Novel technological approaches to enhance stress tolerance of Bifidobacterium longum NCC2705 cells using continuous cultures

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NOVEL TECHNOLOGICAL APPROACHES TO ENHANCE STRESS TOLERANCE OF
BIFIDOBACTERIUM LONGUM NCC2705 CELLS USING CONTINUOUS CULTURES

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presented by

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Summary

The efficient delivery of live cultures in high concentrations at their site of action represents a major challenge in probiotic product development. Application of probiotics as food additives is hampered by their fastidious production and their sensitivity to environmental stresses. Possibilities to produce more robust probiotics include exposition of the cells to sublethal stresses during production, and isolation of resistant strains under selective pressure. Based on the observation that continuous cultures have been used for producing cells with constant physiology over time (Hoskisson and Hobbs 2005) and also in combination with selective pressure for selection of resistant strains to antibiotics (Noack et al. 1988; Butler et al. 1996), two hypotheses were tested in this dissertation. First, that continuous culture could be used to produce cells with constant physiology over time to efficiently screen for sublethal stresses. Secondly, that continuous culture in combination with immobilized cell technology and selective pressure could be used for selection of stress resistant strains.

The prerequisite for using continuous culture in stress screening is the physiological stability of continuously produced cells. In Chapter 2, the potential of continuous culture mode with conditions set to produce late exponential growth phase cells for screening of sublethal stresses was assessed using *Bifidobacterium longum* NCC2705. Physiological parameters as viable cell counts, production of metabolites, susceptibility to antibiotics, and stress conditions showed either stable or only moderate changes over 211 h culturing time. The comparison of gene transcription profiles between samples collected after 31 h of continuous culturing, and those collected after 134 h, and 211 h revealed only limited changes in expression profiles i.e., 1.0 and 3.8 % of total genes, respectively. These minimal changes in time showed that continuous culture can be used to produce physiological stable bacterial cells suitable for fast and efficient screening of sublethal stress conditions.

In Chapter 3, a stable 2-stage continuous culture of *B. longum* NCC2705 was used for fast screening of sublethal stresses and to test the effects of such stresses on cell survival to lethal stresses. Different stress pretreatments of 42 min were tested in the second stage: combinations of pH (6.0, 5.0 and 4.0), temperature (37, 45 and 47°C) and NaCl concentration (0, 5 and 10%) were tested using a 3 by 3 factorial design. This 2-stage
continuous culture design allowed fast and efficient screening of several stress pretreatments during the same culture experiment, up to four different stress pretreatments tested per day with conditions of this study. Of all tested combinations, only those with pH 4.0 significantly affected cell viability compared to control conditions (37°C, pH 6.0, 0% NaCl), and thus could not be considered as sublethal stresses. Pretreatments with 5 or 10% NaCl had a negative effect on cell viability after gastric lethal stress. A significant improvement in cell resistance to a heat lethal stress (56°C, 5 min) was observed for cells pretreated at 47°C. In contrast, heat pretreatments negatively affected cell viability after freeze drying and osmotic lethal stresses. Selected stress pretreatments (pH 4.0; 47°C; 10% NaCl; 47°C+10% NaCl; and pH 4.0+10% NaCl) applied to early stationary phase cells during batch cultures produced similar effects compared to continuous culture, showing that continuous culture permits to select sublethal stress conditions which can be then applied for traditional batch cultures.

