the parental strain when cultivated under acidic condition (156).

Molecular Mechanisms of Acid Stress Response

Carbon metabolism. Acid stress exposure, adaptation and metabolic conditioning influence the expression levels of enzymes involved in the central carbon metabolism, the fermentation end product profile, and the carbohydrate utilization of *Bifidobacterium* strains. Sublethal stress treatment (pH 4.5) of *B. longum* BBMN68 and acid adaptation of *B. longum* NCIMB 8809 resulted in a decreased expression of Xfp at the translational level compared to both the nonstressed and the parental strain (157, 158). However, upon lethal stress treatments (pH 3.5), the production of the Xfp protein was increased in *B. longum* BBMN68, regardless of prior exposure to sublethal acid stress (pH 4.5) (157). In the prestressed *B. longum* BBMN68 cells, additional enzymes involved in carbohydrate and energy production were significantly upregulated at the translational level upon lethal acid stress (157). These results are consistent with findings of another study that observed faster carbohydrate utilization and increased glycoside-hydrolyzing activities of strains belonging to the species *B. longum* and *B. catenulatum* after acid adaptation (159). Enhanced glycoside-hydrolyzing activities might further provide an advantage in the gastrointestinal tract where oligo- and polysaccharides are abundant (159). Taken together, these observations suggest that an enhanced efficiency of carbohydrate dissimilation could be one possible mechanism of bifidobacteria to fulfill the energy requirements needed for an adequate response to acid stress.

When cultivated under acidic conditions (pH 4.8), *B. longum* NCIMB 8809 and its acid-resistant (pH 4.0) derivative showed an increased formate yield on consumed glucose, whereas no strong effects on the ethanol yield or on the acetate/lactate ratio were observed (158). Despite the minor changes in the acetate/lactate ratio, an increased expression of lactate dehydrogenase and reduced expression of the bifunctional acetaldehyde/alcohol dehydrogenase were detected at the translational level in both strains when cultivated under acidic conditions (158). In contrast, acquisition of acid resistance (pH 3.2) of *B. breve* BB8 was accompanied by decreased constitutive expression of lactate dehydrogenases and an upregulation of the bifunctional acetaldehyde/alcohol dehydrogenase (EC 1.2.1.10 and EC 1.1.1.1) at the transcriptional level (160). The differential expression of the two genes compared to that in the parental strain suggests a displacement of the carbon flux toward increased ethanol production and decreased lactate production. Increased ethanol formation at the expense of lactate increases the ATP yield in bifidobacteria (43), since it diverts the carbon flux toward acetate production, which is linked to additional ATP formation (Fig. S1). Additional ATP might be required for an effective acid stress response in the strain. However, as the fermentation end product profile was not reported (160), it is unclear if the changes on the expression level eventually influenced the carbon flux distribution.

The proton-translocating F_1F_o-ATPase. The membrane bound ATP synthases (F_1F_o-ATPases; EC 3.6.1.34) of bacteria have two central physiological functions. Besides catalyzing the synthesis of ATP using the energy of an electrochemical proton gradient across the cell membrane, they can function as proton-translocating ATPases under

low-driving-force conditions (161). In nonrespiring bacteria, such as bifidobacteria, the F_1F_o-ATPase actively pumps protons out of the cytoplasm using ATP produced by fermentative substrate-level phosphorylation and thus generates the proton motive force (149), composed of the proton gradient and membrane potential.

In multiple *Bifidobacterium* strains, enhanced F_1F_o-ATPase activity was suggested to contribute to pH homeostasis under acid stress by enhancing active extrusion of protons. The F_1F_o-ATPase is encoded by the *atp* operon, which has been characterized in *B. animalis* subsp. *lactis* DSM 10140 (162). As in many other bacteria, the *atp* operon of *B. animalis* subsp. *lactis* DSM 10140 comprises eight open reading frames with the gene order *atpBEFHAGDC*. Two different transcripts of the *atp* operon were detected in *B. animalis* subsp. *lactis* DSM 10140; one transcript included all eight genes, indicating polycistronic expression, whereas another comprised only genes of the ATP synthase unit F_1 (*atpAGDC*) (162).

The expression of the *atp* operon was found to be induced upon exposure to pH 3.5 in *B. animalis* subsp. *lactis* DSM 10140 and was suggested to be controlled at the transcriptional level or by mRNA degradation (162). Consistent with this, the subunits AtpA and AtpD of the F_1F_o-ATPase operon were overexpressed at the translational level in *B. longum* NCIMB 8809 and its acid-resistant (pH 4) derivative when grown at pH 4.8, with a more pronounced increase in the mutant strain (158). The higher production of the two F_1F_o-ATPase subunits in the mutant strain matched its enhanced ability to maintain the intracellular pH under acidic condition (158). In *B. longum* BBMN68, sublethal acid stress treatments (pH 4.5) resulted in the upregulation of all subunits F_1F_o-ATPase at the transcriptional level, linked to an increased F_1F_o-ATPase activity (163). Moreover, whether or not it was subjected to prior sublethal acid stress, *B. longum* BBMN68 showed an increased F_1F_o-ATPase-activity upon lethal acid stress at pH 3.5 (157). In line with their higher level of F_1F_o-ATPase activity, prestressed cells showed improved ability to maintain pH homeostasis under acidic conditions compared to the nonconditioned cells. In both experimental groups, the ATP content decreased upon lethal acid stress, emphasizing the energy dependence of the stress response (157). A study on the F_1F_o-ATPase activity of 17 *Bifidobacterium* strains further showed a tendency of acid-resistant strains of the species *B. animalis* to have enhanced F_1F_o-ATPase activity at lower pH (pH 4.0 versus pH 5.0), whereas the activity of F_1F_o-ATPase in most acid-sensitive strains appears to decrease with decreasing pH (153). Taken together, these results suggest that an elevated activity of F_1F_o-ATPase represents an important molecular mechanism that may enable bifidobacteria to cope with acidic stress.

However, in the acid-resistant strain *B. dentium* Bd1, the expression of the F_1F_o-ATPase operon was not upregulated upon acid stress (pH 4) (151). Moreover, the expression of four of eight F_1F_o-ATPase subunits as well as F_1F_o-ATPase activity was lower in an acid-resistant derivative of *B. breve* BB8 than in its parental strain under nonstressed conditions in the stationary phase (160). Thus, the role of F_1F_o-ATPase in acid tolerance seems to vary among *Bifidobacterium* strains, and, as discussed below, additional molecular mechanisms contribute to acid tolerance in bifidobacteria, such as amino acid degradation (151, 160).

Amino acid metabolism. Several studies have suggested that enzymes involved in the amino acid metabolism in *Bifidobacterium* strains might contribute to the maintenance of the intracellular pH upon acid stress due to the formation of ammonia (NH_3). The formed NH_3 can scavenge protons in the cytoplasm and thereby buffer the internal pH during formation of ammonium ions (NH_4^+) (149).

(i) Branched-chain amino acids. In the acid-sensitive strain *B. longum* NCIMB 8809, exposure to acidic conditions resulted in an increased level of enzymes responsible for BCAA biosynthesis, including ketol-acid reductoisomerase (EC 1.1.1.86) and BCAA aminotransferase (BCAT; EC 2.6.1.42), as well as increased production of glutamine synthetase (GS; EC 6.3.1.2) (158). Based on similar results from studies on *Streptococcus mutans* (164), it was proposed that the deamination of glutamine to glutamate catalyzed by GS contributes to NH_3 formation, while glutamate is subsequently converted

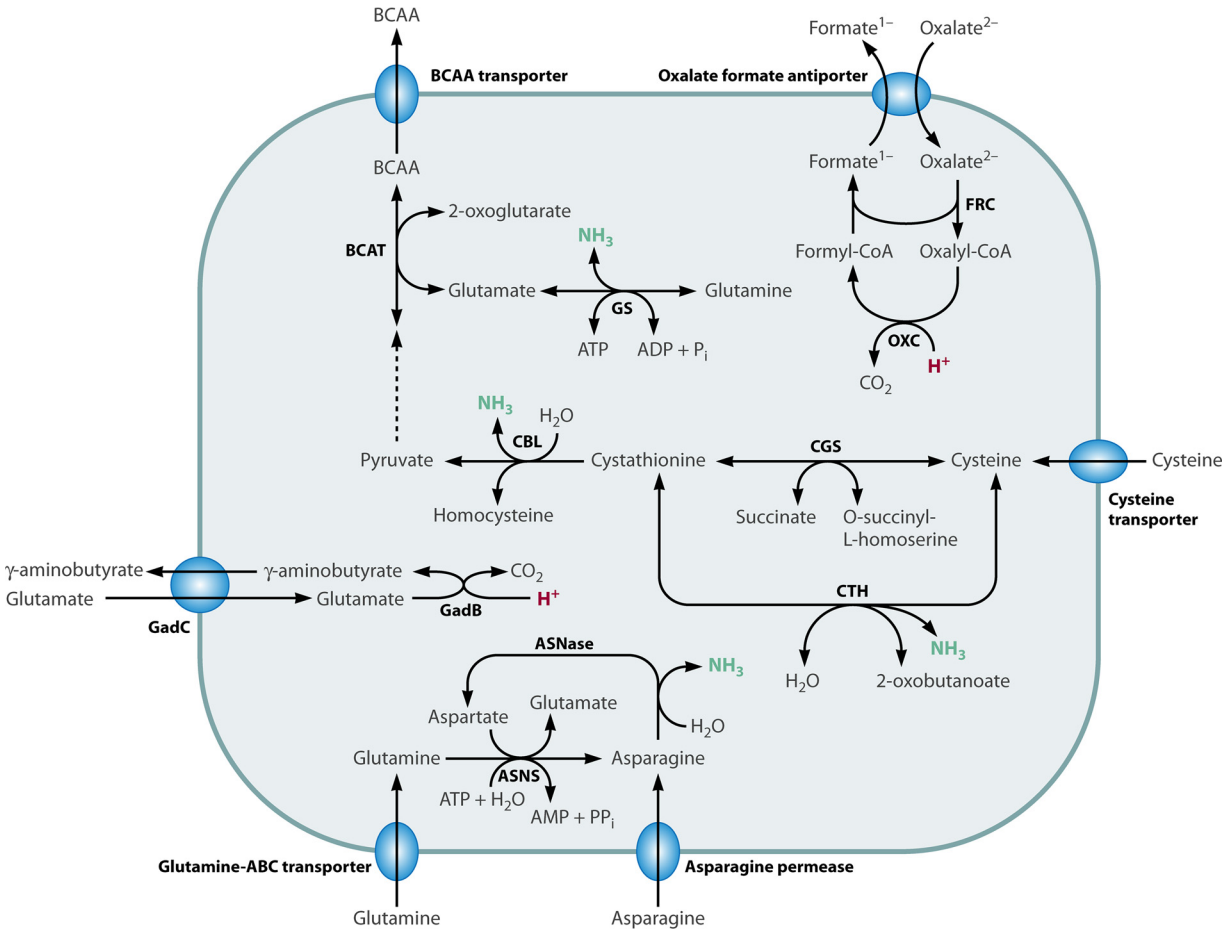

FIG 5 Visualization of mechanisms suggested to be involved in the acid stress response in *Bifidobacterium* strains and that include multiple enzymatic reactions. The proposed mechanisms are based either on the formation of ammonia (NH_3), which can accept protons and yield ammonium ions (NH_4^+), or on the direct export or consumption of protons (H^+). Additional mechanisms involved in the acid stress response are described in the text. ASNase, asparaginase; ASNS, asparagine synthetase; BCAA, branched-chain amino acid; BCAT, BCAA aminotransferase; CBL, cystathionine β-lyase; CGS, cystathionine γ-synthase; CTH, cystathionine γ-lyase; FRC, formyl-CoA transferase; GadB, glutamate decarboxylase; GadC, glutamate/γ-aminobutyrate antiporter; GS, glutamine synthetase; OXC, oxalyl-CoA decarboxylase; P_i, free phosphate; PP_i, pyrophosphate.

to 2-oxoglutarate in the final step of synthesis of valine, leucine, and isoleucine (Fig. 5) (158). This hypothesis was supported by physiological data showing increased NH_3 and valine concentrations in the cell extract of *B. longum* NCIMB 8809 upon acid stress (158). However, it should be noted that the reaction of GS is normally driven by ATP hydrolysis to the direction of amidation of glutamine to glutamate and might therefore be linked to NH_3 consumption instead. In addition, acetylornithine aminotransferase (EC 2.6.1.11), which could alternatively regenerate glutamate from 2-oxoglutarate formed in the biosynthesis of BCAA, was also overproduced under acidic conditions in *B. longum* NCIMB 8809 (158). Moreover, no increase in intracellular valine and less elevated NH_3 concentrations were detected in an acid-resistant (pH 4.0) derivative of *B. longum* NCIMB 8809, in which ketol-acid reductoisomerase and GS were also upregulated upon acid stress exposure (158). In the acid-sensitive strain *B. longum* BBMN68, the transcription of multiple genes encoding enzymes of the BCAA synthesis pathway was upregulated upon sublethal acid stress treatment (pH 4.5), including ketol-acid reductoisomerase and BCAT, whereas neither acetylornithine aminotransferase nor GS was induced (163). At the translational level, ketol-acid reductoisomerase was the only overproduced enzyme involved in BCAA synthesis upon sublethal stress treatment (157). Nevertheless, an elevated cellular NH_3 content was measured in the prestressed cells compared to nonstressed cells (157). Lethal stress treatments of the prestressed

cells further induced the production of GS; however, a decrease in NH_3 content was observed (157). As in the acid-sensitive strain *B. longum* NCIMB 8809, the transcriptional expression of *argD*, coding for acetylornithine aminotransferase, was also induced in the acid-resistant strain *B. dentium* Bd1, isolated from carious lesions in the human oral cavity, upon acid stress (151). However, no enzymes involved in BCAA metabolism were upregulated, and the catabolism of other amino acids was suggested to be determinative for the strain's acid tolerance, as discussed below (151). Further evidence is needed to clarify the role of the proposed mechanism of NH_3 formation via GS.

