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ORIGINAL ARTICLE

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Processing Effects on the Nutritional Advancement of Probiotics and Prebiotics

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OBJECTIVES

The overall objective of the works performed within PROTECH (Nutritional enhancement of probiotics and prebiotics: Technology aspects on microbial viability, stability, functionality and on prebiotic function, QLK1-CT-2000-30042) is to address and overcome specific scientific and technological hurdles that impact on the performance of functional foods based on probiotic–prebiotic interactions. Such hurdles include the lack of a clear knowledge of the primary factors responsible for probiotic viability, stability and performance. Limited information is available on the impact of processing, storage and food matrices or food constituents on probiotic viability, stability and functionality. Furthermore, there are insufficient data about the interactions between probiotics and prebiotics in starter cultures or in functional foods prior to consumption. As a result of achieving this objective, selected prebiotics in combination with tailored manufacturing processes can not only contribute to probiotic functionality but also improve viability and stability of probiotic cultures within food matrices during processing and storage.

METHODOLOGIES

Figure 1 illustrates the objects of investigation and how the partners are interlinked in the multitude of tasks. In the fermentation study, development of a fermentation medium as well as optimization of harvesting time with respect to post-harvesting stability were pursued. In the drying study, freeze-drying and spray-drying were evaluated in terms of identification of suitable processing regimes, performance of different protectants in offering high survival during drying and storage, factors governing stability during storage, etc. The use of sub-lethal stress to improve technological behaviours (i.e. heat and oxygen tolerance), as well as to enhance resistance against extreme conditions in the gastrointestinal tract (bile tolerance) was evaluated. A proteomic approach was applied to examine the specific response of the cells. The survival of probiotics in yogurt was assessed to determine critical factors governing survival in a well-established food system and how modifications of this system could be made to reduce cell death, especially by incorporating prebiotics. The possibility of enzymically modifying prebiotics into a more complex structure, thus

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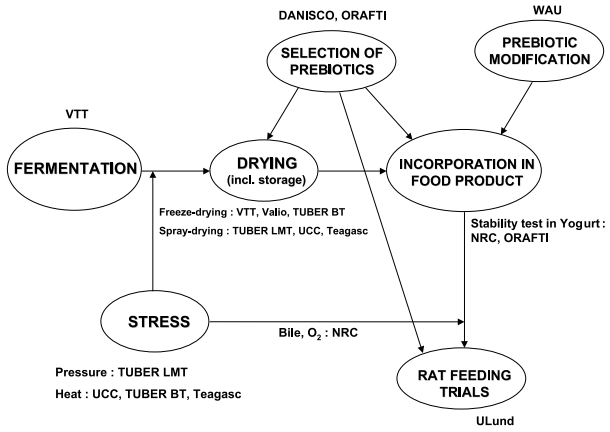

Fig. 1. General and specific objects of investigation, which were accomplished by partners participating in the PROTECH project along with the network of interaction among partners. Acronyms in this figure correspond to those used in the list of authors and affiliations on the title page.

making them less fermentable, was investigated. Feeding trials of commercial prebiotics supplemented with probiotics were performed to assess whether these properties could induce beneficial changes in the composition of short-chain fatty acids in different parts of the rat colon.

The probiotic strains included in the strain panel are the following; *Lactobacillus rhamnosus* VTT E-97800 (E800), *L. rhamnosus* LGG, *Bifidobacterium longum* NCC490, *Bifidobacterium animalis (lactis)* Bb-12 and *Lactobacillus salivarius* UCC500.

GENERAL ACHIEVEMENTS

Fermentation study

It was endeavoured to develop a universal medium to replace MRS, which is unsuitable for industrial applications because of the high cost of the ingredients. Furthermore, some of its components are not food-grade. The fermentation (production) costs form a substantial part of the total costs. Also the components of the medium are crucial in food applications, for example, bitter flavours must be absent from the probiotic ingredient. Moreover, probiotics are widely used in milk-based products and culture preparations typically contain milk-derived carriers (1–3). However, there is a demand for lactose- and milk protein-free products as well as for a wider range of probiotic-containing product applications.

The developed fermentation medium (GEM) contained food-grade components only. This universal medium is composed of glucose, soy peptone, yeast extract, $MgSO_4 \times 7\ H_20$ and KH_2PO_4. The CFUs obtained were in most cases better than or as good as in the MRS broth. GEM supported the growth of the probiotic lactobacilli equally as well as MRS. Of the two bifidobacteria studied, strain NCC490 had a clearly better cell yield in GEM than in MRS, whereas strain Bb-12 showed somewhat better growth in MRS than in GEM. The culture medium was further developed for each strain to improve the cell yield. Cells of LGG and E800, which were grown in GEM with a supplement of Tween 80 (1 ml l^{-1},) were more stable against freeze-drying and the subsequent accelerated storage at 37°C (Fig. 2). The fatty acid composition of the strains may be altered when growing with Tween 80. This will be a subject for further studies.

Drying studies

Freeze-drying. The key objective in this work was to optimize strain-specific fermentation and the freeze-drying procedure to produce highly active probiotic preparations economically in the pilot scale. In addition to good viability the probiotic product has to be stable and it has to retain its

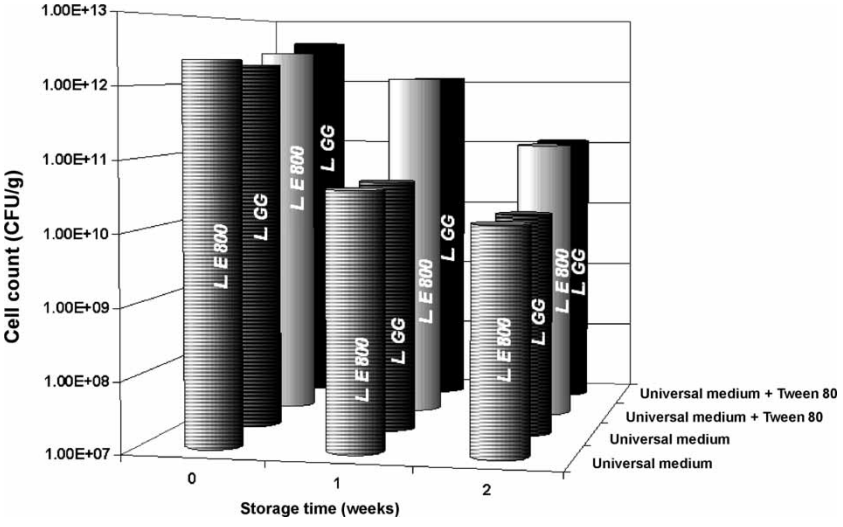

Fig. 2. Effect of supplementing Tween 80 in universal medium (GEM) on the storage stability of freeze-dried *L. rhamnosus* E800 and LGG at 37°C. Cultures were grown in universal medium (GEM) with or without supplement of Tween 80 (1 ml/l) at 37°C, harvested and freeze-dried with 0.9% NaCl. The lyophilisates were stored at 37°C under limited exclusion of air and moisture, away from sunlight in 2-ml Eppendorf vials. Every value is the mean of three independent experiments.

typical probiotic characteristics (2). The selection of appropriate and often strain-dependent medium and growth conditions forms the basis for obtaining active and stable probiotic cultures.