Another possibility to produce more robust probiotics is by selection of resistant strains during growth under selective pressure. Chapter 4 presents a new method to isolate resistant strains to oxidative stress using continuous culture. Continuous culture with selective pressure was combined with immobilized cell technology, which allowed to achieve very high cell densities in the bioreactor of $10^{13}$ CFU l$^{-1}$ without stress application. The continuous culture gradually adapted to increasing H$_2$O$_2$ concentrations, as shown by the optical density of culture effluent which dropped after each increase of H$_2$O$_2$ concentration and then increased again. However, at day 9 after increasing the H$_2$O$_2$ concentration to a high value of 130 ppm the OD of the culture decreased to 0. Full wash out was prevented by immobilization of cells in gel beads. Hence after stopping the stress, it was possible to re-grow the cells that survived the lethal level of H$_2$O$_2$ and to isolate two adapted variants (HPR1 and HPR2). In contrast to HPR1, HPR2 showed stable characteristics over at least 70 generations. HPR2 exhibited higher also tolerance to O$_2$ than non adapted wild type cells. Preliminary characterization showed that 2 genes coding for a protein with unknown function possessing trans-membrane domains and an ABC-type transporter protein were overexpressed in HPR2 cells. This study showed that continuous culture with cell immobilization is a powerful approach for selecting cells adapted to hydrogen peroxide.
In this dissertation, two novel approaches using continuous cultures for improving cell robustness of probiotic microorganisms were designed and experimentally validated. Continuous culture was successfully applied for screening sublethal stresses and, together with immobilized cell technology and selective pressure, was used for selection of *Bifidobacterium* cells resistant to oxidative stress. Our study opens new doors for technological optimization of sensitive strains and their utilization in food products.
Riassunto


Basandosi su queste osservazioni, questa dissertazione di dottorato presenta l’esame di 2 ipotesi per l’uso delle colture continue di probiotici. La prima ipotesi postulò che la coltura continua potesse essere usata per produrre cellule di probiotici con fisiologia costante nel tempo per fare uno screening di stress sub-letali. La seconda fu che la coltura continua in combinazione con la tecnologia di cellule immobilizzate e una pressione di selezione potesse essere usata per isolare ceppi resistenti allo stress. Il prerequisito per utilizzare la cultura continua per fare uno screening sugli stress è la stabilità fisiologica delle cellule prodotte con questo sistema.

Dopo un’introduzione al tema, il secondo capitolo di questa dissertazione presenta la verifica di questo prerequisito: il potenziale del metodo di coltura continua con parametri scelti per produrre cellule alla fine della fase esponenziale di crescita per fare uno screening di stress sub-letali è stato testato usando il batterio *Bifidobacterium longum* NCC2705. Il risultato principale fu che i parametri fisiologici come il numero di cellule vive, la produzione di metaboliti, la suscettibilità agli antibiotici e alle condizioni di stress rimasero stabili o mostraron solamente cambiamenti minori sulle 211 h di coltivazione. Inoltre, il paragone tra i profili d’espressione genetica tra i campioni raccolti dopo 31 h di coltura continua e quelli raccolti dopo 134 h e 211 h hanno rivelato solo lievi
cambiamenti nei profili d’espressione: solo, rispettivamente, 1.0 e 3.8 % del totale dei geni espressi erano indotti o repressi. Questi cambiamenti minimi nel tempo mostrano che la coltura continua può essere utilizzata per produrre cellule batteriche con fisiologia stabile adatte per fare uno screening veloce ed efficiente sugli stress sub-letali.

Nel terzo capitolo della dissertazione viene descritta questa ricerca di stress sub-letali atti a produrre bifidobatteri più robusti. Il primo di due stadi di un bioreattore permise di mantenere una coltura continua di *B. longum* NCC2705. Questi, in seguito, vennero sottoposti a diversi pretrattamenti di stress nel secondo stadio. L’efficacia di questo trattamento venne stabilita misurando la sopravvivenza delle cellule a stress letali. I pretrattamenti di stress consistevano in diverse combinazioni di pH (6.0, 5.0 e 4.0), temperatura (37, 45 e 47°C) e concentrazione di NaCl (0, 5, e 10 %). Queste combinazioni vennero applicate per 42 min usando uno schema fattoriale 3X3. La coltura continua a 2 livelli ha permesso di fare uno screening in modo veloce ed efficiente poiché, nelle condizioni usate in questo studio, fino a 4 pretrattamenti di stress potevano essere testati per giorno sulla stessa coltura . Tra tutte le combinazioni testate, quelle con pH 4.0 hanno influito sulla viabilità cellulare in paragone alle condizioni di controllo (37°C, pH 6.0, 0 % NaCl), e per questo non possono essere considerate come stress sub-letali. Pretrattamenti con 5 o 10 % NaCl hanno invece avuto un effetto negativo sulla viabilità cellulare unicamente dopo stress gastrici letali. Un miglioramento nella resistenza cellulare ad uno stress letale termico (56°C per 5 min) è stato osservato per cellule pretrattate a 47°C. Pretrattamenti di calore hanno avuto però un effetto negativo sulla viabilità cellulare dopo liofilizzazione e dopo un stress osmotico letale. Specifici pretrattamenti di stress (pH 4.0; 47°C; 10% NaCl; 47°C+10% NaCl; and pH 4.0+10% NaCl) vennero anche applicati su cellule all’inizio della fase stazionaria di crescita in colture in lotto (batch). Questo procedimento produsse effetti simili a quelli riscontrati in coltura continua, mostrando che essa permette di trovare gli stress sub-letali applicabili in seguito pure a tradizionali colture in lotto.