In another transcriptomics study, the acid-resistant derivative of *B. breve* BB8 showed higher expression of GS and glutamate synthase (EC 1.4.1.13), as well as comparatively low expression of genes linked to synthesis of BCAAs, under nonstressed conditions than its parental strain (160). As BCAAs serve as precursor for branched-chain fatty acids (BCFA), the authors suggested that decreased BCAA biosynthesis might reduce the BCFA content of the cell membrane and thereby change the membrane profile toward lower fluidity (160), potentially contributing to increased acid tolerance in bacteria (165, 166). Even though the cell membrane composition of the parental strain and its derivative was analyzed in a later study, the BCFA content was not examined (167).

Overall, these results suggest that glutamine/glutamate and BCAA metabolism play a role in the stress response in *Bifidobacterium* strains, but the underlying mechanisms remain unclear.

(ii) Cysteine. Based on transcriptomic and proteomic data, enzymes involved in the metabolism of sulfur-containing amino acids might also play a role in the acid stress response in bifidobacteria. The acid-resistant (pH 4.0) derivative of *B. longum* NCIMB 8809 showed higher levels of enzymes involved in the metabolism of methionine and cysteine under nonstressed conditions than its parental strain (158). Genes that were upregulated encode 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (EC 2.1.1.14; annotated as methionine synthase in that publication), *o*-acetylhomoserine (thiol)-lyase (EC 2.5.1.49), and cystathionine γ-synthase (CGS; EC 2.5.1.48) (158). In keeping with this, higher constitutive expression of CGS at the transcriptional level was detected in an acid-tolerant derivative of *B. longum* JDM301 than in its parental strain (168). Simultaneously, lower expression levels of cystathionine γ-lyase (CTH; EC 4.4.1.1) and cystathionine β-lyase (CBL; EC 4.4.1.8) were observed in the mutant. When the strains were grown under acid stress (pH 3.5), the expression of CGS was further induced in the mutant but also upregulated in the parental strain (168). CGS catalyzes the formation of cystathionine and succinate from cysteine and *O*-succinyl-L-homoserine. The authors suggested that the differential constitutive expression of the three genes in the mutant strain might result in the accumulation of cystathionine under nonstressed conditions, which can be converted upon the release of NH_3 by the activity of CTH and CBL under acidic conditions (Fig. 5) (168). The formed NH_3 would provide buffering capacity by reacting with protons to form NH_4^+. Similarly, an upregulation of CGS and CBL at the transcriptional level observed in *B. longum* BBMN68 upon sublethal acid stress treatment (pH 4.5) might increase the NH_3 formation in the strain (163). Additionally, cystathionine β-synthase (EC 4.2.1.22) was induced by sublethal stress treatment in *B. longum* BBMN68 (163). Based on the proposed mechanism, cysteine supply might contribute not only to improved O_2 but also to acid tolerance in bifidobacteria (163).

(iii) Consumption of glutamine and asparagine. The genome sequence of two acid-tolerant isolates from water kefir, *B. aquikefiri* CCUG 67145^T and *B. tibiigranuli* TMW 2.2057^T, revealed the presence of genes that might allow the production of NH_3 by conversion of glutamine into aspartate but are not commonly shared among bifidobacteria (127). The proposed combination of enzymes includes a glutamine-ABC-transporter, asparagine synthetase (ASNS; EC 6.3.5.4), asparaginase (ASNase; EC 3.5.1.1), and asparagine permease and would consume one ATP per produced NH_3, which can subsequently remove one proton from the cytoplasm (Fig. 5) (127).

(iv) Glutamate decarboxylase pathway. The glutamate decarboxylase pathway has

been previously described to be induced in lactic acid bacteria upon environmental stress, such as acid stress (169). The pathway comprises a glutamate/γ-aminobutyrate anti-porter (GadC) and glutamate decarboxylase (GadB; EC 4.1.1.15). GadB adds an intracellular proton to imported glutamate, forming γ-aminobutyrate (GABA), which is subsequently transported out of the cell in exchange with another glutamate by GadC (Fig. 5). By consuming an intracellular proton, the pathway can contribute to pH homeostasis under acidic conditions (149). The formed CO_2 might be converted by carbonic anhydrase to form bicarbonate, which can also buffer the intracellular pH, as described for the CO_2 that is formed in the malolactic fermentation pathway in lactic acid bacteria (169). In 2009, genome sequencing of the acid-resistant *B. dentium* Bd1 revealed the presence of a gene locus encoding GadB and GadC, whose expression was strongly induced in response to acid stress at the transcriptional level (151). Later, the presence of GadB and GadC was found to be a rather rare characteristic among *Bifidobacterium* species (170). To date, the ability to produce GABA has been described for strains of only eight *Bifidobacterium* species: *B. adolescentis*, *B. angulatum*, *B. dentium*, *B. longum subsp. longum*, *B. merycicum*, *B. moukalabense*, *B. ruminantium*, *B. stercoris* (170–172).

Additional metabolic responses to acid stress. (i) Ammonium transport. A gene encoding an ammonium transporter of the Amt family was found to be overexpressed upon acid stress in *B. longum* JDM301 (168). The authors suggest that the transporter might contribute to ammonium uptake (168). The nature of the substrate of Amt transporters and their mechanism are still under debate (173). However, based on the rationale presented above, they might hypothetically facilitate the uptake of NH_3, which can also cross the membrane by passive diffusion, or contribute to the export of NH_4^+, resulting from the protonation of intracellularly produced NH_3, thereby contributing to intracellular pH homeostasis under acid stress.

(ii) Oxalate/formate metabolism. The acid-tolerant strain *B. dentium* Bd1 showed an upregulation of genes encoding formyl-CoA transferase (FRC; EC 2.8.3.16) and oxalyl-CoA decarboxylase (OXC; EC 4.1.1.8) at the transcriptional level upon exposure to acidic conditions (151). FRC catalyzes the transfer of CoA from formyl-CoA to oxalate, releasing formate, while OXC catalyzes the decarboxylation of the formed oxalyl-CoA, consuming an intracellular proton (Fig. 5). Thereby, the enzymes catalyze the degradation of oxalate, a relative strong organic acid (pK_a = 1.25), present in plant-based food (174). In agreement with the findings for *B. dentium* Bd1, acidic conditions were found to promote oxalate consumption in *B. animalis* subsp. *lactis* BI07 (175). A permease encoded next to the *oxc* gene was suggested to serve as an oxalate transporter in bifidobacteria (175). The upregulation of the oxalate/formate metabolism might be essential for the survival of these strains in their natural habitat.

(iii) Polyphosphate. The accumulation of polyphosphates, which are polyanionic inorganic biopolymers of a few to hundreds of phosphate molecules linked by phosphoanhydride bonds, has been shown to contribute to stress resistance in bacteria (176). The accumulation of polyphosphate in bacteria is mainly associated with the activity of polyphosphate kinase (PPK1; EC 2.7.4.1), which catalyzes the reversible ATP-dependent linking of a polyphosphate chain and a phosphate (176). The expression of PPK and the accumulation of polyphosphates in the cytoplasm have been suggested to be induced by oxidative stress and starvation and were linked to increased acid tolerance of *B. scardovii* BAA-773 (pH 3.0) (177). Similarly, transcription of the *ppk1* gene was found to be upregulated in *B. longum* BBMN68 upon sublethal acid stress treatment (pH 4.5) (163). Besides other biological functions, accumulated polyphosphate might protect the cells under acidic conditions by functioning as a buffer to counteract pH changes (178–180). The ability to accumulate polyphosphates appear to be widespread across *Bifidobacterium* species. So far, it has been detected for strains of the species *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, *B. longum*, *B. pseudolongum*, and *B. scardovii* (177, 179, 181).

Synthesis and integrity of DNA and protein. The effect of acid stress on the expression of heat shock proteins, proteases, and enzymes of the DNA repair system has not been studied in detail. Nevertheless, transcriptomic and proteomic analyses of the acid stress response in *B. longum* strains revealed the induction or downregulation of members of the PQC and DNA repair systems upon exposure to low pH (Table 1). Again, various conditions were used to study the stress-induced changes in expression of the studied strains, hampering the comparison of the individual results. Overall, genes of the *dnaK* operon, the *groEL* gene, and the *groES* gene appear to be commonly induced upon acid stress. However, no general induction pattern can be identified, suggesting that effects are strain and condition dependent.

Increased production of the Xaa-Pro aminopeptidase PepP (EC 3.4.11.9) was detected in *B. longum* NCIMB 8809 when cultivated at pH 4.8 in comparison to levels detected at pH 7 (158), as well as in *B. longum* BBMN68 upon lethal stress treatment (pH 3.5) if the cells were conditioned by prior sublethal heat stress treatment (pH 4.5) (157). These findings suggest that PepP might contribute to the delivery of free amino acids, essential for protein synthesis and repair, from peptides under acidic conditions.

Moreover, acid-conditioned (pH 4.5) *B. longum* BBMN68 with increased survival at pH 3.5, compared to untreated cells, showed an upregulation of glutamate-cysteine ligase (EC 6.3.2.2) at the transcriptional level (163). Glutamate-cysteine ligase, previously named γ-glutamylcysteine synthetase, catalyzes the formation of γ-glutamyl cysteine, which can be converted to glutathione by glutathione synthetase. Due to the absence of glutathione synthetase in *B. longum* BBMN68, the authors suggested that γ-glutamyl cysteine might have a protective role itself under acid stress by forming mixed disulfides with thiol groups of proteins (protein S-thiolation) and thereby preventing protein damage under acidic conditions (163). However, this hypothesis remains to be confirmed.

Inducible acid resistance by sublethal stress treatment was suggested to be linked to a decrease in protein synthesis in two *B. longum* strains. In *B. longum* BBMN68, sublethal acid stress treatment resulted in the upregulation of a gene encoding GTP pyrophosphokinase (EC 2.7.6.5), which is involved in the production of guanosine-tetraphosphate (ppGpp) (163). Accumulation of ppGpp is known to downregulate growth-associated processes while upregulating survival processes (182). Similarly, several tRNAs were downregulated upon exposure to sublethal acid stress in *B. longum* BBMN68, and cells that had not been exposed to sublethal acid stress showed improved resistance to lethal acid stress (pH 3.5) when protein synthesis was inhibited by a chloramphenicol treatment (163). Consistently, in *B. longum* ATCC 15707, sublethal acid stress treatment in the exponential phase caused a significant reduction of the total number of cellular proteins (154). Reduced protein expression might decrease the overall burden imposed on the chaperone system, which is essential for the repair of misfolded proteins under stress conditions, as well as preserving free energy for the stress response in the cell.

Cell envelope adaptations. (i) Peptidoglycan. Enzymes involved in peptidoglycan biosynthesis were upregulated in *B. longum* BBMN68 upon sublethal acid stress treatments (pH 4.5) at the transcriptional level as well as at the translational level in conditioned cells (pH 4.5) upon lethal stress treatment (pH 3.5) (157, 163). These findings were supported with physiological data showing a 35% increase in peptidoglycan content in the cells after preconditioning (157). Moreover, this observation agrees with the finding that genes linked to peptidoglycan biosynthesis were upregulated at the transcriptional level in *B. longum* JDM301 upon acid stress, most notably in the acid-resistant derivative of the strain, *B. longum* JDM301AR (168). Additionally, the transcription of genes related to peptidoglycan synthesis was higher in an acid-resistant derivative of *B. breve* BB8 (pH 3.2) than in its parental strain (160). Since peptidoglycan and its synthesis machinery are located on the cell surface, it is directly exposed to environmental stressors. Therefore, the overexpression of enzymes responsible for peptidoglycan might attempt to compensate for damage of peptidoglycan caused by acid or could further lead to changes in the fine structure of peptidoglycan.

(ii) Cell membrane composition. Acid-resistant derivatives of *B. longum* JDY1017

TABLE 1 Overview of differentially expressed genes involved in protein quality control and DNA repair in *Bifidobacterium* strains upon acid stress[a]

Strain	Molecular player[b]	Condition	Method; technique	Reference
B. longum NCIMB 8809	Downregulation of DnaJ2, GroES and RecA, upregulation of PepP	Growth at pH 4.8	Proteomics; 2D gel electrophoresis	158
Acid-resistant (pH 4) derivative of *B. longum* NCIMB 8809	Upregulation of GroES	Growth at pH 4.8	Proteomics; 2D gel electrophoresis	158
B. longum ATCC 15707	Upregulation of GroES, GrpE, and Dps	Nonstressed, stationary growth phase cells (higher acid tolerance) vs exponential growth phase cells (lower acid tolerance)	Proteomics; 2D gel electrophoresis	154
B. longum BBMN68	Upregulation of DnaK, DnaJ1, and GroEL	After lethal stress treatment at pH 3.5	Proteomics; 2D gel electrophoresis	157
B. longum BBMN68, conditioned by sublethal heat stress at pH 4.5 with improved survival at pH 3.5	Downregulation of LexA, upregulation of GroEL and GroES, Hsp20, Zn-dependent protease (HtpX), *clpP* operon and DnaJ1, NER-Uvr and NER-Vsr system, RecO[c]	After sublethal stress at pH 4.5 (comparison to nonstressed cells)	Transcriptomics; RNA-seq	163
B. longum BBMN68, conditioned by sublethal heat stress at pH 4.5 with improved survival at pH 3.5	Downregulation of PepP, upregulation of GrpE and UspA	After sublethal stress at pH 4.5 (comparison to nonstressed cells)	Proteomics; 2D gel electrophoresis	157
B. longum BBMN68, conditioned by sublethal heat stress at pH 4.5 with improved survival at pH 3.5	Downregulation of GrpE and UspA, upregulation of DnaK, PepP	After lethal stress treatment at pH 3.5 (comparison to prestressed cells)	Proteomics; 2D gel electrophoresis	157

[a]2D, two-dimensional; RNA-seq, transcriptome sequencing; NER, nucleotide excision repair system.
[b]Expression changed by at least 1.5-fold.
[c]RecO is a DNA repair protein.