The general fermentation medium (GEM) was used for cell production in studies where the effect of fermentation time and freeze-drying medium on freeze-drying survival of probiotic lactobacilli and bifidobacteria were investigated. It was demonstrated that cell viability, storage stability and probiotic properties (acid/bile tolerance) could be influenced by fermentation technology and downstream processing.

For strain E800, although harvesting time (15–23 h) did not have a pronounced effect on the survival of the strains during freeze-drying (50–65%), it significantly affected the storage stability. A fermentation time of 15–17 h was found to be optimal. Presumably all fermentable carbohydrates (initial concentration 4%) were consumed after 17 h and after this period the cells started to die due to the lack of fermentable carbon. No difference with respect to the storage stability was found when milk-based or milk-free cryoprotectant (20% w/v) was added to concentrate. The harvesting time appeared to affect acid tolerance. Exposure of strain E800 to pH 3 or 4 resulted in higher acid tolerance for cells harvested after 15 h in contrast to harvesting after 22 h.

A high variability among probiotic strains was observed concerning the effect of fermentation time (between 16 and 23 h) on the freeze-drying survival and probiotic functionality. Harvesting time did not affect survival of strain LGG after freeze-drying, whereas with strain Bb-12 prolonged fermentation tended to result in poorer survival. For both of these probiotic strains, no systematic differences in acid and bile tolerance between different fermentation times could be identified.

When the effect of adjustment of pH of the concentrate prior to freeze-drying was examined, studies on strain E800 showed that pH 7.0 was optimal in the improvement of stability during accelerated storage at 37°C. In contrast to this, neutralization prior to freeze-drying did not have any significant impact on the freeze-drying survival and acid/bile tolerance of strains LGG and Bb-12.

Sucrose has been one of the most commonly used cryoprotectants for microbes (4). Glycine betaine is a compatible solute which microbes accumulate when extracellular osmolality rises (5), and as it also helps to maintain membrane fluidity at low temperatures it has been hypothesized to have cryoprotective effects (6). When betaine and sucrose were applied as cryoprotectants, sucrose turned out to be far superior. The effect of the cryoprotectants on the freeze-drying survival was strain-dependent. Although no marked difference in the freeze-drying survival of strain LGG was observed between sucrose and betaine-protected cells, clearly better survival of Bb-12 cells was obtained with sucrose than with betaine (Fig. 3). Furthermore, strain LGG dried in the presence of sucrose tolerated bile better (bile extract 1.5% for 3 h) and also tended to have better acid tolerance (pH 2.5 for 2 h) than betaine-protected cells. Cell preparations had excellent stability during storage at −20°C.

Several other cryoprotectants (combinations of gelatine, glycerol, maltodextrin, lactose and/or sucrose) have also been tested in freeze-drying. Of the mixed protectants, a carrier combination containing gelatine, glycerol, maltodextrin and lactose gave the best results as regards both survival during freeze-drying and storage stability of strain E800.

Carrier properties of starch were tested for strains LGG, E800 and Bb-12 (Fig. 3). The major advantage of adding the starch carrier was the excellent consistency of the freeze-

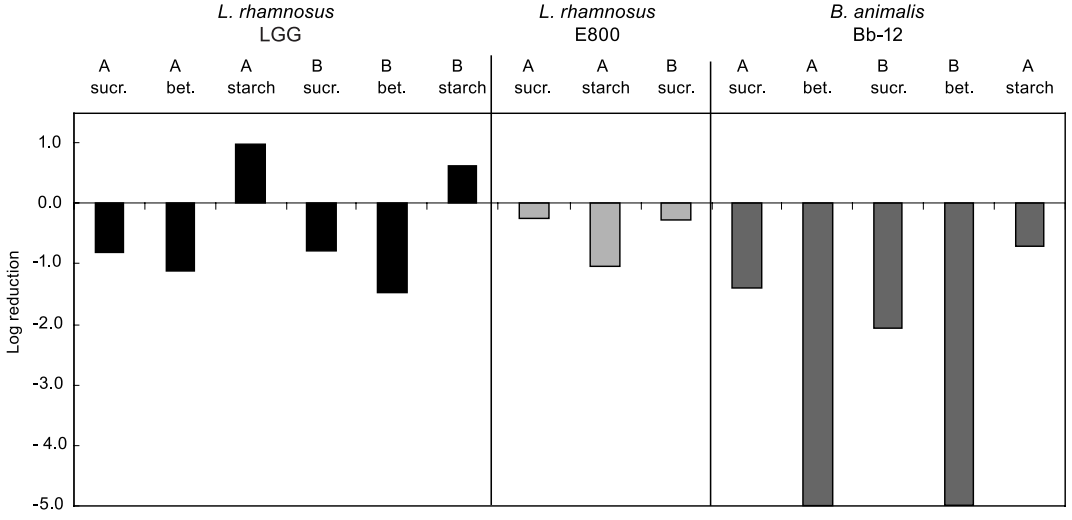

Fig. 3. Freeze-drying survival of *L. rhamnosus* LGG, *L. rhamnosus* E800 and *B. animalis* Bb-12 freeze-dried with sucrose (sucr.), betaine (bet.) or starch carriers in non-neutralized (A) or pH 7-neutralized (B) cells.

dried product. Starch added formulation is granular and light in colour whereas the commercial freeze-dried preparation changed colour from light beige to pink during storage at ambient temperature and moisture for 3 months. This allows the use of the formulation even in light-coloured products. However, starch did not improve the storage stability of the powders. No marked difference was observed in the storage stability between strains E800 and Bb-12 protected either with starch or sucrose.