Un’altra possibilità di produrre probiotici più resistenti è l’isolamento di ceppi genetici durante una coltura sotto pressione di selezione. Nel capitolo 4 viene presentato un nuovo metodo per isolare ceppi resistenti allo stress ossidativo usando colture continue. Partendo dal presupposto che sottoporre i bifidobatteri a stress porterà all’apparizione di
ceppi più resistenti e che una densità cellulare più elevata porta ad una maggiore probabilità di mutazione; la coltura continua con pressione di selezione è stata combinata con la tecnologia di cellule immobilizzate, che ha permesso di raggiungere densità cellulari molto alte nel bireattore di $10^{13}$ CFU l$^{-1}$ senza applicazione di stress. Sottoposta a crescenti concentrazioni di perossido d’idrogeno ($H_2O_2$), la coltura continua si è adattata gradualmente, come mostrato dall’evoluzione della densità ottica. Questa diminuiva dopo ogni aumento in concentrazione di $H_2O_2$ ed in seguito aumentava nuovamente. Dopo 9 giorni, dopo aver aumentato la concentrazione di $H_2O_2$ a 130 ppm, la densità ottica della coltura è scesa a 0. La completa eliminazione delle cellule dal bioreattore è stata evitata grazie all’immobilizzazione delle cellule in sfere di gel. Quindi dopo aver interrotto lo stress è stato possibile ricoltivare le cellule sopravvissute e di isolare 2 ceppi (HPR1 e HPR2) adattati allo stress di $H_2O_2$. Paragonato a HPR1, HPR2 ha dimostrato di possedere caratteristiche stabili per almeno 70 generazioni. Inoltre, HPR2 ha anche dimostrato di possedere una più alta tolleranza all’$O_2$ che cellule del ceppo originale non adattate allo stress. Una caratterizzazione preliminare ha mostrato che HPR2 mostra un’espressione indotta di due geni che codificano uno per una proteina con regioni transmembranari e funzione sconosciuta e l’altro per una proteina da trasporto del tipo ABC. Questo studio ha mostrato che la coltura continua con cellule immobilizzate è un approccio efficace per selezionare cellule adattate al $H_2O_2$.

In conclusione, questa dissertazione descrive la progettazione e la validazione sperimentale di due nuovi approcci con coltura continua per migliorare la resistenza cellulare dei microorganismi probiotici. La coltura continua è stata applicata per fare uno screening di stress sub-letali e, insieme alla tecnologia di cellule immobilizzate e a una pressione di selezione, è stata usata per isolare cellule di *Bifidobacterium* resistenti allo stress ossidativo. Il nostro studio apre nuove vie per l’ottimizzazione tecnologica di ceppi probiotici sensibili e per la loro utilizzazione in prodotti alimentari.
1 Introduction

1.1 Human microbiota of the gastro-intestinal tract

The gastrointestinal tract (GIT) consists of stomach, small intestine (duodenum, jejunum and ileum) and large intestine (cecum, colon and rectum). The stomach generates a fasting gastric pH of 1.5, which increases to 3.0-5.0 during alimentation (Cotter and Hill 2003). It is therefore a hostile environment for most bacteria. It contains bacteria ingested with the food and those dislocated from the mouth, with bacterial counts of approximately $10^3$ to $10^6$ cells g$^{-1}$ of contents after meals and lowest (frequently undetectable) after digestion (Baron et al. 1996). In the duodenal and jejunal fluids cell counts of $10^3$ ml$^{-1}$ are found in most individuals. Further along the jejunum and into the ileum, bacterial counts begin to increase (Baron et al. 1996). The majority of the gastrointestinal microbiota is found in the large intestine where it reaches densities of $10^{11}$–$10^{12}$ cells ml$^{-1}$ of luminal content (Whitman et al. 1998). Bacteria of the gastro intestinal microbiota are estimated to be ten fold more numerous than the total number of cells in the human body (Palmer et al. 2007).