(pH 3.5) and *B. breve* BB8 (pH 3.2), evolved in medium containing Tween 80, showed a greater increase in acid tolerance than their parental strain when grown in medium with Tween 80 than when grown in the absence of Tween 80 (167). In the presence of Tween 80, both derivatives showed increased octadecenoic acid ($C_{18:1}$) and cyclopropane fatty acid (cyc $C_{19:0}$) content in their cell membrane, consistent with the detected higher expression of cyclopropane-fatty-acyl-phospholipid synthase at the transcriptional level under the given condition (167). The changes in the fatty acid profiles of both strains were accompanied by a higher mean fatty acid chain length and decreased membrane fluidity, which might decrease the cells' susceptibility to acidic damage (167). Overall, these findings suggested that the increased acid tolerance of the acid-resistant derivatives could be linked to their ability to use Tween 80 as an exogenous fatty acid source to modify their fatty acid profile (167). Also in *B. longum* BBMN68, sublethal stress treatments in a medium containing Tween 80 resulted in the upregulation of cyclopropane-fatty-acyl-phospholipid synthase expression at the transcriptional level, suggesting that changes in the fatty acid profile might be a result of metabolic conditioning to acid stress in bifidobacteria (163). Moreover, the acid-resistant derivative of *B. longum* JDM301 showed an increase of $C_{14:0}$ content in its membrane upon exposure to lethal acid stress (pH 3.5) in Tween 80-containing medium, whereas no significant change in its fatty acid profile was detected in its parental strain when exposed to acid stress (168). In addition, a gene encoding a long-chain fatty acid coenzyme A ligase (EC 6.2.1.3) was upregulated in the acid-resistant derivative at the transcriptional level, which was thought to contribute to the incorporation of exogenous fatty acids, provided by Tween 80, into the cell membrane (168). Overall, the ability to use exogenous fatty acids and modify the fatty acid profile of the cell membrane appears to be an important mechanism to cope with acid stress in bifidobacteria.

(iii) Exopolysaccharides. Genome sequencing of an acid-resistant (pH 2.5) mutant of *B. longum* BBMN68 revealed a mutation in the gene encoding galactosyl transferase CpsD, a key enzyme in EPS production (183). The mutated gene was expressed at a reduced level in the mutant strain, which showed lower EPS production and improved cell aggregation ability. The stress resistance of the mutant was decreased by addition of EPS produced by its parental strain, while the addition of the EPS produced by the mutant could not increase the acid stress resistance of the parental strain. These results suggested that the improved acid resistance of the derivative was due to lower EPS production that improved cell aggregation, which in turn might reduce the penetration of acids into the cells (183).

In contrast, genes encoding enzymes involved in the production of EPS were found to be upregulated at the transcriptional level in *B. longum* BBMN68 after sublethal acidic treatments (163), and the increased acid tolerance of an acid-resistant derivative of *B. breve* BB8 was found to be accompanied by enhanced EPS production (160). Moreover, acidic condition showed a more detrimental effect on EPS-negative mutants of *B. breve* UCC2003 than on the EPS-producing parental strain (24). Additionally, for 31 strains of the species *B. breve*, *B. bifidum*, *B. longum*, and *B. pseudocatenulatum*, a correlation between high EPS production and acid resistance along with bile salt resistance could be established (184). These results suggest a protective function of the EPS layers against the penetration of protons into the cell (24, 160, 163, 184). Thus, while reduced EPS production was suggested to improve acid resistance by promoting cell aggregation, increased EPS production was also found to be linked to enhanced acid tolerance in bifidobacteria.

BILE STRESS

Besides being exposed to technological stressors, bifidobacteria must cope with stressors present in the gastrointestinal tract of the host (Fig. 1). Bile, or bile salt, stress is the best studied of all gastrointestinal tract stressors in terms of its effect on the physiology and metabolism of bifidobacteria, most likely as bile tolerance represents a formal functional criterion for the selection of probiotics (185). Bile resistance mechanisms in

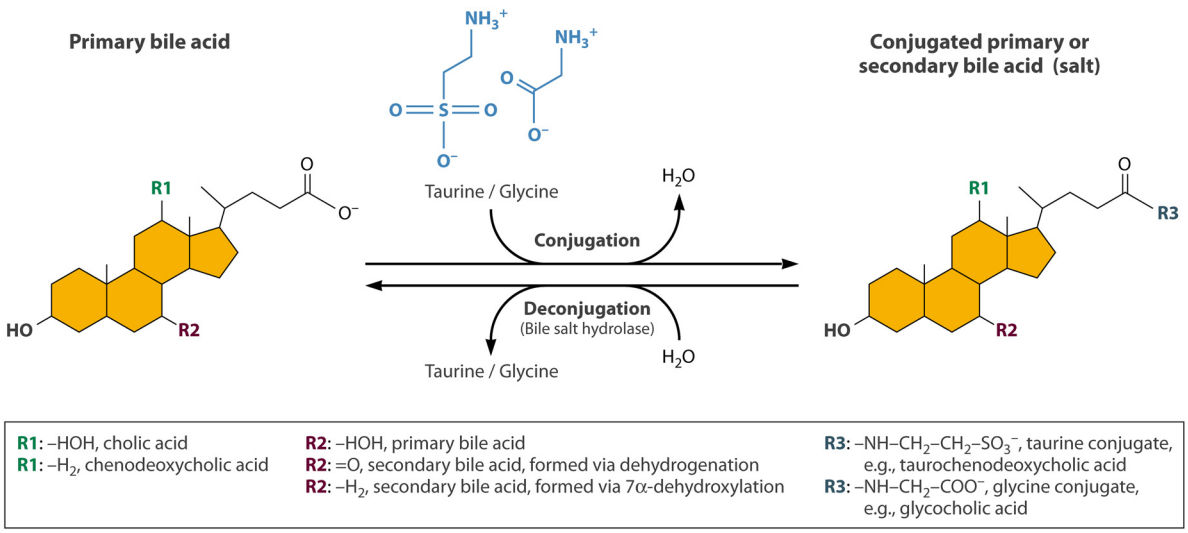

FIG 6 Overview of primary and secondary bile acids. Primary bile acids are synthesized in the liver from cholesterol and can be conjugated with a molecule of glycine or taurine before being secreted. In the intestine, the primary bile acids can be modified by the microbiota, for example by deconjugation, dehydrogenation, and 7α-dehydroxylation. Bile salts are bile acids associated with potassium or sodium ions.

bifidobacteria were reviewed in 2013 by Ruiz et al. (186). Below, we combine previously reviewed knowledge with newly gained insights on the bile stress response in bifidobacteria.

In humans, the liver continuously synthesizes bile, which is concentrated and is stored in the gallbladder (187). From the gallbladder, or directly from the liver, bile is released into the small intestine, where it functions as a biological detergent to emulsify and solubilize dietary lipids, such as fat-soluble vitamins and cholesterol (187, 188). One major component of bile is bile salts (187). The concentration of bile salts in the human intestine can range from 0.05% to 2% (186). In the liver, primary bile acids, which are C_{24} cyclopentanephenanthrene sterols (cholic acid or chenodeoxycholic acid), are synthesized from cholesterol (189). Prior to secretion from the liver, primary bile acids are conjugated with either of the amino acids taurine and glycine via an amide bond (Fig. 6) (189). The conjugation lowers the pK_a of the sterols from around 4.9 to 5.1 to around 4.3 to 4.5 for glycine-conjugated bile acids and to around 0.7 to 1.0 for taurine-conjugated bile acids (190). Therefore, conjugated primary bile acids have a higher solubility than their corresponding unconjugated bile acids (191). The term "bile salts" is often used interchangeably with "bile acids" (187). However, since conjugated bile acids are almost fully ionized at physiological pH (189), they should rather be termed "bile salts." While most bile that enters the small intestine is recycled, a small fraction ends up in the large intestine, where the primary bile salts can be modified by gut bacteria (189). Biotransformation of bile salts includes deconjugation, 7α-dehydroxylation, and dehydrogenation (Fig. 6) (187). The deconjugation of bile salts regenerates primary bile acids, while the dehydroxylation and dehydrogenation results in the formation of so-called secondary bile acids (salts), mainly deoxycholic acid, lithocholic acid, and 7-oxolithocholic acid (187). Bile acids are highly amphipathic; i.e., they possess a hydrophilic and a hydrophobic site (187). Their amphipathic character is essential for their physiological function but also confers strong antimicrobial activity (187). Above a critical micellar concentration, bile salts form micelles (188). The antimicrobial activity of high concentrations of bile salts is attributed to solubilization of membrane lipids and dissociation of membrane proteins, resulting in immediate cell death due to a loss of membrane integrity (187). Also at low concentrations, bile salts can affect membrane characteristics such as permeability and fluidity, as well as physical-chemical properties of the cell surface (187). Besides their destructive effect on the

cell membrane, bile salts can damage macromolecules, including RNA, DNA, and proteins (187).

Phenotypic Effects of Bile Stress

The intrinsic bile tolerance of *Bifidobacterium* strains varies significantly between species and strains (152, 184, 192, 193), with *B. animalis* strains commonly showing the highest resistance (98, 194–196). The presence of bile salts results in a significant decline of growth of most bifidobacteria *in vitro*, with a positive correlation between the inhibitory effect and bile salt concentration (87, 184, 192, 193, 195–198).

The toxicity of bile salts for bifidobacteria varies among different types of bile salts. *Bifidobacterium* strains were suggested to be more sensitive to unconjugated than to conjugated bile salts, with *B. animalis* ATCC 25527 being an exception (195, 199). In addition, glycine conjugates were shown to be more detrimental than taurine conjugates (195, 199). Moreover, the toxicity of glycodeoxycholic acid was further found to increase with decreasing pH (195). The toxicity of a bile salt and the pH-dependent manner of inhibition were suggested to be determined by the acidity of the bile salt, which determines its protonation state at physiological pH ranges. Conjugated bile salts are more acidic than unconjugated bile salts and are completely dissociated over a wide pH range. In contrast, unconjugated bile acids are weak acids and can pass through the cell membrane in a protonated state through diffusion (200).

Hence, the mechanism of growth inhibition by weak bile acids is similar to the mechanism by which weak acids in general exhibit toxicity, as described in the section above. In addition, the stress mechanism of bile acids further includes their membrane-damaging effect. A concentration-dependent effect of the unconjugated cholic acid on *Bifidobacterium* strains has been proposed based on findings with *B. breve* JCM 1192^T (201). At concentrations up to 2 mM, cholic acid accumulates according to the transmembrane proton gradient of the energized cell. Inside the cell, the dissociation of the unconjugated acid decreases the pH of the cytoplasm. The dissociated bile acid can no longer passively cross the cell membrane and therefore accumulates until an equilibrium of the undissociated cholic acid inside and outside the cell is reached (200). At increased concentrations between 2 mM and 4 mM, cholic acid starts to disrupt the cell membrane integrity (201). The MIC of cholic acid, i.e., the concentration required to inhibit cell growth by 100%, is reached between 3 mM to 9 mM and is below its critical micellar concentration (201). At this elevated cholic acid concentration, the proton motive force is dissipated, and cellular components start to leak out, eventually causing the loss of cell viability (201). Consistent with this, the application of multiparametric flow cytometry revealed physiological heterogeneity within a population of *B. animalis* subsp. *lactis* DSM 10140 upon treatment with unconjugated bile salts, with subpopulations of viable cells, dead cells, and injured cells (202).

In addition to acidity, the hydrophobicity of bile acids influences their toxicity (203). Bile acids with fewer hydroxy groups (e.g., deoxycholic acid) were suggested to have a more detrimental effect on bifidobacteria due to their higher hydrophobicity, which accelerates their trans-bilayer movement compared to less hydrophobic bile acids (e.g., cholic acid) (201, 203).

Not only the properties of the bile acids but also external factors were found to affect the susceptibility of bifidobacteria to bile salts. Intracellular accumulation of short-chain fatty acids was shown to reduce accumulation of cholic acid in *B. breve* JCM 1192^T, probably by causing reduction of the transmembrane proton gradient (200). Interestingly, short-chain fatty acids themselves did not influence membrane integrity or the viability of the strain (201). In addition, the presence of soy protein was found to reduce bile inhibition of bifidobacteria, presumably due to their ability to bind and aggregate bile salts (204). Similar to what was reported for acid stress resistance in bifidobacteria, survival of bile salt-sensitive *Bifidobacterium* strains upon bile salt stress was found to be improved in the presence of a fermentable carbon source, in particular in the presence of polymeric carbohydrates (196, 199). Interestingly, the addition of carbohydrates had no significant effect on the survival of *B. animalis* ATCC

25527 in the presence of bile salts, which already has a relatively high bile salt tolerance (196). The increased survival in the presence of a carbon and energy source indicates that most *Bifidobacterium* strains possess an energy-dependent defense mechanism against bile salt toxicity (196).