Spray-drying. It has been reported that to be of benefit to the consumer, high levels of probiotic bacteria need to be consumed daily. Stable dried cultures with a high cell population would offer major advantages in the production of probiotic products. These cultures could potentially be added directly to a finished food or could be consumed in tablet form. Dried cultures would also offer advantages for use as starters in the production of fermented probiotic foods, as the work involved in maintaining liquid stocks would be eliminated. Spray-drying, which has high processing rates and low operating costs, would be an ideal method for the production of dried cultures; however, spray-drying often results in high loss of cell viability. In this sub-task, the effects of spray-drying conditions on survival and storage stability of the probiotic cultures were investigated.

Initially, the thermal tolerance of strains E800, UCC500 and LGG in reconstituted skim milk (RSM) was compared in order to predict the spray-drying suitability of these probiotic strains. At 59°C, a decrease of 1.92 $\log_{10}$ CFU/ml of strain LGG was obtained, while the two other strains were more heat-resistant at this temperature. At 61°C, strain E800 was the most thermal tolerant (a decrease of 1.59 $\log_{10}$ CFU/mL), while strains UCC500 and LGG showed decreases of 2.99 $\log_{10}$ CFU/mL and 3.70 $\log_{10}$ CFU/ml, respectively. D-values calculated from these data confirmed strain E800 as the most thermal tolerant, and following exposure to 61°C its D-value was 2.76 min. In contrast, D-values for strains LGG and UCC500 were 1.32 and 1.64 min, respectively.

As exposure to acid, as encountered in various food systems and during gastric conditions, can severely impair probiotic culture viability, systematic work has been initiated to identify the strain-specific acid resistance and to monitor the processing effect (spray-drying, storage, etc.) on this particular probiotic functionality. Studies were also carried out to evaluate how the addition of dairy-based media constituents could improve acid tolerance. At the initial stage different probiotic strains were exposed to simulated gastric juice. Strain LGG was the greatest survivor in simulated gastric juice (a decrease of 0.51 $\log_{10}$ CFU/ml, compared with 2.68 and 6.62 $\log_{10}$ CFU/ml for strains UCC500 and E800, respectively). A component analysis of the simulated gastric juice showed that glucose (19.4 mM) was responsible for enhancing survival by approximately 4.5 $\log_{10}$ CFU/ml (Fig. 4). Since the contribution of glucose to acid resistance was of utmost importance, studies related to the design of composition of effective protectant should consider the presence of glucose in the matrix. Other components such as arginine, histidine and monosodium glutamate did not provide enhanced survival effects comparable to glucose. Confirmation that membrane-bound F_0F_1ATPase complex is involved in acid protection in lactobacilli was obtained from experiments where the enzyme was inhibited by the F_0F_1ATPase inhibitor, N,N′-dicyclohexylcarbodiimide.

Initial spray-drying experiments were designed to investigate the effect of process parameters, primarily outlet temperatures and carrier media (skim milk and skim milk-based mixture) on bacterial survival rate and residual moisture content. The solid content of the RSM was held

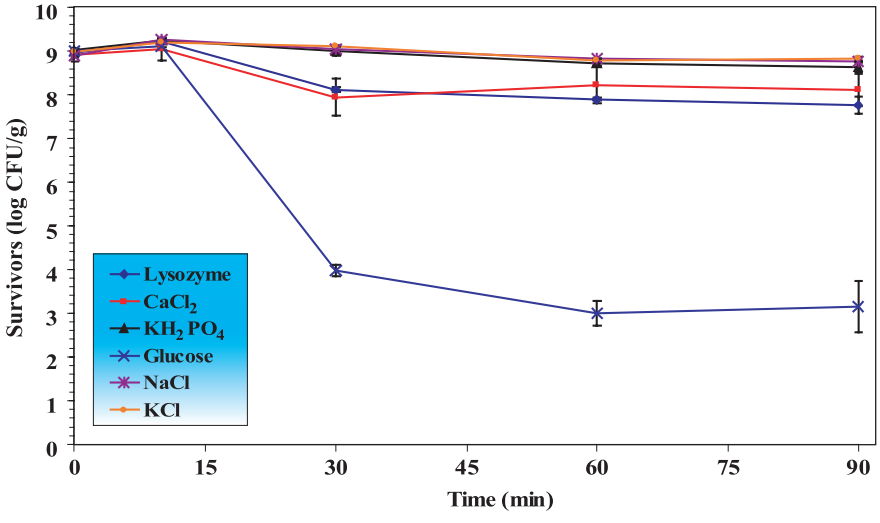

Fig. 4. Effect of removing various components of simulated gastric juice (pH 2.0 at 37°C) on survival kinetics of *L. rhamnosus* LGG on stationary growth phase. Each data point represents the survivor number after incubation of strain LGG in the gastric juice made under exclusion of the associated component.

constant at 20% w/v. In previous spray-drying studies this solid concentration was frequently used and has been regarded as optimal for assuring high residual viability (7–10). A range of outlet temperatures between 70°C and 100°C was used in the preliminary experiments. It was observed that survival rate was inversely proportional to outlet temperatures. On the other hand, residual moisture was gradually reduced at higher temperatures. Thus, a compromise had to be made for determining optimal drying temperatures, as a high outlet temperature is detrimental to cell viability, but if the outlet temperature is too low the process results in powders that are too moist, which in turn impairs the storage stability. A residual moisture of 4% was regarded as a good quality parameter of dried dairy products (11). Residual moisture contents of around 4% were achieved upon spray-drying at an outlet temperature of 80°C.

The resistance against spray-drying was strain-dependent. Upon drying at outlet temperature of 85–90°C, the survival of strains LGG and E800 was 50% and 25% in RSM, respectively. In contrast to the *L. rhamnosus* strains, strain UCC500 experienced >99% loss in viability during spray-drying in RSM. Furthermore, strain UCC500 survivors exhibited a greater degree of injury than *L. rhamnosus* strains, as indicated by the inability of a subpopulation of strain UCC500 to grow in the presence of 5% (w/v) NaCl.

The applicability of spray-drying in the production of skim milk-based preparations containing probiotic bacteria was evaluated. Various inulin/oligofructose-based or polydextrose-based prebiotic substances were also included in the carrier matrix to assess their protection capacity. When reconstituted skim milk was used as spray-drying carrier, a microbial survival rate of 60% was achieved at an outlet temperature of 80°C. The ability of probiotic bacteria to survive spray-drying using 10% RSM + 10% prebiotic as the carrier medium was compared to cells dried in 20% RSM as a control.

One of the prebiotics investigated was polydextrose (DANISCO). Although the substitution of 10% (w/v) polydextrose for 10% (w/v) RSM did not give any significant improvement in survival during spray-drying, a high level of survivability comparable to RSM could still be achieved (Fig. 5). Compared to cells spray-dried in RSM, the storage stability of strain LGG spray-dried in RSM/polydextrose was not significantly impaired. These encouraging results gave new perspective on the possibility of producing symbiotic powder with a high viability level of incorporated probiotics.