In humans at birth, the sterile intestine of newborns is contaminated by the mothers genital flora and/or by the environment. Alternatively, new studies indicate that bacterial transfer from the gut to mammary gland of the mother may be a source for maternal milk contamination and therefore of colonization of the intestine of newborns (Gueimonde et al. 2007; Perez et al. 2007). At first, the intestine of newborns is colonized by aerobic or facultative anaerobic bacteria. They change the gut environment by consuming oxygen after which anaerobic bacteria start colonization. The first anaerobes establishing in the gut are *Bifidobacterium*, *Clostridium* and *Bacteroides*. As more oxygen-sensitive species establish, the population sizes of aerobic and facultative aerobic bacteria decline (Palmer et al. 2007).

The human intestinal microbiota is a complex microbial environment. The main phylotypes found in the adult human intestinal tract includes Firmicutes, Bacteroidetes, Proteobacteria, Verrucomicrobia, Actinobacteria, Cyanobacteria and Fusobacteria (Eckburg 2005; Turroni et al. 2008). From an analysis of prokaryotic ribosomal RNA
gene sequences from intestinal samples, Eckburg et al. (2005) detected a majority of members of the Firmicutes and Bacteroidetes phyla, most (95%) of the Firmicutes sequences were members of the Clostridia class.

Gut bacteria interact with the host in a symbiotic relationship, where the host provides a nutrient-rich habitat and bacteria can bring important benefits on host’s health (Shanahan 2002). The GIT microbiota contributes to host nutrition by increasing the efficacy of energy harvest from diet (e.g. complex sugar breakdown) as well as by synthesizing essential vitamins (Turroni et al. 2008). Moreover, it provides a barrier against exogenous pathogenic bacteria, by occupying available habitats (adhesion sites) at the mucosal level, competing for metabolic substrates and producing antimicrobial factors including lactic acid, H$_2$O$_2$ and bacteriocins (Hao and Lee 2004; Shanahan 2002). On the other hand, GIT microbiota can also negatively influence host’s health by producing carcinogenic substances. Such substances can be produced from the anaerobic metabolism of peptides and proteins, degradation of sulfur-containing amino acids or bile acids deconjugation (Blaut and Clavel 2007). Besides, certain gastrointestinal diseases, such as inflammatory bowel disease, have been linked to malfunctioning GIT microbiota (Turroni et al. 2008).

### 1.2 Probiotics

#### 1.2.1 Definition

Probiotic comes from Greek and means “for life” and was initially used as an antonym of the word ‘‘antibiotic’’. However, it has been given different meanings over the years. Initially, probiotics were described as substances that promote health of malnourished patients, stimulate growth of other microorganisms, or contribute to intestinal microbial balance. More recent definitions commonly state that probiotics should include live microorganisms (Vasijevic and Shah 2008), as e.g. the frequently used definition of Fuller (1992), who defines probiotics as ‘‘a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance’’. According to recommendations of a FAO/WHO working group on the evaluation of probiotics in food, probiotics are defined as ‘‘live microorganisms that when administered in adequate amounts confer a health benefit on the host’’ (FAO/WHO 2002).
1.2.2 Criteria for selection of probiotics

There are a number of factors that are important for the selection of probiotic (Table 1). First of all probiotics need to be safe. Taxonomic classification gives an indication about the origin, habitat and physiology of a strain and is therefore a major criterion for the selection of probiotics. However, because the intestine of newborns is sterile and the origin of the human intestinal microbiota is therefore unclear (Vasijevic and Shah 2008), a FAO/WHO (2002) expert panel has suggested that the specificity of probiotic action is more important than the source of microorganism. Another important safety aspect is the presence of antibiotic resistance genes in probiotics strains. These genes, especially those encoded on plasmids, can be transferred to other microorganisms in the gastrointestinal tract, potentially leading to selection of antibiotic resistant pathogens in the GI tract.