Molecular Mechanisms of Bile Stress Response

Several studies have focused on deciphering the defense mechanism of bile resistance in bifidobacteria. It is important to note that most of these studies use experimental conditions that simplify and modify the dynamic gastric environment so that collected data might not represent the strain's *in vivo* behavior. Most studies have applied model bile solutions or bile from animals to investigate the effect of bile salts on growth and survival of bifidobacteria. The choice of a bile solution is crucial as different bile reagents have very different toxicity (205). In addition, many different experimental conditions are applied, including the use of different types of bile and media among studies (187). Moreover, the response of bifidobacteria to bile stress is very complex and comprises several cellular mechanisms. However, common detoxification mechanisms for different bile salts appear to exist, as bile salt-tolerant derivatives of multiple strains from four *Bifidobacterium* species were shown to display cross-resistance to other bile salts than those that they were selected for (206).

Carbon metabolism. The exposure as well as the adaptation to bile stress was found to induce changes in carbon metabolism and end product formation in *Bifidobacterium* strains. In general, bifidobacteria seem to aim for improved synthesis of ATP under bile stress, which most likely fuels energy-dependent protective mechanisms against bile salt toxicity. In *B. longum* NCIMB 8809 bile stress induced a global upregulation of enzymes of the bifid shunt at the translational level, allowing the accelerated conversion of glucose into lactate, as indicated by an increased glucose consumption rate and a reduced acetate/lactate ratio (207). In contrast, a study of the bile stress response in *B. animalis* subsp. *lactis* IPLA 4549 and its bile-resistant derivative indicated that in these strains the presence and adaptation to bile salt induces a diversion of the bifid shunt toward acetate, formate, and ethanol formation, which allows extra ATP generation compared to the formation of lactate (49, 208). In bile salt-resistant derivatives of *B. bifidum* CECT 4549 and *B. longum* subsp. *infantis* CECT 4551, a combination of more efficient usage of glucose, due to increased fructose-6-phosphoketolase activity, and an increased acetate/lactate ratio have been detected (198). Taken together, these findings indicate that when exposed to bile stress, bifidobacteria increase the rate of ATP formation by increasing the metabolic flux in the bifid shunt and/or by increasing the catabolic ATP yield.

Besides improving the efficiency or effectiveness of the central carbon metabolism in terms of energy production, bifidobacteria seem to change their preference of carbohydrate utilization in response to bile salt exposure. Various changes have been reported for individual strains, but a tendency toward preferred utilization of polymeric carbohydrates can be observed. In *B. animalis* subsp. *lactis* IPLA 4549, bile adaptation was accompanied by the constitutive upregulation of genes linked to the utilization of oligo- and polysaccharides at the translational level, including α-galactosidase (EC 3.2.1.22) and α-glucosidase (EC 3.2.1.20) and UDP-glucose 4-epimerase (EC 5.1.3.2) (208). Growth on glucose, but not on maltose, was impaired compared to the parental strain (49). Also in eight additional strains of the species *B. bifidum*, *B. breve*, and *B. longum*, acquisition of bile resistance was coupled with significant changes in their glycoside-hydrolyzing activities (206). Thus, bile salt may induce adaptation of the metabolism of bifidobacteria to efficient utilization of polymeric carbohydrates, which might provide a selective advantage in their natural habitat, where oligo- and polysaccharides are abundant. It was suggested that the utilization of oligo- and polysaccharides is further linked to a higher ATP yield than the utilization of monosaccharides and thus improves the supply of energy necessary for bile detoxification (49, 196, 199).

In *B. longum* BBMN68, bile salt stress was found to activate genes related to the utilization of xylose, the pentose monomer of xylan present in plant biomass, at the

transcriptional level (87). Other genes for oligo- and polysaccharide utilization were, however, downregulated at the transcriptional or translational level (87). The authors suggested that this might be due to less efficient utilization of these complex sugars by the strain compared to glucose (87). In *B. bifidum* CECT 4549, the acquisition of cholate resistance was linked to loss of the ability to ferment galactose, lactose, and fructose (209). However, whether there is a link between the changed sugar utilization profile of the mutant and its increased cholic acid resistance remains questionable.

The proton-translocating F_1F_o-ATPase. As reported for acid stress, enhanced proton-translocating F_1F_o-ATPase activity of bifidobacteria might contribute to bile stress tolerance (155). Upon exposure to bile stress, the production and the ATP hydrolysis activity of the H^+-translocating F_1F_o-ATPase were found to be induced in *B. animalis* subsp. *lactis* IPLA 4549 and its bile-resistant derivative (155). Both strains maintained the intracellular pH above 6.0 when exposed to pH 6.0 and pH 5.0. However, at pH 4.0, the intracellular pH of the mutant cells was higher than that of the parental strain, regardless of whether they had been exposed to bile. Overall, the derivative had a higher intracellular ATP content at pH 6.0 and pH 5.0 than the parental strain, independently of whether bile was present. ATP levels decreased in both strains upon exposure to bile stress, with a more pronounced decrease in the parental strain. These findings indicate that bile adaptation of *B. animalis* subsp. *lactis* IPLA 4549 resulted in an enhanced cellular production of ATP, fueling the proton-translocating F_1F_o-ATPase under acidic and bile stress. Under bile stress, increased F_1F_o-ATPase activity may allow the removal of protons from the cytoplasm that accumulate due to intracellular dissociation of unconjugated bile acids. Thereby, the enzyme may contribute to the maintenance of a physiologically beneficial intracellular pH (155).

Bile salt hydrolase. One enzyme which has been extensively studied in the context of bile salt resistance is the bile salt hydrolase (BSH; EC 3.5.1.24). This enzyme hydrolyzes the amide bonds of primary and secondary bile salts and thereby deconjugates them to unconjugated bile salts and the corresponding amino acid residues. Deconjugation appears to be the prevailing type of conversion of bile salts in bifidobacteria, while conversion of primary to secondary bile salts has been observed only occasionally (210, 211). BSH activity is generally widespread among *Bifidobacterium* strains; however, the level of activity as well as its substrate specificity was found to vary between species and strains (193, 195, 199, 210, 212, 213). BSH was found to be located intracellularly in *B. longum* BB536 (214). Whereas a single study suggested that BSH in bifidobacteria recognizes only secondary bile salt derivatives (199), other studies reported that bifidobacteria can also deconjugate primary bile salts (210, 212). Characterized bifidobacterial BSHs show a broad substrate range but often prefer glycine-conjugated bile salts (213, 215, 216).

The mechanism and importance of BSH in bile tolerance are still not fully understood (186), as experimental findings supporting a pivotal contribution of BSH activity to bile resistance in bifidobacteria stand in contrast with observations suggesting that it has no key role in the detoxification of bile salts.

To begin with, the importance of BSH in bile detoxification can be questioned, since unconjugated bile salts, which can freely pass through the cell membrane, show a higher toxicity than conjugated bile salts (186). Moreover, several *B. bifidum* and *B. longum* strains were found to grow in the presence of bile salts for which they showed no or only limited deconjugation activity, suggesting that BSH activity is not decisive for bile salt tolerance in bifidobacteria (193). Whereas one previous study suggested a positive correlation between the growth-inhibiting effect of bile salts and their deconjugation by BSH activity (195), another study showed higher sensitivity of four *B. animalis*, *B. bifidum*, and *B. longum* strains to the conjugated primary bile salts which could not be deconjugated by the studied strains than to the conjugated secondary bile salts which they were able to deconjugate (199).

Interestingly, three strains of *B. animalis*, *B. longum*, and *B. breve* with BSH activity were found to survive better in the presence of 1 mM glycodeoxycholic acid under

acidic conditions (pH 4.5 and pH 5.5) than a strain of *B. coryneforme* lacking BSH activity, suggesting that BSH activity is beneficial for survival in the presence of bile salt under acidic conditions (195). Under acidic conditions, glycine-conjugated bile salts are partially protonated and may enter the cells by passive diffusion. It was suggested that intracellular deconjugation by BSH will convert the bile salt into a weaker acid that might recapture and export the cotransported proton and thus contribute to pH homeostasis (217). However, this mechanism would result in the accumulation of toxic unconjugated bile salts in the extracellular space (217). It has been suggested that a possible detoxification mechanism of the deconjugated bile salts *in vivo* might be its coprecipitation with cholesterol, which would diminish their toxicity (218–220). Another indication that BSH plays a key role in bile salt tolerance in bifidobacteria is the increased BSH expression at the translational level and increased activity that were observed in bile-tolerant derivatives of *B. animalis*, *B. bifidum* (two strains), and *B. longum* (199, 208).

BSH activity was further suggested to provide a nutritional advantage for strains, since glycine or taurine released by deconjugation may be used as a nitrogen source (87, 187). This hypothesis is supported by the finding that the *bsh* gene in *B. longum* SBT2928 seems to be cotranscribed with a homolog of *glnE* gene that encodes a glutamine synthetase adenylyltransferase (EC 2.7.7.42), which regulates the assimilation of nitrogen by glutamine synthetase activity (87, 216). In contrast, BSH in *B. bifidum* appears to be transcribed monocistronically (221).

Different results were observed when the expression of BSH across *Bifidobacterium* strains and different conditions was studied. In *B. animalis* subsp. *lactis* BB-12, *B. longum* NCC2705, and *B. longum* BS49, the *bsh* gene was found to be expressed under nonstressed conditions at the transcriptional and/or translational level (109, 222, 223). Permanent expression of BSH was suggested to allow an immediate response to bile salts (222); however, it could also indicate that BSHs have an alternative function in the absence of bile salts, e.g., serving as a plasminogen-binding protein (224). In response to bile stress (0.75 g L^{-1} ox-bile), *B. longum* BBMN68 showed increased BSH expression at both the transcriptional and translational levels (87). In contrast, no significant change in the gene expression of the *bsh* gene was observed in *B. animalis* subsp. *lactis* BB-12 in the presence of 0.1% bovine bile (222). Moreover, in *B. longum* NCC2705, the expression of BSH was upregulated under *in vivo* conditions in a rabbit intestine compared to *in vitro* conditions, likely due to the presence of bile in the rabbit intestine (225).

BSH expression in *Bifidobacterium* strains was further reported to be affected by other stressors than bile; however, no consensus results have been obtained. In *B. longum* BBMN68, the expression of BSH was found to be upregulated at the transcriptional level (80), but downregulated at the translational level, upon oxidative stress (3% O_2) (101). In addition, sublethal acid stress (pH 4.5) was found to induce BSH expression in *B. longum* BBMN68 at the translational level (157). In *B. dentium* Bd1, acid stress (pH 4.0) caused an upregulation of BSH at the transcriptional level (151). These results indicate that in some strains, BSH activity might be stimulated by acidic conditions. However, adaptation to acidic conditions of *B. longum* NCIMB 8809 was accompanied by constitutive lower expression of BSH and lower BSH activity (158). Whereas exposure to acid stress had no effect on the BSH expression and activity of the acid-resistant derivative, a decrease of both was detected in its parental strain under acidic conditions (158).

In summary, the role and importance of BSH in bile tolerance specifically, and in the stress response in general, remain elusive.

Bile efflux. The bile detoxification mechanism in bifidobacteria includes active efflux of bile acids (226–229). Several export systems have been identified in bifidobacteria that may or were proven to contribute to the detoxification of bile, comprising the multidrug resistance (MDR) transporters BetA, BbmAB, BmrAB, and Ctr (BbmR). In *B. breve* UCC2003, the induction profile of individual transport systems at the transcriptional level varied upon exposure to subinhibitory concentrations of different bile and bile components,

suggesting a complementary role of the different transport systems due to different substrate specificities or regulation mechanisms (228).

(i) BetA. Upon exposure to cholate or oxgall, the bile efflux transporter A (BetA) gene showed the highest induction level among the studied MDR-encoding genes in *B. breve* UCC2003 (227, 228). In agreement with this, strong upregulation of the transcription of the *betA* gene was observed in *B. longum* NCC2705 and *B. longum* BBMN68 upon bile stress (87, 227). In *B. breve* UCC2003, the *betA* gene is transcribed monocistronically and is mainly induced by cholic acid and glycocholic acid (228, 229). The genes encoding BetA in *B. breve* UCC2003 and *B. longum* NCC2705 share high protein sequence identity with putative efflux transporters from other strains of the genus (227). Inactivation of *betA* in *B. breve* UCC2003 resulted in increased sensitivity to cholate (229), and heterologous expression of the *betA* genes of *B. breve* UCC2003 and of *B. longum* NCC2705 in *L. lactis* and *E. coli*, respectively, promoted bile resistance of the hosts (227, 228). These observations provide further evidence of the importance of BetA in bile efflux and bile tolerance.

(ii) BbmAB. Additional transporter genes which were slightly upregulated upon bile stress in *B. breve* UCC2003 encode the heterodimeric ABC-type MDR transporter BbmAB (227), previously suggested to contribute to antimicrobial resistance (230).