Different forms of inulins, i.e. Raftilose®P95, Raftilose®Synergy1, Raftiline®GR and Raftiline®HP from ORAFTI were also included in the spray-drying medium with strain LGG. Survival during spray-drying in the presence of inulin was comparable to RSM control powders. Similar rates of decline in probiotic counts in inulin and control

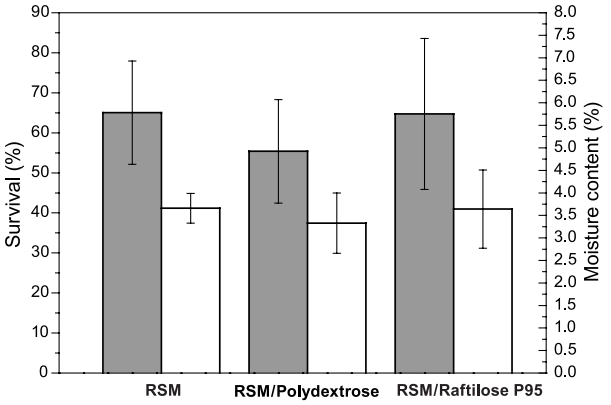

Fig. 5. Comparison of the survival rates (grey bars) of *L. rhamnosus* LGG spray-dried with different carrier formulation at an outlet temperature of 80°C along with the corresponding residual moisture (unfilled bars). Reconstituted skim milk (RSM) at 20% w/v was regarded as reference media. Commercial prebiotic substances (Raftilose P95 and polydextrose) were incorporated in the RSM carrier by substituting half of RSM solid with each prebiotic. The final solid concentration of spray-drying medium was maintained constant at 20% w/v.

powders were obtained at 15°C. On the other hand, at storage temperatures of 25 and 37°C, long-term stability was negatively affected upon partial substitution of milk fraction by Raftilose P95.

Furthermore, attempts were made to verify whether the difference in the capacity of the drying media in conferring storage stability correlated with the glass-forming capability of the constituents, as entrapment of a living system in a glassy matrix upon dehydration was suggested to be responsible for their long-term stability (12). The glassy structure of the external matrix is a highly effective environmental barrier with an extremely low molecular mobility. With respect to the preservation of bacteria this condition leads to a suppression of unexpected deteriorating events on bacterial membranes, which constitute the interface to the surroundings and are predominantly exposed to various environmental abuses. Among all possible degradation events, lipid oxidation of membrane fatty acid was mainly deemed responsible for cell death during storage (13). As translational diffusion is drastically restricted in the glassy state, the diffusion of oxygen, which precedes oxidative damage, could most likely be limited. Other degradative events such as fusion of membranes, protein unfolding, etc. could also be prevented (14). Each constituent of the carrier medium was further calorimetrically characterized in terms of their glass transition temperatures. Although all evaluated carriers were in the glassy state, differences were observed in their capacity for conferring protection for probiotics. The presence of the dried sample in a glassy state seems not to enhance bacterial stability. Although required, the formation of a glassy state in itself is not sufficient for preservation. The deficiency in conferring storage stability might be caused by incompat-

ibility of the saccharides constituent of Raftilose P95 in adequately replacing water molecule in the dehydrated state; thus the maintenance of structural and functional integrity was not as effective as in the presence of milk alone.

The combination of RSM and glycine as a carrier for spray-drying was evaluated with strains UCC500 and E800. Glycine facilitated minimal retention of moisture but afforded less protection to the cells compared with RSM. Stability of strain E800 cells dried in 10% RSM +/− glycine during storage was also examined. Protection from oxygen (vacuum packaging) during storage further improved the storage stability of spray-dried powders. Systematic study on the effect of various environmental factors of storage stability revealed that best retention of viability was observed when cells were dried in 10% RSM + 10% glycine at inlet/outlet temperatures of 150°/70°C, which were then vacuum packed and stored at −20°C.

The effect of culture growth phase on survival during spray-drying and powder storage was investigated using strain LGG. Lag phase cultures performed poorest (1.7% survival), with early log phase cultures yielding intermediate (14% survival) and stationary phase cultures yielding best survival (50%) following spray-drying. Using stationary phase cultures powders in excess of 10^9 CFU g^{-1} were generated in each case. Furthermore, lag phase cultures also exhibited greatest membrane damage (indicated by an inability to grow in the presence of 5% NaCl) during spray drying. However, early log phase cultures were the poorest survivors during powder storage.

Furthermore, flow cytometric assessment in combination with functional dyes (cFDA/PI) was applied as a diagnostic tool to evaluate the type of cellular injuries that occurred upon spray-drying. With the help of flow cytometric analysis, measurement at single cell level of particle size, surface properties, interaction between fluorochrome and cells, membrane potential, etc. could be achieved. By using functional dyes morphological and physiological changes in response to the processing conditions can be probed. A wide range of fluorescent dyes is available nowadays, which are aimed at specific cellular targets, like DNA, enzyme activities, internal pH, or the cytoplasmic membrane (15, 16). The cellular injury sites and compromised metabolic activity as affected by spray-drying could be precisely identified.

In general, metabolically active cells are capable of converting non-fluorescent cFDA into a fluorescent product, cF, with intracellular esterases. However, during flow cytometric measurement the green fluorescence as a result of enzymatic activity could not be registered unless the fluorescence product was accumulated in the cytoplasm. Thus, a high degree of membrane intactness was necessary to retain the fluorescent dye and only cell population fulfilling both cellular requirements was scored as viable. On spray-dried strain LGG it was observed that the population with intact membranes – actively accumulating cF and not propidium iodide (PI)-labelled – was decreasing at higher outlet temperatures. Simultaneously the population labelled with PI increased. Both sets of evidence were consequences of increased damage to cellular membranes at higher drying temperatures. Cells that experienced membrane damage could not accumulate cF and counterstained by PI, which were expelled by cells with intact membrane but can diffuse into cells with damaged membranes. This finding confirmed literature data, which mainly proposed damage to cellular membranes as the underlying mechanism responsible for cell death during drying (17, 18). Cell death upon drying was caused mainly by damage to cell membranes and the degree of membrane disintegration was progressively increased at higher outlet temperatures.