Secondly, strains used as probiotics need to be suitable from a technological point. They must be culturable, viable and stable under industrial production conditions. Furthermore, probiotics need to fulfill some functional criteria including resistance to bile and acid and be able to survive and proliferate in vivo. Additionally, probiotics should show one or more desirable physiological criteria and health effects (Table 1).
Table 1 Selection criteria for probiotic strains (Klaenhammer and Kullen 1999; Vasijevic and Shah 2008)

<table>
<thead>
<tr>
<th><strong>Safety criteria</strong></th>
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<tr>
<td>Origin</td>
<td>Accurate taxonomic identification</td>
</tr>
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<td>Non-toxic, non-pathogenic, GRAS status</td>
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<td>Intrinsic properties: antibiotic resistances and metabolic activities</td>
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<th><strong>Technological suitability</strong></th>
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<td>Amenable to mass production and storage</td>
<td>Viability at high population density (preferred at $10^6$ – $10^8$ CFU ml$^{-1}$)</td>
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<td>Provides desirable organoleptic qualities (or no undesirable qualities)</td>
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<td>Genetically stable</td>
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<td>Phage resistant</td>
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<th><strong>Functional criteria</strong></th>
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<td>Resistant to bile and acid</td>
<td>Adhesion to mucosal surface</td>
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<td>Capable of survival, proliferation, and metabolic activity at the target site <em>in vivo</em></td>
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<td>Able to compete with the microbiota, including the same or closely related species, potentially resistant to bacteriocins, acid and other antimicrobials produced by residing microbiota</td>
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<td>Able to exert one or more clinically documented health benefits</td>
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<th><strong>Desirable physiological criteria and health effects</strong></th>
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<td>Antagonistic toward pathogenic/carcinogenic bacteria</td>
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<tr>
<td>Production of antimicrobial substances (bacteriocins, hydrogen peroxide, organic acids or other inhibitory compounds)</td>
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<tr>
<td>Production of bioactive compounds (enzymes, vaccines, peptides)</td>
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<td>Amelioration of the immune response</td>
<td></td>
</tr>
<tr>
<td>Alleviation of lactose intolerance</td>
<td></td>
</tr>
<tr>
<td>Decrease in serum cholesterol level</td>
<td></td>
</tr>
<tr>
<td>Prevention of certain types of diarrhea</td>
<td></td>
</tr>
</tbody>
</table>
1.2.3 Beneficial effects of probiotics

Various probiotic strains have been shown to alter (temporarily) the intestinal microbiota and/or to hinder colonization of the gut by (potential) pathogens, as well as translocation of pathogenic bacteria through the intestinal wall and the infection of other organs (de Vrese and Schrezenmeir 2008). Proposed beneficial health effects of probiotics, which are documented for certain strains, include prevention of certain types of diarrhea especially caused by rotavirus, reduction of antibiotic associated symptoms, decrease in the prevalence of allergy in susceptible individuals, amelioration of the immune response, relief of lactose intolerance and decrease in unfavorable metabolites, e.g., ammonium and procancerogenic enzymes in the colon (Schrezenmeir and de Vrese 2001; Saxelin et al. 2005; Parvez et al. 2006). There is some evidence of health effects through the use of probiotics for the following: prevention or reduction of the effects in chronic intestinal inflammation, reduction of the risk of acute diarrhoea in children, reduction of the risk of respiratory infections, suppression of Helicobacter pylori and reduction of serum cholesterol levels (Schrezenmeir and de Vrese 2001; Saxelin et al. 2005; Parvez et al. 2006).