(iii) BmrAB. In *B. longum* BBMN68, another bile-induced MDR transporter system was described recently (231). The transport system was found to be encoded within a polycistronic operon that consists of three genes, encoding the heterodimeric ABC transporter BmrAB and the MarR-family transcriptional regulator BmrR (231). *In silico* structural analysis and DNA-binding analysis of BmrR indicated that the expression of the *bmrRAB* operon is autoregulated by BmrR, which functions as a repressor. Under nonstress conditions, BmrR is assumed to bind to an inverted repeat sequence (ATTGTTG-N_6-CAACAAT) in the promoter region of the operon, while being released from the promoter when some component of the bile interacts with BmrR and causes conformational changes in the DNA binding site (231). Heterologous overexpression of the *bmrA* gene and *bmrB* gene of *B. longum* BBMN68 in *L. lactis* significantly increased the host's resistance to ox bile (231). Similarly, in *B. breve* UCC2003, the expression of three consecutive genes encoding a MarR regulator and the heterodimeric ABC transporter were also induced upon bile stress, mainly in the form of oxgall (228). We found that BmrAB and BmrR of *B. longum* BBMN68 share low sequence identity with the upregulated proteins in *B. breve* UCC2003 (BmrA, 32% identity and 94% coverage; BmrB, 36% identity and 83% coverage; and BmrR, 45% identity and 94% coverage). Surprisingly, the genome of *B. breve* UCC2003 contains another set of consecutive genes encoding a MarR regulator and an ABC transporter with 92 to 97% protein sequence similarity to the genes in the *bmrRAB* operon of *B. longum* BBMN68, but this set was not reported to be upregulated upon bile stress in the previous study (228).

(iv) BbmR/Ctr. Yet another MDR transporter potentially involved in bile export was characterized in *B. longum* NCIMB 702259^T (226). The heterologous expression of the MDR transporter gene *ctr* from *B. longum* NCIMB 702259^T conferred 16-fold-increased cholate resistance, promoted active efflux of cholate, and increased the resistance to other antimicrobial agents by 2- to 4-fold in *E. coli* (226). The studied Ctr transporter is an ortholog of the Ctr transporter of *B. longum* NCC2705 (226). We detected 95% protein sequence identity of the Ctr transporter of *B. longum* NCC2705 with the MDR transporter BbmR identified in *B. breve* UCC2003 (232), suggesting that these are homologous proteins. The expression of the *ctr*/*bbmr* gene was only slightly upregulated in *B. breve* UCC2003 and not at all induced in *B. longum* NCC2705 in the presence of subinhibitory oxgall bile concentration (227). In addition, the *bbmr* gene from *B. breve* UCC2003 contributed to macrolide resistance, but not to bile salt or bile extract resistance, when expressed in *L. lactis* (232). The reason for the varying specificities of the Ctr/BbmR transporter in *E. coli* and *L. lactis* could be linked to the slight sequence variation of the cloned genes. Overall, the contribution of Ctr/BbmR to bile resistance in bifidobacteria remains questionable.

Additional genes encoding transporters were upregulated in *B. longum* BBMN68 and *B. breve* UCC2003 at the transcriptional level upon bile stress (87, 228). The role of these transporters in bile export needs further investigation. In *B. animalis* subsp. *lactis* BB-12, which shows high bile tolerance (6), none of the studied putative MDR genes showed induction due to the presence of bile (227), which indicates that this strain harbors an additional mechanism to cope with bile stress.

Additional (metabolic) responses to bile stress. **(i) Oxalate/formate metabolism.** In the presence of bile salt, *B. animalis* subsp. *lactis* IPLA 4549 and its derivative showed an increased production of formyl-CoA transferase, involved in the degradation of the strong organic acid oxalate, as discussed above regarding acid stress (208). Accordingly, oxalate consumption was significantly promoted in the mutant strain in the presence of bile (208).

(ii) SenX3-RegX3 system. In *B. longum* BBMN68 bile stress induced the transcription of the two-component system *senX3-regX3*, which was found to bind upstream of the phosphate-binding protein PstS of the high-affinity phosphate transporter Pst (87). The gene cluster *pstSCAB* encoding Pst lies downstream of *senX3-regX3* in bifidobacteria (87). This result suggests that the upregulation of *senX3-regX3* accelerates the acquisition of inorganic phosphate for increased ATP synthesis under bile stress (87).

(iii) Nitrogen metabolism. Like acid stress, bile stress was reported to affect the expression of proteins involved in amino acid metabolism in *Bifidobacterium* strains. In *B. longum* NCIMB 8809 an increased expression of BCAT was reported at the translational level upon bile salt stress (207). In *B. longum* BBMN68, enzymes involved in the aromatic amino acid biosynthesis were upregulated at the transcriptional and partially also at the translational level, whereas the expression of ketol-acid reductoisomerase was downregulated at both levels (87). These results suggest there are deviating regulatory mechanisms of the amino acid biosynthesis in *Bifidobacterium* upon bile exposure (87). However, elevated synthesis of both aromatic and BCAAs were suggested to contribute to the synthesis of proteins with high hydrophobicity required in the presence of bile (87, 207).

(iv) Oxidative stress response. Accumulation of bile salt was suggested to induce the production of ROS in the gastrointestinal tract (187, 233). Indeed, bile stress was found to induce the expression of proteins assigned to the oxidative stress response at the transcriptional or translational level, including thioredoxin-dependent thiol peroxidase in *B. animalis* subsp. *lactis* IPLA 4549 (208), Dps in *B. breve* UCC2003 (228), and nitroreductase (homolog of NPOX) in *B. longum* BBMN68 (87).

Maintenance of DNA and protein synthesis and integrity. As described for other stressors, bile stress was found to induce the expression of genes linked to the PQC system, with various extents of induction among different *Bifidobacterium* strains (Table 2). Overall, genes of the *dnaK* operon, the *groEL* gene, and the *groES* gene appear to be commonly induced upon bile stress in bifidobacteria.

In *B. longum* BBMN68, exposure to sublethal bile salt stress resulted in differential expression of several transcription regulatory genes, including a significant upregulation of the alternative ECF sigma factor RpoE at the transcriptional level (87). Bile salt stress was further found to promote the expression of genes linked to the translational machinery. In *B. longum* NCIMB 8809, bile stress resulted in the overexpression of tRNA synthetases or their subunits at the translational level (207). In addition, the strain showed increased accumulation of five ribosomal proteins, responsible for ribosome assembly and stability, upon bile exposure (234). In *B. longum* BBMN68, bile stress strongly induced the expression of a gene annotated as ribosome-associated protein Y at both the transcriptional and translational levels (87), which was also upregulated as part of the oxidative stress in the strain (101). The same gene was upregulated at the transcriptional level in *B. breve* UCC2003 when grown in the presence of bile (228). Moreover, when growing in the presence of 0.6 g L^{-1} ox bile, *B. longum* NCIMB 8809 showed an increase in subunits of dipeptide and oligopeptide transporters in the cell envelope proteome, suggesting an improved peptide uptake upon bile stress (234).

TABLE 2 Overview of upregulated genes involved in protein synthesis, protein quality control, and DNA repair in bifidobacteria upon bile stress

Strain	Upregulated molecular player(s)	Condition	Method; technique	Reference
B. adolescentis NCC251	DnaK	0.1% bile	Dot-blot hybridization	65
B. animalis subsp. *lactis* BB-12	*dnaK* operon, GroEL, GroES, HrcA and Clp	0.1% bile (bovine bile)	Transcriptomics; microarray	222
B. animalis subsp. *lactis* IPLA 4549	GrpE, GroES, GroEL, ClpB, DnaK, HtrA and Dps	3 g L^{-1} oxgall	Proteomics; 2D gel electrophoresis	208
Oxgall-resistant derivative of *B. animalis* subsp. *lactis* IPLA 4549	GrpE, GroES and GroEL	10 g L^{-1} oxgall	Proteomics; 2D gel electrophoresis	208
B. breve UCC2003	GroES, ClgR (Bbr_1183) and Aminopeptidase C (PepC1[a]), UspA, Ribosome-associated protein Y and Dps	0.06% cholic acid and 0.15% oxgall	Transcriptomic; microarray	228
B. longum 3A	DnaK, HtrA and GroEL	0.1% bile salts	Proteomics; 2D gel electrophoresis	37
B. longum BBMN68	GroES, GroEL,[b] Hsp20,[b] UspA,[c] several proteases/peptidases[b] (DegQ,[d] PepO,[e] PepDA1[f]), DNA helicase,[b] and ribosome-associated protein Y	0.75 g L^{-1} ox bile	Transcriptomics, RNA-seq and/or proteomics, 2D gel electrophoresis	87
B. longum NCIMB 8809	DnaK and GroEL; more pronounced at 0.6 g L^{-1}	0.6 g L^{-1} and 1.2 g L^{-1} ox bile extract LS55	Proteomics; 2D gel electrophoresis	207

[a]Aminopeptidase C.
[b]Upregulated only at the transcriptional level.
[c]Upregulated only at the translational level.
[d]Trypsin-like serine protease.
[e]Endopeptidase.
[f]Dipeptidase.

Physiological data on the uptake of dipeptides and tripeptides showed that the uptake rate was accelerated for one of five peptides. Taken together, these results indicate that in the presence of bile, the uptake of specific peptides is induced, which might fuel protein synthesis and repair (234).

On the other hand, bile stress in bifidobacteria appears to inhibit pyrimidine synthesis. In *B. longum* BBMN68, the transcription of seven genes encoding proteins in pyrimidine biosynthesis was downregulated in the presence of bile, while the levels of two of the corresponding proteins also decreased (87). Moreover, the expression of CTP synthase (EC 6.3.4.2), which catalyzes the formation of CTP, was induced in *B. animalis* subsp. *lactis* IPLA 4549, *B. longum* NCIMB 8809, and *B. longum* BBMN68 at the translational level upon exposure to bile (87, 207, 208). This indicates a dedicated decrease in pyrimidine biosynthesis, since CTP is known to inhibit L-aspartate carbamoyltransferase (EC 2.1.3.2), which catalyzes the first reaction in pyrimidine biosynthesis (235).

Morphological and cell envelope adaptations. Several studies have investigated the effect of bile salt on the cell envelope of bifidobacteria, which is the first point of physical contact and one of the main targets of bile salt toxicity. Optical microscopy provided visual evidence that bile distorts the cell surface of *B. animalis* subsp. *lactis* IPLA 4549 strains and induces the formation of vesicle-like structures, which might be membrane vesicles that allow the removal of toxic misfolded protein (23, 236). Bile-resistant mutants of *B. bifidum* CECT 4549 appeared smaller and more regular in size than their parental strains (209).

(i) Cell surface properties. It has been observed that exposure and adaptation to bile can modulate the surface potential, the hydrophobicity, and the adhesion and autoaggregation behavior of *Bifidobacterium* strains. Effects of bile on the cell surface properties were found to be more pronounced on growing than on nongrowing cells (237). Overall, the modifications of cell surface properties appear complex and difficult to interpret, as they vary depending on the applied bile solution (bile component and whole bile) and on the strain, possibly due to differences in their intrinsic surface properties (237–240). For example, most studied strains of *B. bifidum* and *B. pseudolongum* showed a reduced adhesion to human colon adenocarcinoma cells (Caco-2) as well as decreased autoaggregation and surface hydrophobicity when grown in the presence of ox bile (237). In contrast, increased adhesion to human colon adenocarcinoma cells and cell surface hydrophobicity, still coupled with decreased autoaggregation, was detected for *B. longum* BBMN68 when grown under ox bile stress (87). In addition, autoaggregation of cells grown from immobilized *B. longum* NCC2705 cells during continuous cultivation was suggested to be the reason for an improved bile tolerance of the produced cells (241). Changes in the cell surface properties induced by bile might not solely be a bile resistance mechanism but rather facilitate the colonization of the human gut, e.g., by the differential expression of moonlighting proteins (242), such as DnaK, that can have an adhesive function when displayed on the cell surface (224, 234, 243, 244).

(ii) Cell membrane composition. Bile stress induces changes in the expression of genes linked to fatty acid biosynthesis in *Bifidobacterium* strains, suggesting an effect of bile on the cell membrane composition. Upon exposure to bile salts, several genes responsible for fatty acid biosynthesis were downregulated in *B. animalis* subsp. *lactis* BB-12 (222) and *B. longum* BBMN68 at the transcriptional level (87) and in *B. animalis* subsp. *lactis* IPLA 4549 along with its bile-tolerant mutant at the translational level (208). At the same time, individual steps of the fatty acid biosynthesis have been reported to be upregulated in *Bifidobacterium* strains upon bile salt stress. In *B. longum* BBMN68, the presence of bile was found to result in the upregulation of cyclopropane-fatty-acyl-phospholipid synthase at the transcription level, but no upregulation was detected at the translational level (87). In contrast, a decrease of $C_{19:0}$ cyclopropane fatty acid was detected in the membrane of *B. animalis* subsp. *lactis* IPLA 4549 and its bile-resistant derivative upon bile stress exposure, and an increase of BCFA was detected (236). These results indicate that bile stress induces strain-specific adaptations

of the cell membrane composition in *Bifidobacterium* strains, most likely to reduce the membrane susceptibility and permeability for bile salts. Accordingly, increased membrane viscosity (decreased lateral diffusion of fatty acids) was observed in *B. animalis* subsp. *lactis* IPLA 4549 upon adaptation and exposure to bile salt (236).