Stress studies

When bacteria encounter unfavourable conditions they activate stress response mechanisms, which enable them to survive under stress. This response can be detected as an enhanced production of stress-related proteins that are involved in the repair or degradation of stress-damaged proteins. This phenomenon can be exploited to improve the stability and viability of the cells. In contrast to starter cultures, which often lose viability after fermentation, probiotic bacteria are expected to remain viable and active in the gastrointestinal tract. As a consequence of this, it is important to understand the stress response of probiotic strains. Attempts have also been made to identify specific stress markers that can be used to monitor bacterial stability and viability, and furthermore to define optimal growth and process parameters for the production of probiotic culture concentrates.

Bifidobacteria were stressed with bile salts, as the bacterial response to bile stress is so far poorly understood. Furthermore, bifidobacteria, when used as probiotics, have to overcome this bile stress in the small intestine of the human gastrointestinal tract. For this purpose strains Bb-12 and NCC490 were stressed during growth with bile salts. This is of importance, as probiotic strains are selected for bile salt resistance and bacterial deconjugation of bile salts is considered to have a health-promoting effect. Protein extracts of bile-stressed and non-stressed cells were fractionated and subjected to a proteomic analysis.

When analysing soluble extracts from bifidobacteria by 2-D gel electrophoresis several proteins were identified, which were altered upon bile stress (Fig. 6). For instance, the general heat-shock protein, GroEL, was clearly induced, while enzymes with high molecular weight like the fatty acid synthase (FAS) became less abundant, indicating the stressed status of the cells. To go more into detail, membrane proteins from the same cells were extracted

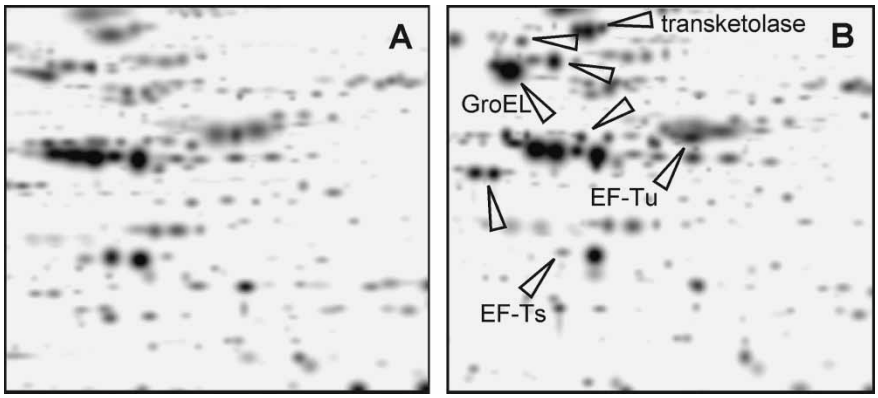

Fig. 6. Two-dimensional electrophoretic profiles of crude bifidobacterial proteins from cells cultured with bile salts (B) or without bile salts (A); 50 mg proteins were separated in the first dimension from pH 4 to 7. Several of the silver-stained proteins were identified by LC-MS/MS. Altered spots are indicated by arrowheads. GroEL, chaperone GroEL ; EF-Tu, elongation factor Tu ; EF-Ts, elongation factor Ts.

and analysed by 1-D PAGE, 2-D LC-ESI MS/MS. To compare relative protein levels, output scores with the Sequest software package were taken, reflecting the quality and quantity of identified peptides after trypsination and LC-MS/MS. After reproducing the results with independent protein analysis experiments, an upregulation was observed for an oxalate/formate antiporter, polyphosphate kinase, ABC-like cation transport system, chaperone DnaJ, multidrug resistance transporter, ABC-like amino acid transporter, universal stress protein, etc. These results strongly suggest that membrane(-associated) proteins play a crucial role in counterbalancing the stress caused by bile salts. Furthermore, when analysing the fatty acid composition of the cells, bile-stressed bacteria possessed a higher amount of unsaturated fatty acids then the non-stressed bacteria. Taken together, the exposure of the cells to bile causes changes to the cytoplasma, the membrane proteins and the fatty acid composition.

The debate about bacterial bile salt resistance and possible beneficial effects of bile salt deconjugation by probiotic strains has been ongoing for many years; nevertheless, knowledge about molecular mechanisms is still limited. Here, different compartments of the bifidobacterial cell were analysed upon bile treatment, providing the basis for further detailed analyses. Genome analyses and microarray technology will provide further information about the stress response. It will be interesting to see if and how bile salt resistance/metabolism influences probiotic function/activity and if the bile stress response overlaps with other adaptation mechanisms.

The effect of moderate pressure stress on induction of heat tolerance of strain LGG was also evaluated. Related work on the stress response of *Escherichia coli* to elevated hydrostatic pressure showed that upon increase of pressure the synthesis of many proteins occurs at an elevated rate and these proteins are also induced by heat or cold shock (19). In the light of previous findings, it was observed that pressure-induced protein synthesis was involved in the increase of bacterial heat tolerance. LGG cells pressure pre-treated at 100 MPa at 37°C for 10 min showed higher survivability than untreated ones when exposed to heat challenge at 60°C (20). To gain more insights on the cellular mode of action of pressure-induced heat tolerance, flow cytometric analysis was applied in combination with the functional dye LIVE/DEAD®BacLight™ bacterial viability kit. Dot plot analysis showed that a lower degree of membrane damage was observed at pressure pretreated cells upon heat treatment at 60°C for 3 min. These data suggest that pressure-induced thermotolerance occurred as a consequence of stabilization of cellular membranes, which in turn led to an enhanced transient protection against degradative effects of heat on cell membrane. Evaluation of heat inactivation kinetics of strain LGG previously pressure pretreated in the presence of chloramphenicol, a protein synthesis inhibitor, pointed out the potential contribution of pressure-induced protein biosynthesis in the enhancement of bacterial heat tolerance. This observation gives evidence about the role of proteins expressed during pressure adaptation in the prevention of thermal degradation of cell membrane.

The effect of cross-protective action of unrelated stress factors on the improvement of spray-drying survival was evaluated. Pre-exposure to sub-lethal stresses such as bile (1 mM GDCA), NaCl (5%), heat (48°C) or heat (48°C)+NaCl (5%) increased survival of both strains by as much 40%, when strains UCC 500 and E800 were dried at an outlet temperature of 90°C (Fig. 7).

Feeding trials

The aim of the work was to identify prebiotic carbohydrates and probiotic/prebiotic combinations that favour the generation of specific short-chain fatty acids (especially propionic and butyric acid) in the human colon, in order to improve colonic health and reduce the prevalence of colonic diseases, such as ulcerative colitis and, in the long term, colonic cancer.