Resuming probiotic bacteria may affect the intestinal microbiota and other organs by modulating immunological parameters, intestinal permeability, or by providing bioactive metabolites. Possible mechanisms of action were reviewed by de Vrese and Schrezenmeir (2008) and include: reduced intestinal pH; production of bactericidal substances (e.g. organic acids, H₂O₂ and bacteriocins); agglutination of pathogenic microorganisms; strengthening barrier function of the intestinal mucosa competition for fermentable substrates or receptors on the cellular surface of the mucosa; absorption and metabolism of potentially pathogenic, toxic, or cancerogenic metabolites and enzymes; modulation of immunological mechanisms; stimulation of the intestinal motility and mucus production.
1.3 Bifidobacteria

1.3.1 Family and genus Bifodobacteriaceae

In 1899 Tissier observed and isolated a Y shaped bacterium in the faeces of breast-fed infants and called it "Bacillus bifidus communis" (lat. Bifidus: Cleft, divided) (Biavati and Mattarelli 2001). However, due to similarities to lactobacilli, bifidobacteria were included in the genus Lactobacillus (Breed and Murray 1957) and only since the VIIIth edition of Bergey’s Manual of Determinative Bacteriology, they were reclassified as a separate genus (Buchanan 1974). Bifidobacteria are Gram-positive, anaerobic, non-motile, non-spore forming rods, which are usually curved and often in Y- or V-shapes. Unlike lactobacilli, they possess a fructose-6-phosphate phosphoketolase, no aldolase or glucose-6-phosphate dehydrogenase, and have a high G+C DNA content between 55 and 67%. Bifidobacteria are catalase-negative, with the exception of B. indicum and B. asteroides (Biavati and Mattarelli 2001).

The genus Bifidobacterium, consisting of 32 species (type species Bifidobacterium bifidum, Tissier; Orla- Jensen, 1924), and the genus Gardnerella (Greenwood and Pickett, 1980), with Gardnerella vaginalis as the only species, belong to the family of Bifidobacteriaceae. This family is classified in the order of Bifidobacteriales, in the subclass of Actinobacteridae, in the class of Actinobacteria, in the lineage of Firmicutes, and in the domain of Bacteria (Stackebrandt et al. 1997; Biavati and Mattarelli 2001).

1.3.2 Habitat

The habitat of bifidobacteria is mainly the intestinal tract of warm-blooded animals (Table 2). They are sometimes involved in certain human infective processes (mostly dental caries) but usually they are considered non-pathogenic (Biviati and Mattarelli 2001). Normally, Bifidobacterium species are specific for their human or animals host; the only exception being Bifidobacterium species found in the intestinal microbiota of both suckling calves and breast-fed infants (Table 2). Additionally bifidobacteria were found in sewage and in fermented milk products.
The sterile intestine of newborns humans is colonized by contamination with the mother’s genital flora and/or with the environment. Alternatively, new studies indicate that bacterial transfer from the gut to mammary gland of the mother may be a source for maternal milk contamination and therefore of colonization of the intestine of newborns (Gueimonde et al. 2007; Perez et al. 2007). The intestine of neonates has a positive redox potential and is then first colonized by facultative anaerobe bacteria (enterobacteria and Gram-positive cocci) which lower the redox potential (Favier et al. 2003). The decrease in redox potential then permits the colonization of the gastro intestinal tract (GIT) by bifidobacteria (Bezirtzoglou 1997).

Studies using Fluorescence In Situ Hybridisation (FISH) technology revealed that bifidobacteria comprise up to 75 % of the total faecal microbiota of formula-fed infants and up to 91 % of the breast-fed infants (Harmsen et al. 2000). The amount of bifidobacteria in the human GI-tract decreases during lifetime. The faecal microbiota of children is similar to that of an adult after approximate two years and bifidobacteria make up 10% of the gut flora in adults when measured by classic culture technique, and 1-3% when analyzed using molecular methodologies (Tannock 2002). In elderly people the bifidobacteria number is lower whereas the number of faecal clostridia rises (Roissart and Luquet 1994).
Table 2 References and habitat of species of the genus *Bifidobacterium* (Biavati et al. 2000; Ventura et al. 2004; Leahy et al. 2005; Klaassens 2007)