(iii) Exopolysaccharides. In addition to the modifications of the cell surface properties and cell membrane composition, the formation of a protective coat of EPS was identified to be a part of the defense mechanism of *Bifidobacterium* strains against bile stress (23, 24, 184). In *B. animalis* subsp. *lactis* IPLA 4549 and its bile-tolerant derivative, the exposure to bile induced the expression of a putative glycosyltransferase at the transcriptional level and enhanced the production of EPS, with a more pronounced effect at higher bile concentrations (23). In corroboration, a positive correlation between high levels of production of cell surface-associated EPS and bile salt resistance was detected for *B. breve* UCC2003 and 31 additional strains of the species *B. breve*, *B. bifidum*, *B. longum*, and *B. pseudocatenulatum*, as mentioned above (24, 184). In line with this, in *B. breve* UCC2003, the surface-associated EPS was found to improve *in vivo* persistence in the mouse gut (24). As mentioned above, EPS might serve as a protective coat for bacteria when they are exposed to acid stress and bile stress in their natural environment (24, 184).

(iv) Biofilm formation. Linked to EPS production, biofilm formation was strongly induced by high concentrations of porcine bile in strains of the species *B. adolescentis*, *B. breve*, *B. dentium*, *B. longum*, and *B. pseudolongum* (245). Multiple mutants of *B. breve* UCC2003 that formed less biofilm demonstrated reduced survival upon bile stress (245). However, in general, cells embedded in biofilms showed good survival after exposure to 0.5% porcine bile for 24 h (245). Based on these results, biofilm formation was suggested to have a protective function in the presence of bile (245).

OSMOTIC STRESS

When used as probiotics, bifidobacteria can be exposed to osmotic stress during fermentation, downstream processing, and storage, as well as upon administration (Fig. 1). During fermentation, pH control can result in elevated sodium, potassium, or ammonium concentrations. In addition, organic acids produced by the cells can contribute to osmotic stress in bifidobacteria at neutral pH (150). When preserved via drying, industrial *Bifidobacterium* strains are subjected to osmotic stress due to the decrease of water activity (28). In addition, their application in food products exposes the cells to osmotic stress, and upon administration, the cells have to cope with changing osmolality both along the gastrointestinal tract and due to fluctuations in the diet (26).

Growth of *Bifidobacterium* strains becomes inhibited at an osmolality around 850 to 1,300 mosM kg^{-1} in complex medium at neutral pH (150). The MICs of sodium chloride (NaCl), sodium acetate, sodium lactate, and mixed salts for *Bifidobacterium* strains were found to be between 0.3 M and 0.5 M at neutral pH (150).

One of the functions of the cell wall in Gram-positive bacteria is to withstand the turgor pressure of the cells. Upon hyperosmotic stress, the positive turgor pressure decreases due to dehydration. The main function of the osmotic stress response is therefore to maintain the turgor pressure within a range that allows maintenance of cell viability and active metabolism by either accumulation or efflux of compatible solutes. Whereas the accumulation of osmoprotectant compounds, such as glycogen, trehalose, and glycine betaine, has been detected and studied in other probiotic genera in response to high osmolality (28), only one system for the efflux of solutes has been reported in bifidobacteria to date. In *B. longum* BBMN68, a small conductance mechanosensitive channel (MscS) was upregulated at the transcriptional level in the presence of bile, which might facilitate solute efflux to protect cells from increased turgor pressure that might be caused by membrane damage due to bile salts (87). However, the specificity of the transporter remains to be investigated.

Osmotic stress also affects the expression of molecular players of the PQC and DNA repair systems. Current knowledge of the role of chaperones in the osmotic stress

response in bifidobacteria was mainly collected by transcriptomic studies on *B. breve* UCC2003, which showed diminished growth under osmotic stress (51). Osmotic stress treatments (0.5 M NaCl to 0.7 M NaCl) of the strain were shown to result in induction of the *dnaK* operon (66), the *clpB* gene (60) (HspR regulon) and the *hsp20* gene (74), while no induction of the *clpP* operon (64), the *clpC* gene (61) (ClgR regulon), and the *groES* and *groEL* genes (HrcA regulon) was detected (51) (Fig. 3). In addition, the expression of the bicistronic operon comprising the *hrcA* gene and the *dnaJ2* genes was found to be upregulated in *B. breve* UCC2003 upon osmotic stress (51, 83). From the DNA repair system, the *lexA* gene and *impB* gene were induced (51). With the exception of the upregulation of *dnaJ2* expression, which was exclusively detected upon osmotic stress, the described expression pattern revealed a strong overlap between the osmotic stress response and the severe heat shock response in *B. breve* UCC2003 (83). The different expression patterns of the genes encoding DnaJ1 and DnaJ2 upon heat stress and osmotic stress in *B. breve* UCC2003 suggest deviating physiological functions of the two proteins in that strain (83). The induction profile of heat shock proteins upon osmotic stress in *B. breve* was found to vary depending on the level of osmotic stress. For example, DnaK was significantly induced only upon exposure to NaCl concentrations between 0.5 M and 0.7 M, not at lower or higher NaCl concentrations (66). Likewise, expression of DnaK was only slightly induced in *B. adolescentis* NCC251 at the transcriptional level upon mild osmotic stress (0.3 M NaCl) and not at all in *B. longum* NCC481 (65). The effect of elevated osmotic stress treatments was not tested with these two strains.

Despite the similar induction patterns of genes involved in protein control and DNA repair upon heat and osmotic stress in *B. breve* UCC2003, sublethal heat stress treatment of *B. longum* NCC2705, which provided increased protection against subsequent lethal heat stress, did not offer cross-protection to lethal osmotic stress (246). This indicates that the osmotic stress response in *B. longum* NCC2705 does not fully overlap its heat stress response (246), maybe because single genes (such as *dnaJ2*) that are crucial for a sufficient osmotic stress response or for some other physiological adaptations are not induced by heat stress.

ORGANIC SOLVENT STRESS

During fermentation, bifidobacteria can produce small amounts of ethanol (Fig. S1). In addition, probiotics can be exposed to ethanol upon administration due to alcohol consumption by the host. Many organic solvents can be toxic to bacteria due to their ability to accumulate inside the cell membrane, causing severe disruption of membrane function and integrity. Different mechanisms against organic solvent toxicity in Gram-positive bacteria have been described, for example, the induction of general stress regulons, deactivation and excretion of organic solvents, and changes in cell morphology, cell surface, and cell membrane composition (247).

Like other stresses, solvent stress (8% ethanol) was found to reduce the growth rate and induce genes of the PQC system in *B. breve* UCC2003 (51). In a whole-genome microarray analysis of the strain's response to treatment with 8% ethanol, members of the ClgR, HspR, and HrcA regulons were found to be upregulated (84), while *groEL* became the most transcribed gene under this condition, which was also observed for mild to severe heat stress treatments (51, 84). In addition, genes involved in DNA repair were found to be induced by organic solvent stress, including RecA, RecX, LexA, and MutY (51). Contrary to the other stress treatments, solvent stress caused a significant upregulation of the ClgR-regulated genes, including *clpP1*, *clpP2*, and *clpC*, but did not result in the upregulation of the small heat shock protein Hsp20 in *B. breve* UCC2003 (51).

In *B. breve* NCFB 2258, a FAD-dependent fatty acid hydratase (oleate hydratase; EC 4.2.1.53) was suggested to contribute to organic solvent stress tolerance (16% ethanol) based on the observation that an insertion mutation in the corresponding gene resulted in increased sensitivity to organic solvent (248). Heterologous expression of the oleate hydrase gene in *L. lactis* NZ9800 and *Corynebacterium glutamicum* ATCC

13032 increased the resistance of both strains to 3% butanol (249). The oleate hydratase in *B. breve* NCFB 2258 is responsible for the hydration of oleic acid ($C_{18:1}$) to 10-hydroxyoctadecanoic acid (248). How the FAD-dependent fatty acid hydratase contributes to solvent tolerance has not been revealed; however, its effect on the membrane fatty acid composition might be crucial for solvent tolerance. Unsaturated fatty acids, such as oleic acid, are toxic to bacteria due to their ability to deteriorate their cellular membranes (250, 251), and the characterized oleate hydratase might contribute to oleic acid detoxification in bifidobacteria (248).

MECHANICAL STRESS

Throughout their production, probiotic bifidobacteria are exposed to mechanical stress, mainly in the form of shear forces that can result in a loss of viability by causing damage to the cell wall and membrane. During fermentation, mechanical stress originates from mixing and hydrostatic pressure changes, and during downstream processing, cells are exposed to mechanical stress from pumping, centrifugation, or membrane filtration (252). Wall shear stress in a membrane bioreactor was shown to affect the viability of *B. longum* severely (253). Moreover, bifidobacteria are exposed to mechanical stress during preservation and in food manufacturing processes (2, 254, 255). For example, ice crystals that are formed in the surrounding environment or inside the cells during freezing can damage the membrane of the cells (2).

The molecular response of bifidobacteria to mechanical stress has not been described. However, lower membrane fluidity, facilitated by a lower ratio of unsaturated to saturated fatty acids in the cell membrane of *B. longum* R0175, was suggested to be the reason for slightly higher freezing resistance of the strain in the stationary growth phase than in the exponential growth phase (256). Increased membrane rigidity may contribute to higher mechanical resistance during removal of bound water during freeze-drying (257). In contrast, no effect of the harvesting time point on freeze-drying resistance could be detected for *B. animalis* subsp. *lactis* BB-12, implying that the growth phase-related differences in membrane fluidity might have been small in this case (205). The results from the two strains might deviate because of higher intrinsic stress resistance of *B. animalis* subsp. *lactis* BB-12 compared to *B. longum* R0175 (256) or because the harvesting points of *B. animalis* subsp. *lactis* BB-12 were too close together (late exponential and early stationary growth phases) to see a significant difference.

NUTRIENT LIMITATION

In the intestines, nutrient availability is constantly changing. While in the small intestine mono- and disaccharides, such as glucose and fructose, are available in abundant quantities, mainly indigestible oligosaccharides and fibers remain as the prevailing carbohydrates in the large intestine (99). In addition, bifidobacteria must compete with other microorganisms in the intestine for the available nutrients. Bifidobacteria are equipped with a considerable number of carbohydrate-modifying enzymes, such as glycosyl hydrolases, and hence can ferment various complex carbohydrates in the gastrointestinal tracts of their host (1, 108). This ability probably allows them to adapt to variations in the carbohydrate supply and may also constitute a competitive advantage over other microorganisms in their natural environment (1, 75, 108). Moreover, their ability to compete with other bacteria for additional nutrients, such as vitamins, most likely determines their survival in their natural habitat.

So far, little is known about the specific stress response in bifidobacteria to carbohydrate starvation. Limited nutrient availability was found to influence the morphology of bifidobacteria. Carbohydrate-starved cells of *B. pseudolongum* subsp. *globosum* RU809/1 showed a reduced size, which might be a potential strategy to conserve energy (258). Moreover, carbohydrate starvation caused plasmid curing in *B. pseudolongum* subsp. *globosum* RU809/1 (with various results for different carbohydrates) (258). In *B. longum* NCC2705, starvation by entry into the stationary growth phase resulted in the upregulation

of multiple heat shock proteins and proteases, such as Hsp20 and DnaK at the transcriptional level (75).

Iron starvation was shown to diminish growth of most *Bifidobacterium* strains (259). Transcriptomic analysis of the stress response in *B. breve* UCC2003 to iron-limiting conditions revealed the induction of a potential high-affinity ferrous iron uptake system, BfeUOB, which shows high homology to the iron transport system EfeUOB in *E. coli* (259). In a later study, the *bfeuB* gene was assigned to the iron uptake system *sifABCDE*, encoded directly downstream of the *bfeUO* operon (260). Both iron transport systems were found to be crucial for growth of *B. breve* UCC2003 under iron starvation conditions but differ in their substrate specificity (260). While BfeUO was suggested to import Fe^{3+} and Fe^{2+}, the *sifABCDE* system appears to transport solely Fe^{2+} (260). The two iron transport systems are highly conserved across bifidobacteria (260).

Like iron limitation, phosphate limitation reduced growth of *B. breve* UCC2003 and induced changes in the global gene expression profile of the strain (261). Under phosphate-limiting conditions, heat shock proteins, including DnaK, ClpB, and GroEL, as well as a two-component regulatory system, PhoRP, were found to be upregulated in *B. breve* UCC2003 (261). We found that the genes encoding the PhoRP system in *B. breve* UCC2003 are homologs of *senX3* (82% identity, 99% coverage) and *regX3* (98% identity, 92% coverage) in *B. longum* BBMN68, which have been described to be upregulated upon bile stress (87). In line with the findings in *B. longum* BBMN68, PhoRP was suggested to activate the expression of the Pst phosphate transport system (PstSCAB, located directly downstream of *phoRP*) in *B. breve* UCC2003 during phosphate limitation (261). Moreover, PhoRP appeared to be autoregulated and regulated the expression of a gene encoding a putative phosphate-responsive regulatory protein (*phoU*) in *B. breve* UCC2003 (261). The critical role of the studied PhoRP system was evidenced by the finding that a *phoP* insertion mutant of *B. breve* UCC2003 showed increased sensitivity to phosphate-limiting conditions compared to its parental strain (261).

STRESS CAUSED BY DIGESTIVE ENZYMES

In the oral cavity and gastrointestinal tract, bifidobacteria are exposed to various digestive enzymes, such as lysozyme and proteases. The effect of digestive enzymes on *Bifidobacterium* strains has been investigated in only a few studies. The adherence of *B. animalis* subsp. *lactis* BB-12 to mucus appeared not to be affected when this strain was treated with different digestive enzymes, such as trypsin (262). A study on gastrointestinal transit tolerance indicated that the resistance of some *Bifidobacterium* strains to human gastric conditions increases in the presence of milk proteins and mucin (194). It was suggested that the protective effect of milk proteins and mucin on the survival of the strains not only is based on their buffering capacity but also might be due to an inhibitory effect on proteases which protects the cells against this enzymatic activity (194). Other studies indicated that the digestive endopeptidase pepsin might further protect *B. animalis* subsp. *lactis* BB-12 at low pH (205, 263, 264). The protective effect of pepsin during exposure to acidic conditions appears to be species dependent and was not seen in strains of other species with higher acid sensitivity than *B. animalis* subsp. *lactis* (264). The evidential value of these studies is clearly weakened by the fact that the applied pepsin solution may contain impurities, like gastric mucin, that actually cause the observed effect (205, 264).