Lactitol and various types of fructose-containing oligosaccharides [lactulose, Raftilose®P95 (P95), Raftilo-

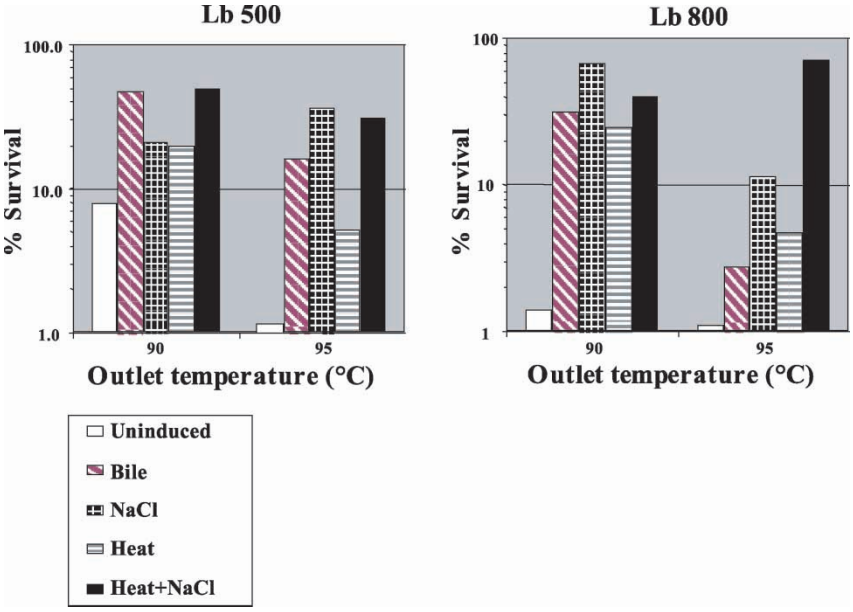

Fig. 7. Effect of induced sub-lethal stress on the ability of strains UCC500 (Lb500) and E800 (Lb800) to survive the spray-drying process. Cells were spray-dried at an outlet temperature of 90°C with reconstituted skim milk 20% w/v as protective media.

se®SYNERGY1 (Synergy), Raftiline®HP (HP), Raftiline®HPX (HPX)] were included in the first study. In a second study, where the focus was on prebiotic and probiotic interactions, three potential prebiotic carbohydrates (HPX, Pectin 1400 and lactitol) were combined with two probiotic strains (Bb-12 and UCC500), to see if the composition of the microflora could be changed and hence the production of short-chain fatty acids.

A rat model that has been shown to correlate well with human experiments concerning total fermentability was used. The diets investigated contained similar amounts of protein, fat, sucrose, minerals and vitamins. The prebiotic carbohydrates tested were included in the diets to give a total concentration of indigestible carbohydrates of 8 g/100 g diet (calculated on dry weight basis). A basal diet with no prebiotic carbohydrates was also prepared. In the second experiment the probiotic strain was included to give each rat an amount of strains Bb-12 and UCC500 corresponding to 1×10^{10} CFU/day or 1×10^{9} CFU/day, respectively.

The rats were adapted to the diet for 7 days, then a 5-day experimental period followed when faeces were collected. At the end of the experiment the rats were killed. Faeces, caecum and the colon (distal and proximal part) were analysed for short-chain fatty acids. Contents of indigestible carbohydrates in the diets and faeces were measured by gas-liquid chromatography to allow calculations of hindgut fermentability of the indigestible carbohydrates. The bulking index (i.e. the increase in faecal wet or dry weight in g/g of indigestible carbohydrates eaten) of the oligosaccharides could be ascribed to the physico-chemical properties of the carbohydrates. Thus, HP and HPX, with the highest molecular weights, revealed higher faecal wet and dry weight increments than the other oligosaccharides. Further, HPX with a high crystallinity seemed to be comparatively more resistant to microbial degradation, as judged by the higher faecal dry weight increment with this oligosaccharide than with the others. Interestingly, HPX also gave the lowest caecal pool of short-chain fatty acids, while the faecal excretion of short-chain fatty acids with this substrate was similar to that with HP and Synergy, suggesting that HPX is mainly fermented in the distal part of the hindgut. Further, a comparatively high proportion of total acids excreted comprised butyric acid. This might be important from a nutritional point of view, as most colonic diseases occur in the distal part of the colon. HPX was also the substrate that gave the highest caecal pH (6.9 versus mean 6.6 ± 0.1 for the other oligosaccharides), providing further evidence for a higher resistance against caecal fermentation of this oligosaccharide than the others.

Considerable differences in the short-chain fatty acid concentrations in the caecum of rats could be seen with lactitol and the different fructose-containing oligosaccharides. Acetic acid was the major anion formed, followed by propionic and butyric acid. Figure 8 illustrates that the highest proportions of propionic acid were seen in rats fed Synergy and HP (34% and 30%, respectively, versus mean 23 ± 1% for the other oligosaccharides), while rats fed P95 generated the highest proportions of butyric acid (29% versus 14 ± 1% for the other substrates). Interestingly, similar tendencies could also be seen with these substrates in other parts of the hindgut and in faeces. High proportions of propionic and butyric acid could also be seen in the distal part of colon and in the faeces of rats fed HPX. Rats fed lactitol and lactulose generally had rather low propor-

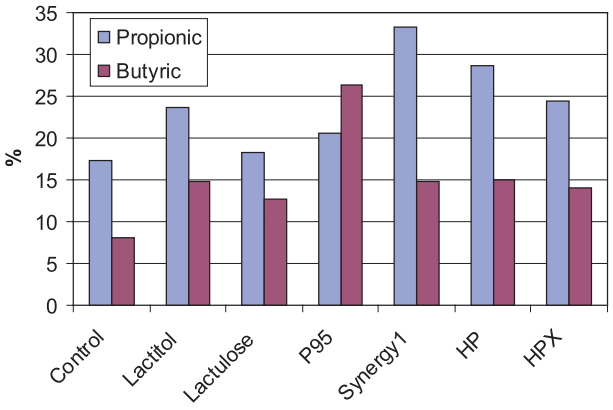

Fig. 8. Proportion of propionic and butyric acid in caecum as percentage of total short-chain fatty acids (%) with various prebiotic substances in the diet of rats. The explanation of the codes used in this figure to identify the prebiotics can be found in the section 'Feeding trials'.

tions of propionic and butyric acid and high proportions of acetic acid.

Adding strain Bb-12 to the test diets increased the caecal formation of short-chain fatty acids in rats fed HPX and lactitol ($p < 0.05$), while rats fed pectin were unaffected. The increase was due to an increase of propionic and acetic acid. In faeces, however, there was also an increased formation of short-chain fatty acids (propionic and acetic acid) with pectin, whereas lactitol instead showed a decreased formation (acetic, propionic and butyric acid). No increase in short-chain fatty acid formation could be seen with strain UCC500.