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Found in human GI</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. adolescentis</em></td>
<td>ATCC15703T</td>
<td>Infant and adult feces, appendix, dental caries and vagina</td>
<td>(Reuter 1963)</td>
</tr>
<tr>
<td><em>B. angulatum</em></td>
<td></td>
<td>Adult feces</td>
<td>(Scardovi and Crociani 1974)</td>
</tr>
<tr>
<td><em>B. bifidum</em></td>
<td>DSM 20215 JCM 1254 NCIMB 41171</td>
<td>Infant and adult feces and vagina</td>
<td>(Orla-Jensen 1924)</td>
</tr>
<tr>
<td><em>B. breve</em></td>
<td>UCC2003</td>
<td>Infant feces and vagina</td>
<td>(Reuter 1963)</td>
</tr>
<tr>
<td><em>B. catenulatum</em></td>
<td></td>
<td>Infant and adult feces and vagina</td>
<td>(Scardovi and Crociani 1974)</td>
</tr>
<tr>
<td><em>B. dentium</em></td>
<td>Bd1</td>
<td>Human dental caries, oral cavity and adult feces</td>
<td>(Scardovi and Crociani 1974)</td>
</tr>
<tr>
<td><em>B. gallicum</em></td>
<td></td>
<td>Adult feces</td>
<td>(Lauer 1990)</td>
</tr>
<tr>
<td><em>B. longum</em> bv. <em>infantis</em></td>
<td>ATCC 15697</td>
<td>Infant feces and vagina</td>
<td>(Reuter 1963; Sakata et al. 2002)</td>
</tr>
<tr>
<td><em>B. longum</em> bv. <em>longum</em></td>
<td>DIO10A</td>
<td>Infant and adult feces and vagina</td>
<td>(Reuter 1963; Sakata et al. 2002)</td>
</tr>
<tr>
<td><em>B. pseudocatenulatum</em></td>
<td>NCC2705</td>
<td>Human intestine</td>
<td>(Schell et al. 2002)</td>
</tr>
<tr>
<td><em>B. scardovii</em></td>
<td></td>
<td>Infant feces</td>
<td>(Scardovi et al. 1979)</td>
</tr>
<tr>
<td><em>B. thermophilum</em></td>
<td></td>
<td>Adult urine and blood</td>
<td>(Hoyles et al. 2002)</td>
</tr>
<tr>
<td><em>B. thermophilum</em></td>
<td></td>
<td>Piglet, chicken and calf feces and rumen, infant</td>
<td>(Mitsuoka 1969; von Ah et al. 2007)</td>
</tr>
<tr>
<td><strong>Found in animal GI</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. animalis</em> ssp. <em>animalis</em></td>
<td></td>
<td>Rat, chicken, rabbit and calf feces</td>
<td>(Scardovi and Crociani 1974; Masco et al. 2004)</td>
</tr>
<tr>
<td><em>B. asteroides</em></td>
<td></td>
<td>Honeybee</td>
<td>(Scardovi and Trovatelli 1969)</td>
</tr>
<tr>
<td><em>B. bouom</em></td>
<td></td>
<td>Rumen and piglet feces</td>
<td>(Scardovi et al. 1979)</td>
</tr>
<tr>
<td><em>B. choerimum</em></td>
<td></td>
<td>Piglet feces</td>
<td>(Scardovi et al. 1979)</td>
</tr>
<tr>
<td><em>B. coryneforme</em></td>
<td></td>
<td>Honeybee</td>
<td>(Biviati et al. 1982)</td>
</tr>
<tr>
<td><em>B. cuniculi</em></td>
<td></td>
<td>Rabbit feces</td>
<td>(Scardovi et al. 1979)</td>
</tr>
<tr>
<td><em>B. gallinarium</em></td>
<td></td>
<td>Chiken feces</td>
<td>(Watebe et al. 1983)</td>
</tr>
<tr>
<td><em>B. indicum</em></td>
<td></td>
<td>Honeybee</td>
<td>(Scardovi and Trovatelli 1969)</td>
</tr>
<tr>
<td><em>B. longum</em> bv. <em>suis</em></td>
<td></td>
<td>Piglet feces</td>
<td>(Matteuzzi et al. 1971)</td>
</tr>
<tr>
<td><em>B. magnum</em></td>
<td></td>
<td>Rabbit feces</td>
<td>(Scardovi and Crociani 1974)</td>
</tr>
<tr>
<td><em>B. merycicum</em></td>
<td></td>
<td>Rumen</td>
<td>(Biviati and Mattarelli 1991)</td>
</tr>
<tr>
<td><em>B. pseudolongum</em> ssp.</td>
<td></td>
<td>Piglet, calf, rat, rabbit, lamb</td>
<td>(Biviati et al. 1982)</td>
</tr>
</tbody>
</table>
### 1.3.3 Physiology