GENERAL STRESS RESPONSE

Multiple molecular mechanisms have been described to be involved in the stress response in bifidobacteria. When the mechanisms of bifidobacterial responses to various stressors are compared, it becomes apparent that some elements are shared across the defense mechanisms against different stressors, such as the participation of chaperones and proteases to maintain and control protein quality. In particular, the small heat shock protein Hsp20 appears to play a crucial role in the response to multiple stressors (74–76). In addition, the WhiB-like protein WblE was suggested to be part of

the general stress response in bifidobacteria (265). WhiB-like family proteins are putative transcriptional regulators of essential cellular processes, such as the stress response, in *Actinobacteria* (266). Two types of *whiB*-like genes were discovered during genomic analysis of 36 *Bifidobacterium* strains of 11 species: WhiB2 and WblE (265). While all studied strains possessed *wblE* orthologs, *whiB2* orthologs were detected in all strains except *B. animalis* subsp. *lactis* and *B. gallicum* strains. Moreover, some strains contained additional *whiB*-like genes of various lengths and low sequence similarity (265). The expression of the *wblE* gene in *B. longum* subsp. *longum* B 379M was induced as part of the response to various stressors, including heat, osmotic, oxidative, antibiotic, and bile salt stresses as well as nutrient starvation (265), whereas the expression of the *whiB2* gene was not induced (265). However, in *B. breve* UCC2003, the expression of the *whiB2* gene was upregulated upon osmotic stress, the *wblE* gene was induced upon osmotic and severe heat stress, and another *whiB*-like gene was induced upon severe heat stress at the transcriptional level (see the supplemental material in reference 51).

Even though some mechanisms appear to be involved in the response to multiple stressors, as described in this review, bifidobacteria seem to be equipped with many different specific responses to individual stressors, which further appear to vary between *Bifidobacterium* species and strains. In Fig. 7, we provide an overview of the best-studied stress response strategies of bifidobacteria, and all stress responses described in this review are summarized in Table S1 in the supplemental material.

Recently, we analyzed the prevalence of 76 stress-associated genes in the genomes of 171 *Bifidobacterium* strains and compared the results to their stress physiology to assess the extent to which the distribution of these genes across the genus can explain the phenotypic diversity of *Bifidobacterium* strains (20). As expected, the analysis revealed clear differences in the presence/absence pattern of stress-associated genes among previously suggested phylogenetic groups of bifidobacteria (267, 268) and among species. For example, the presence of genes encoding putative transcriptional regulators of stress responses, including multiple *whiB*-like genes and alternative sigma factors, were shown to vary significantly across the studied species and strains (20). Overall, the obtained stress response gene profiles could only partly explain the different stress tolerance of *Bifidobacterium* strains. Therefore, differences in the regulation of gene expression and protein activity must also contribute to the highly diverse stress physiology across the genus (20).

APPROACHES FOR IMPROVING THE STABILITY OF BIFIDOBACTERIA

Poor stability might hamper the commercialization of *Bifidobacterium* strains with very promising health-beneficial characteristics. Understanding the molecular mechanisms underlying the stress response in bifidobacteria as well as identifying biomarkers for stability and/or robustness could enable the rational screening for novel, stable probiotic *Bifidobacterium* strains. Moreover, it is also of interest to establish approaches that can improve survival or enhance the stability and robustness of a specific strain.

The choice of process parameters is one important component in delivering viable strains. For example, during fermentation, a standard practice to minimize oxidative stress is to remove O_2 from the broth either by sparging the medium with nitrogen prior to inoculation or by flushing the headspace of the reactors throughout the fermentation. In addition, oxidative stress can be reduced by addition of a reducing agent, e.g., cysteine, to the medium (75, 97). During storage, the initial O_2 content within the package and the O_2 transmissibility of the packaging material should be as low as possible to minimize the loss of viability of the stored cells due to exposure to oxidative stress (2). Interestingly, exposure to light was reported to enhance the sensitivity of the O_2-sensitive strains *B. longum* subsp. *longum* 46 (DSM 14583) and *B. longum* subsp. *longum* ATCC 15707, suggesting that protection from light should be an additional factor to consider when producing bifidobacteria (269). The stress-promoting effect of light might be due to ROS formation induced by illumination of the cell culture (270).

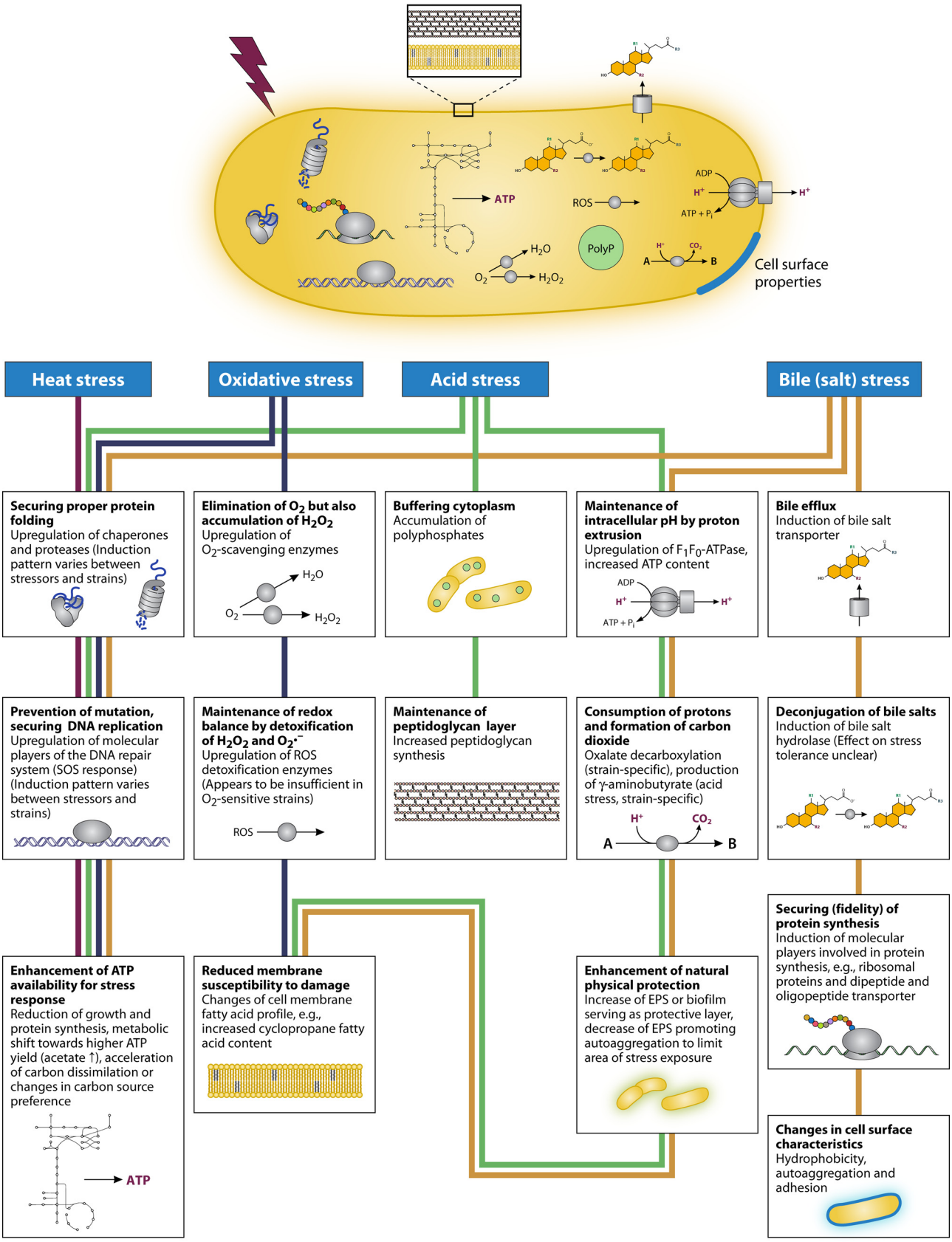

FIG 7 Overview of well-studied strategies in the stress response of bifidobacteria. Strategies shown are supported by physiological data or by genetic engineering approaches and have been observed in two or more *Bifidobacterium* strains (except for "prevention of mutation, securing DNA replication," which was evidenced on the transcriptional and translational level only, albeit in several strains upon exposure to many stressors). All strategies described in this review are summarized in Table S1, including those supported by lower degrees of evidence.

Besides the optimization of process parameters in the production of probiotics, different strategies can be applied to improve the survival of probiotic bifidobacteria throughout their life span. The application of cryoprotectants and microencapsulation aims for the physical protection of the cells from environmental stressors. Adaptive laboratory evolution (ALE) and genetic engineering approaches, on the other hand, target the genetic makeup of a strain to improve its stability, whereas sublethal stress treatments can result in metabolic conditioning of the cells prior to exposure to lethal stress.

Physical Protection of Probiotic Bifidobacteria

Cryoprotectant. Prior to freezing, probiotic cultures are commonly mixed with a cryoprotectant to reduce freezing damage of the cell. Cryoprotectants include penetrating and nonpenetrating compounds (271). Cryoprotectants can minimize the mechanical and hyperosmotic stress of the cells by inhibiting ice crystal formation and/or preventing dehydration due to formation of strong hydrogen bonds with intracellular water (272). Generally, penetrating cryoprotectants that are water soluble and nonreactive, have low toxicity, and do not precipitate at high concentrations are ideal (271). Both single cryoprotectants and mixtures are applied for preservation of microorganisms (272). Glycerol is a commonly used cryoprotectant for the preparation of frozen stocks of bacteria in research (271), including bifidobacteria (49, 143); however, in industry, other types of cryoprotectants are employed.

A few studies on the evaluation and optimization of cryoprotectants for protection of bifidobacteria during freezing and freeze-drying have been published. The effectiveness of cryoprotection appears to be strain dependent (273). Cryoprotectants that were shown to be effective for *Bifidobacterium* strains include 5% (wt/wt) sucrose; 5% (wt/wt) reconstituted skim milk (205); a mixture of 5% dried skim milk and 4% trehalose (274); a mixture of 10 to 20% skim milk and 20% *Abelmoschus esculentus* (L.) Moench polysaccharides (275); a mixture of 5.5% glycine, 0.8% sodium bicarbonate, 7% xylo-oligosaccharides, 4.5% arginine, and 25% skim milk (276); and media containing many cryoprotective compounds, such as sugars and peptides (277).

Microencapsulation. During microencapsulation, cells are entrapped in a protective material, usually natural biopolymers, to provide physical protection against external stressors. Encapsulation was found to increase the survival of probiotic bifidobacteria to various stressors on a laboratory level (278), and companies started to explore microencapsulation for improved cell protection on an industrial scale (279). Recent developments of microencapsulation for physical protection of probiotics, including bifidobacteria, have been summarized and discussed in previous reviews (2, 278, 279). However, industrial application of microencapsulation still remains to be further optimized for high-quality encapsulation at low costs (278, 279).

Metabolic Engineering

Genetic engineering. To date, genetic engineering has been only sparingly exploited for the enhancement of stress tolerance of probiotic *Bifidobacterium* strains. This might be due to the limited availability of suitable molecular tools for functional studies of bifidobacteria until recently (280, 281) as well as the regulatory restrictions and social and ethical concerns regarding the commercialization of genetically modified organisms (GMOs) in many countries. Nevertheless, the few studies that have applied genetic engineering to improve stress tolerance of *Bifidobacterium* strains clearly showed its potential. For example, homologous overexpression of Hsp20 in *B. longum* NCC2705 increased the strain's tolerance to multiple stressors, such as heat and acids (76). The combined introduction of heterogenous catalase and superoxide dismutase genes into *B. longum* NCC2705 significantly improved the strain's tolerance to oxidative stress (133). In addition, the tolerance of *B. breve* UCC2003 to gastric juice and osmolarity was improved by heterologous expression of the betaine uptake system of *Listeria monocytogenes* in the presence of betaine (282), and the heterologous expression of the listerial bile resistance mechanism in *B. breve* UCC2003 resulted in enhanced bile tolerance of the strain as well as improved *in vivo* survival and clinical efficiency (283). A probiotic strain engineered for

TABLE 3 Overview of evolved strains with cross-resistances to stressors other than the ones used for their adaptation, suggesting overlapping mechanisms of tolerance to individual stressors

Strain(s)	Selective stress treatment	Cross-resistance	Reference
B. breve, *B. longum*, *B. bifidum* and *B. infantis* strains	Gradually increasing bile concentration in medium broth; maximum, 1 % (wt/vol)	Acid stress (pH 2)	206
B. longum subsp. *longum* 8809	Acid (pH 4), 16 h at 37°C	Bile salt stress (0.5–3% [wt/vol])	158
B. longum and *B. catenulatum* strains	Acid (pH 4), 16 h at 37°C	Bile stress (1–3% oxgall), osmotic stress (6–10% NaCl), and heat stress (60–70°C, 10 min)	159
B. longum JDM301	Final pH in non-pH-controlled culture, 150 repeats	Osmotic stress (0.5 M NaCl) and oxidative stress (1.25 mM H_2O_2)	168
B. longum NCC2705	H_2O_2 (maximum 130 ppm) in continuous culture	O_2 (7.5–12.5%)	135

improved stress tolerance would need to be tested regarding its functionality and safety aspects before being used in industrial applications.