The increasing evidence of positive effects of some types of short-chain fatty acids (propionic and butyric acid) has resulted in a growing interest in increasing the supply of these acids in the colon, especially in the distal part, by diet. The amount and type of short-chain fatty acids formed in the colon are, however, highly dependent on the type of carbohydrate ingested. Factors of importance have been proposed to be related to the monomeric composition of the carbohydrate as well as the type of glycosidic linkages present. However, other factors may also be of importance such as the degree of polymerization, the solubility and the structural arrangement of the carbohydrates. This work indicates that the degree of polymerization and the crystallinity is of great importance for the total fermentability, the short-chain fatty acid pattern formed and the place of fermentation, at least for fructose-containing oligosaccharides. Further, it may be possible to change the total formation of short-chain fatty acids as well as the short-chain fatty acid pattern by adding certain probiotic strains. Thus, by optimizing processing parameters and in this way tailoring carbohydrates with specific physico-chemical properties it seems possible to obtain a desirable degree of fermentation and short-chain fatty acid pattern.

Survival of probiotics in fermented products

Identification of critical environmental factors. Probiotics are bacteria selected for their ability to survive gastric transit. However, in the case of bifidobacteria, propagation in large numbers and stability throughout product shelf-life remains challenging. A common way to deliver probiotics is via fermented dairy products, which are believed to offer protection during gastric transit. Poor survival of bifidobacteria in these products has been demonstrated, limiting their efficiency as a delivery vehicles. Efforts were made to gain an understanding of the relative impact of the various factors playing a role in survival of strains Bb-12 and NCC490, to ensure better survival.

Factors such as oxygen stress (type of packaging), osmotic pressure (addition of sucrose), storage temperature and low pH/presence of classical yogurt bacteria (i.e. *Lactobacillus delbreuckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) have been implicated as influencing bifidobacterial survival. Here, these factors were studied in concert by creating a pH gradient within the natural boundaries of yogurt, i.e. between pH 4 and pH 5, using a combination of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus* and glucose. Oxygen stress was studied using three types of packaging materials with varying oxygen barriers. These were polystyrol (low oxygen barrier), polypropylene (medium oxygen barrier) and glass (high oxygen barrier). Osmotic pressure was studied by adding 0%, 5% and 10% of sucrose to the yogurt base; 10% was chosen as highest concentration as this corresponds to a level described as consumer preference. Finally three storage temperatures were compared. As an optimal temperature for yogurt storage 4°C was chosen, 10°C mimics a minor temperature abuse system that might occur in chill cabinets when yogurts are stored in supermarkets. As an obvious temperature abuse system 25°C was chosen.

A detailed comparison of various factors that play a role in the stability of bifidobacteria in yogurt revealed that strain Bb-12 was stable under conditions close to the commercial setting. Storage temperature, osmotic pressure and the presence of classical yogurt bacteria were found to play a significant role in the survival of strain Bb-12. The different packaging materials (oxygen stress) were found not to play a significant role, thereby underlining the oxygen tolerance of *B. animalis/lactis*. Overall a dose of 10^9 CFU can easily be delivered, making yogurt an adequate delivery vector for strain Bb-12 and justifying its abundant use for industrial purposes. Under the same conditions the stability of strain NCC490 was poor, indicating a high strain dependency and the viability of strain NCC490 requires further attention. The comparison done here included two bifidobacteria strains; further testing will establish if other *B. animalis/lactis* strains are equably as stable as the one tested here (Bb-12) and if more stable *B. longum* strains can be found. The importance of alternative bifidobacteria

strains is illustrated by the fact that our survey of commercially available bifidobacteria yogurts revealed that all tested products contained only *B. animalis/lactis*.

The discovery of other stable strains will expand the availability of potential probiotic bifidobacteria. Nevertheless certain strains with auspicious health-promoting properties might not be stable. Therefore efforts were made to stabilize strain NCC490, as this information could potentially be used for such strains. One possibility is synbiotics, where strain NCC490 was grown in the presence of a prebiotic and their stability in yogurt improved. We are currently looking into the specific mechanisms of this protective effect. Another possibility we investigated was acid adaptation, which was also found to enhance the stability of strain NCC490 in yogurt. Micro-arrays revealed the underlying molecular responses and further research is being undertaken to fully exploit the bacterial natural defences.

Improvement of viability and vitality by incorporating prebiotics. In this sub-task specific prebiotic–probiotic interaction and the effect of prebiotics on the viability and vitality of probiotic bacteria were to be examined. Viability is identified as the percentage of viable cultures at the end of the shelf-life of a food product and vitality is the ability of the probiotic culture to resist external stress conditions occurring in a food product during its shelf-life, resulting in a higher survival rate during passage in the gastrointestinal tract. The latter concept is developed on the basis of the knowledge that some probiotic cells that are submitted to the stress conditions in a food product may be partially damaged, leading to an absence of the associated health-related effects. The addition of prebiotics Raftilose®P95 and Raftilose®Synergy1 into probiotic yogurt was assessed as to their potential effect in conferring a better survival during gastrointestinal passage.

The evidence for the beneficial prebiotic–probiotic interaction in finished food products was demonstrated in a previous study, where oligofructose (Raftilose®P95), galacto-oligosaccharides and inulin were supplemented in the milk-based growth media of bifidobacteria (21). Growth promotion, enhancement of activity and retention of viability were greatest when bifidobacteria were grown in the presence of Raftilose®P95 at a concentration of 5% (w/v). Furthermore, the effect of Raftilose®P95 on the microflora of fermented milk produced using a commercial starter culture containing strain Bb-12 was evaluated during refrigerated storage (22). The presence of this oligofructose had no significant impact ($p < 0.05$) on survival of either *L. acidophilus* or *S. thermophilus* during storage at 4°C for 42 days, but had a significantly beneficial effect ($p < 0.05$) on the viability of bifidobacteria.

A second aspect was the vitality of probiotic cultures to survive the passage of stomach and intestine. A preliminary test was done on a single sample based on strain Bb-12. Fresh cultures and yogurts with 0%, 1% and 3% Raftilose®P95 were added to artificial intestinal juice containing pancreatin and bile salts. Increasing survival with increasing levels of Raftilose®P95 was detected. Based on the aforementioned result, a further investigation of the influence of Raftilose®P95 and Raftilose®Synergy1 on the survival of probiotic strains during gastrointestinal transit was assessed. An *in vitro* gastrointestinal passage protocol was developed in which a sufficient reduction of each probiotic strain was realized. Thereafter, probiotic yogurts containing 3% Raftilose®P95 or Raftilose®Synergy1 or control yogurt were tested after 1 day, 4 weeks and 10 weeks to evaluate the impact of prebiotics addition on the ability of probiotics to deal with stress during the shelf-life of yogurt.

For the simulation of gastrointestinal transit, probiotic yogurt was initially mixed with gastric juice and incubated for 90 min at 37°C to mimic stomach conditions. Thereafter the amounts of viable probiotic bacteria were determined. The residual gastric sample was transferred to the intestinal juice medium and incubated for 90 min at 37°C. The results of the study demonstrated that for strain LGG the survival during gastrointestinal transit was improved with 1 or 2 log units when adding 3% Raftilose®P95 or 3% Raftilose®Synergy1. The improved survival was only found in yogurt that was stored for 4 or more weeks. This suggests that the viability in fresh yogurt was unaffected and that the protective effect developed during storage of the yogurts.

Enzymatic modification of probiotics

Bifidobacteria play an important role in carbohydrate fermentation in the colon. Carbohydrates can be modified to low molecular weight oligosaccharides or monosaccharides by using a wide range of depolymerizing enzymes. These glycanases can be found extracellularly, associated with the bacterial cell, or intracellularly. An objective of our research is to prepare prebiotics that could give bifidobacteria a selective advantage. The glycosidases present in bifidobacteria are of interest to us, particularly those capable of elongating oligosaccharides, i.e. those that can catalyse transglycosylation reactions. Using enzymes from bifidobacteria itself for generating prebiotics will ensure that these carbohydrates are indeed degraded. As found in the literature (23–26) galacto-oligosaccharides seem to have a large prebiotic potential. So far, not much is known about the enzymic machinery for galactose utilization. In this research the galactan and galacto-oligosaccharide degradation mechanisms were investigated. Understanding the mechanism of degradation determines, to a certain extent, the choice of prebiotic developed.

Two galacto-oligosaccharide-degrading enzymes from *Bifidobacterium adolescentis*, namely a β-galactosidase and an α-galactosidase (27), were cloned into a pBluescript SK(−) vector system and transformed into *E. coli* XL1

blue MRF' cells. Both enzymes are found intracellularly. The enzymes were purified from the cell extracts by anion-exchange chromatography. For the β-galactosidase a consecutive gel permeation chromatography was necessary to obtain a purified enzyme. The physico-chemical properties and substrate specificity of both enzymes were determined. Galacto-oligosaccharides, necessary for characterizing the β-galactosidase's kinetic parameters, were prepared by partial degradation of potato galactan by an endogalactanase from *Aspergillus aculeatus*. At three different time points in the degradation process fractions were collected to get a range of oligosaccharides with different degrees of polymerization.

In total 16 mg of the β-galactosidase were obtained, with an activity of 1200 U (1 U = 1 μmol of galactose liberated per min in 20 mM phosphate buffer, pH 6, at 37°C). The physico-chemical properties of the enzyme are shown in Table I. It was stable at 40°C. A range of substrates was used to determine the substrate specificity of the β-galactosidase. From this experiment it was concluded that the β-galactosidase is highly specific towards β1-4 linked galacto-oligosaccharides.

As the β-galactosidase belongs to a class of retaining enzymes according to the family classification of Davies and Henrissat, its capacity to perform chain elongation by transglycosylation was tested (28). For this, the enzyme was incubated at high galactobiose concentrations, which favour transglycosylation reactions (29–31). It appeared that the β-galactosidase was inhibited under these conditions; therefore, the transglycosylation potential was estimated at lower galactobiose concentrations. At a substrate concentration of 10 mg/ml, only 5% of the enzyme–substrate encounters resulted in a transfer reaction, compared with 41% for bifidobacterium α-galactosidase at similar conditions. From this it was concluded that the β-galactosidase has a low transglycosylation activity.

The purification of the α-galactosidase yielded 3.56 mg of the pure enzyme (SDS-PAGE), which is equivalent to 83 U. The physico-chemical properties of the enzyme are shown in Table I. The substrate specificity of the α-galactosidase was tested with several substrates. The enzyme was able to hydrolyse melibiose, α-D-Galp-(1→3)-D-Galp, raffinose, α-D-Galp-(1→3)-β-D-Galp-(1→4)-D-Glcp, stachyose and verbascose. It was not active towards galactomannan-derived oligosaccharides.

As mentioned before the α-galactosidase possesses a high transferase activity. In a transfer reaction the non-reducing part of the donor molecules (often a monosaccharide unit) will be attached to the non-reducing end of a carbohydrate acceptor. The acceptor specificity for the α-galactosidase has been determined by testing different substrates, with melibiose as the galactose-donor. From these experiments it was concluded that substrates containing a pyranose ring and a CH_2OH group at the C-5 of the pyranose ring are good galactose-acceptors for the α-galactosidase.

The two enzymes discussed above are different enzymes in terms of their substrate specificity. The β-galactosidase has very low transferase activity, whereas the α-galactosidase is able to elongate oligosaccharides with relatively high efficiency. As the genome sequence of *B. longum* is available from the NCBI (www.ncbi.nlm.nih.gov/), much more information about the *Bifidobacterium* glycolytic enzymes is available. For a better understanding of the galactose metabolism the genome sequence will be investigated for galactan- and galacto-oligosaccharide-degrading enzymes. These enzymes will be cloned and over-expressed in *E. coli* cells to investigate their substrate specificity in detail. Subsequently, this information will be used for the development of better prebiotics.

CONCLUSIONS AND OUTLOOK

More insights and key findings on the impact of processing and storage on probiotic viability and stability have been gained. Furthermore, significant data have been accumulated on probiotic stress responses and on prebiotic function. Built on the data collected so far the database in processing-induced probiotic stresses and functionality, as well as on prebiotic function, will be enhanced further. Utilizing these data probiotic viability models and functionality of biomarkers will be established.

Table I

Physico-chemical properties of β-galactosidase and α-galactosidase from Bifidobacterium adolescentis

Properties	β-Galactosidase	α-Galactosidase
Specific activity towards *p*-nitrophenyl-β-D-galactopyranoside/ *p*-nitrophenyl-α-D-galactopyranoside (U/mg)	79.6	388
Molecular weight (kDa; SDS-PAGE)	81	83
Native molecular weight (kDa; Superdex S-200 column)	235	332
Proposed molecular structure	Trimer	Tetramer
Optimum pH	6.0	6.5
Optimum temperature (°C)	37	45

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