Adaptive laboratory evolution. The evolutionary engineering strategy known as ALE has been performed in multiple studies to generate *Bifidobacterium* strains with increased tolerance to heat (77), bile salts (206, 209, 226), acids (158, 159, 167, 183), and H_2O_2 (135). Comparison of the evolved strains and the parental strain can deliver valuable insights in the molecular mechanism of improved tolerance to stressors. In contrast to genetic engineering, ALE does not require prior knowledge of the molecular basis of stability and robustness when used for the optimization of a strain. Moreover, the commercialization of evolved strains selected from ALE experiments is not hampered by regulatory restrictions, since ALE derivatives are not considered GMOs. However, when they are used industrially, it must be ensured that the functionality of the evolved strains is not impaired by its stress adaptation.

Different ALE strategies have been applied for the improvement of *Bifidobacterium* strains. While most studies conducted repeated batch cultivations with a constant or gradually increased stress level (167, 183, 206, 209, 226), other studies cultivated cells under nonstress conditions and applied stress-induced selections between cultivation cycles (77), applied only a single stress treatment before selection of mutants with a stable improved phenotype (158, 159), or conducted continuous cultures under increasing selective pressure with immobilized cells for evolution of stress-tolerant *Bifidobacterium* strains (135). In addition, one study emphasized the possibility of evolving strains for increased acid resistance only by multiple successive cultivations under common non-pH-controlled batch conditions (168). Unfortunately, the authors did not report the final pH of the cultures, which probably represents the actual stress factor in these ALE experiments.

Several studies observed cross-resistance of the evolved strains to stressors in addition to those used for their adaptation, suggesting overlapping mechanisms of tolerance to individual stressors, in particular between bile salt and acid stress (Table 3).

Metabolic conditioning by sublethal short-term stress treatments. Several studies have investigated the effect of sublethal stress treatments on the cellular robustness of bifidobacteria. Inducible stress resistance is linked to the activation of a stress response, resulting in transient physiological and metabolic adaptation to the applied stress condition. Results from various sublethal stress pretreatments of *Bifidobacterium* strains which resulted in improved tolerance of the treated strain to the same stressor that was applied for the sublethal stress treatment (homologous stress condition) or another stressor (heterologous stress condition) are summarized in Table 4.

The triggered stress response might shape the physiology beyond the actual stress treatment and prepare the strain for subsequent exposure to stress. In contrast to cell improvement using ALE, the cells are exposed to the stressor for only a short time, which should not cause mutations in the genome and thus cannot generate a stable phenotype. In an industrial setting, sublethal stress treatments, such as starvation, could be applied after production to enhance the survival of the probiotics during

TABLE 4 Overview of short sublethal stress treatments of *Bifidobacterium* strains that resulted in improved survival of the strains to elevated lethal stress

Stress treatment and strain	Sublethal stress	Improved tolerance	Remarks	Reference(s)
Homologous				
B. longum D2957	H_2O_2 (1.25 mM, 20 min)	H_2O_2 (2.55 mM)	Species- and strain-dependent intrinsic and inducible resistance to H_2O_2. The result depended on the combination of duration of sublethal treatment and subsequent level of lethal stress treatment applied.	143
B. longum NCC2705	H_2O_2 (1.25 mM, 60 min or 20 min)	H_2O_2 (5.25 mM and 2.55 mM after 60 min pretreatment and 2.55 mM after 20 min)	Species- and strain-dependent intrinsic and inducible resistance to H_2O_2. The result depended on the combination of duration of sublethal treatment and subsequent level of lethal stress treatment applied.	143
B. animalis subsp. *lactis* RH-1 and *B. animalis* subsp. *lactis* BL-04	H_2O_2 (1.25 mM, 20 min)	H_2O_2 (5.25 mM)	Species- and strain-dependent intrinsic and inducible resistance to H_2O_2. The result depended on the combination of duration of sublethal treatment and subsequent level of lethal stress treatment applied.	143
B. adolescentis NCC251	Bile (0.1%, oxgall)	Bile (0.3 and 0.4% bile salts, oxgall)	Mechanism that prevents solubilization of membrane proteins might be induced. Effect is stronger in exponentially growing cells. Improved tolerance might be due to the induction of chaperones (e.g., DnaK) by sublethal stress treatment.	65
B. adolescentis NCC251	Heat (45°C, 30 min, or 47°C, 15 min)	Heat (55°C)	Improved tolerance might be due to the induction of chaperones (e.g., DnaK) by sublethal stress treatment.	65
B. longum NCC2705	Heat (47°C)	Heat (50°C, 5 min)	Results varied slightly depending on the operation mode applied for fermentation in which the effect of sublethal stress treatment was tested (batch vs continuous mode).	246
B. longum E-011884 (E1884)	Heat (47°C, 1 h)	Heat (55°C, 1 h)	Only slightly improved tolerance observed after sublethal stress treatment	285
B. longum BBMN68	Acid (pH 4.5)	Acid (pH 3.5)	Improved tolerance might be due to global metabolic adaptation to acid stress.	157, 163
B. animalis subsp. *lactis* (Degussa BioActive)	Decrease of medium from pH 6 to pH 5.2, tested in multiadaptation treatments	Acid (synthetic gastric fluid, pH 3.5)	Decreased pH might induce changes in membrane fatty acid composition or induce production of acid shock proteins.	284

(Continued on next page)

TABLE 4 (Continued)

Stress treatment and strain	Sublethal stress	Improved tolerance	Remarks	Reference(s)
Heterologous				
B. adolescentis NCC251	Salt (1.5% NaCl, 1 h, or 2% NaCl, 1 h)	Heat (55°C)	Improved tolerance might be due to the induction of chaperones (e.g., DnaK) by sublethal stress treatment.	65
B. adolescentis NCC251	Salt (2 % NaCl, 1 h)	Freeze-thawing cycles	Improved tolerance might be due to the production by an intrinsic osmoprotectant.	65
B. longum NCC2705	Osmotic stress (10% NaCl)	Freeze-drying	Improved survival has been observed in continuous setup but not in batch mode.	246
B. longum NCC2705	Acid, pH 4	Gastric juice (0.5% (wt/vol) NaCl, 0.3% (wt/vol) pepsin at pH 2.8, 15 min), bile salts (1.5% [wt/vol] porcine bile extract, 10 min), and osmotic stress (27% [wt/vol] NaCl, 2 h)	Treatment at pH 4 caused high loss of viable-cell count and can therefore not be considered sublethal.	246
B. animalis subsp. *lactis* E-012010 (E2010/BB-12)	Acid (pH 3.5)	Bile (1.5% bile extract, 3 h)	Slightly improved survival observed after sublethal stress treatment; impaired acid tolerance of *B. longum* E1884	285
B. animalis subsp. lactis E-012010 (E2010/BB-12)	Heat (47°C, 1 h)	Bile (1.5% bile extract, 3 h)	Slightly improved tolerance observed after sublethal stress treatment in laboratory scale (3–4 mL) but only minor effect at fermentation scale (7 L)	285
B. longum ATCC 15707	Starvation (30–60 min), tested in multiadaptation treatments	Cold (6°C)	Starvation might induce regulators of stress response or changes in the membrane fatty acid composition (increased cyclic fatty acids). No effect on −80°C survival.	284

downstream processing, storage, and administration. However, establishing cell conditioning for industrial production processes is challenging. The effect of sublethal stress treatment was found to depend on the species and strain (143, 284, 285), the growth state of the treated cells (154), the fermentation mode and scale, and the duration and level of applied stress (143, 246, 285). Moreover, sublethal stress can also result in decreased cell survival of the treated cells (143, 154, 246), and too-high stress levels might significantly reduce the count of viable cells in the culture broth. Taken together, these findings emphasize the need for strain- and process-specific optimizations of sublethal stress pretreatments before they are applied in industry.

CONCLUDING REMARKS

Owing to their health-promoting effects, bifidobacteria are widely used as probiotics in pharmaceutical and food products. In order to function as probiotics, *Bifidobacterium* strains must survive production, formulation, and storage, despite being subjected to various environmental stressors that can affect their viability. Thus, the ability to cope with technological and gastrointestinal stressors is an important selection criterion for *Bifidobacterium* strains in industrial applications. The ability of a strain to survive the exposure to stress is closely linked to its stress response, which allows the strain to adjust its metabolism and physiology to cope with the prevailing stress conditions.

In general, the ability to cope with individual stressors was found to vary among *Bifidobacterium* species and sometimes also among strains. A straightforward explanation for the highly diverse stress tolerance is the deviating conditions in the natural habitats of different species. The presence of certain stressors in their natural environment might have equipped strains of different species with mechanisms to cope with different stressors. For example, the presence of oxygen in the digestive tract of bees has been suggested to be linked to the high oxygen tolerance of bee isolates.

Over the last decades, multiple studies investigated the response of *Bifidobacterium* strains to stressful conditions to gain understanding of the molecular mechanisms underlying stability and robustness. Present knowledge of the stress physiology of bifidobacteria can be exploited in industrial applications in various ways (Box 1). However, whereas the effects of some stressors, such as acid and bile salt, have been investigated in more depth, current knowledge of the response to other stressors, such as low temperatures and high osmolality, is limited. Moreover, studies on individual stressors have addressed different sets of physiological responses. For example, the effect of heat stress on the carbohydrate metabolism of bifidobacteria remains largely uncharted, whereas much insight on its effect on the PQC system has been obtained. Currently, most studies are performed at laboratory scale under conditions that try to mimic industrial or *in vivo* conditions. Moreover, the application of various conditions for stress treatments across studies hampers the comparison between them, which is of particular importance for revealing the differences in the stress responses of stress-tolerant and -sensitive strains. The field would benefit from the adoption of standard conditions for stress treatments, to act as reference positive-control stress conditions, as well as including lab-scale, pilot-scale, and *in vivo* studies.

Some strains of the genus, such as *B. longum* NCC2705 and *B. breve* UCC2003, have been better studied in terms of their response to multiple stressors. However, the apparent variation in resistance of *Bifidobacterium* strains to stressors, on both the species and strain levels, hampers the extrapolation of findings in these well-studied strains to other strains of the genus. Additional strains of the genus representing different species should be subjected to in-depth studies, to assess the genus-wide validity of suggested stress response mechanisms.

The rapid advancement in genome sequencing and omics technologies in the last decade has opened entirely new ways to study the stress physiology of bifidobacteria and has generated a vast amount of system-wide data that need to be integrated and interpreted. In recent years, multiple studies applied transcriptomics and proteomics to investigate how environmental stressors affect the gene expression of *Bifidobacterium*

BOX 1: OUTLOOK

- *Bifidobacterium* strains are equipped with various mechanisms to respond to environmental stressors.
- Variability in the presence and efficiency of these mechanisms among strains and species results in deviating robustness and shelf life stability of probiotic bifidobacteria.

Exploiting present knowledge of the stress physiology of bifidobacteria for industrial applications. Knowledge of the molecular mechanisms underlying the diverse stress physiology of bifidobacteria can be applied in industrial applications as follows.

- Optimization of fermentation media of probiotic strains for enhancing stress tolerance and improving survivability throughout manufacturing and storage
- Selection of process parameters for conditioning the cells for improved robustness and stability
- Identification of biomarkers that can be used for monitoring (or evaluating) robustness and stability
- Rational selection of novel probiotic strains by accounting for genetic and/or cellular characteristics that contribute to higher stress tolerance
- Targeted genome editing of probiotic strains for improved stress tolerance (note that application of genetically engineered probiotics is restricted in multiple countries)

Future development of the field. The future development of our understanding of the multidimensional stress response in bifidobacteria will require the following.

- Deeper investigations of previously less investigated stress responses that can be utilized in industrial settings, like osmotic stress and nutrient effects
- Physiological studies to obtain experimental evidence for more species and strains
- Investigation of the effect of other bacteria and/or food matrix ingredients in commercial formulations on the stress tolerance and stress response in bifidobacteria
- Traditional microbiological methods to validate hypotheses from omics studies
- Further development of molecular biological tools for bifidobacteria to facilitate more rapid generation and validation of physiological data

strains with various stress tolerances. Omics studies have revealed that the stress response of bifidobacteria is a complex and multifactorial phenomenon that involves changes in various cellular functionalities. Not only the presence and absence of stress-associated genes but also the regulatory mechanisms differ between phylogenetic groups of *Bifidobacterium*, even for highly conserved genes. Understanding the connections and cooperation of the molecular players and traits that appear to be involved in the stress response will help to identify the importance of individual elements in the stress physiology of a strain.

However, the interpretation of omics data sets, and the extraction of biological meaning from them, is nontrivial. Some of the necessary developments for making best use of omics data are also highlighted in Box 1. Many hypotheses that have been proposed in recent omics studies remain to be validated in classical physiological studies. To facilitate the correct interpretation of omics data on the bifidobacterial stress response, comprehensive molecular studies of the function of various components are required. To date, interpretation of omics studies of *Bifidobacterium* relies widely on inference from the function of similar genes and their products in lactic acid bacteria, or even less closely related

bacteria. Thus, to drive functional studies of bifidobacteria forward, the application of classical molecular biology approaches, such as gene knockouts and complementation by heterologous expression, remains essential.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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