#### 1.3.3.1 Carbohydrates metabolism

Bifidobacteria are chemoorganotrophs having a fermentative type of metabolism. They produce acid from a variety of carbohydrates, mainly acetic and lactic acids, and no gases. The catabolism of hexoses in *Bifidobacterium* species involves a characteristic pathway known as the fructose 6-phosphate pathway (F6P) (Scardovi, 1964; Scardovi and Trovatelli, 1965; De Vries et al, 1967). The key enzyme of this pathway is fructose-6-phosphate phosphoketolase (EC 4.1.2.2) (Biavati and Mattarelli 2001) that catalyzes the decomposition of pentose phosphates, to acetyl phosphate and glyceraldehyde-3-phosphate. Fructose-6-phosphate phosphoketolase is encoded by the gene *xfp* (Meile et al. 2001). Hexoses undergo a series of cleavage and isomerisation reactions to produce pentose phosphates. The acetyl phosphate is converted to acetate, thereby yielding one ATP, whereas the glyceraldehyde-3-phosphate is converted to pyruvate and further reduced to lactate to regenerate NAD (Degnan and Macfarlane 1994). Initially, the calculated theoretical ratio of the F6P pathway was 3:2 (acetate/lactate) (Figure 1). However, pyruvate can also be converted to formate and acetyl-CoA (Figure 2), which is subsequently reduced to ethanol to oxidize the NADH produced earlier in the metabolic pathway (Devries and Stouthamer 1968).
Figure 1 fructose 6-phosphate shunt adapted from Biviati and Mattarelli (2001)
Figure 2 Schematic diagram of bifidobacterial sugar metabolism adapted from Van der Meulen et al. (2006a).
The control of dissimilation of pyruvate to lactate or ethanol is unclear, but it can be depending on carbohydrate availability. Degnan and Macfarlane (1994; 1995) observed that during carbon limitation, pyruvate is preferentially cleaved to acetyl-CoA and formate since one additional ATP can be formed, whereas with excess of substrate ATP formation is not a problem and pyruvate is reduced to lactate. Van der Meulen et al. (2006a) also observed that when the specific sugar consumption rate increased, relatively more lactic acid and less acetic acid, formic acid, and ethanol were produced, and vice versa.

Bifidobacteria are able to grow on a wide range of carbon sources (Table 3) including mono-, di-, tri- and oligo-saccharides. This gives bifidobacteria an ecological advantage in the intestinal environment where complex carbohydrates are present either because of production by the host epithelium (e.g. mucin) or introduction through the diet. This adaptation to complex carbohydrates is reflected in the genome of *B. longum* that encodes over 40 predicted glycosylhydrolases putatively involved in the degradation of higher order oligosaccharides (Schell et al. 2002; Parche et al. 2007).
Table 3 Growth capacity of *B. longum* NCC2705 on 23 carbohydrates (Parche et al. 2007)

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Growth capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Monocarbohydrates</strong></td>
<td></td>
</tr>
<tr>
<td>Arabinose</td>
<td>++</td>
</tr>
<tr>
<td>Fructose</td>
<td>+</td>
</tr>
<tr>
<td>Galactose</td>
<td>+++</td>
</tr>
<tr>
<td>Glucose</td>
<td>+++</td>
</tr>
<tr>
<td>Glycerol</td>
<td>-</td>
</tr>
<tr>
<td>Mannose</td>
<td>-</td>
</tr>
<tr>
<td>Mannitol</td>
<td>-</td>
</tr>
<tr>
<td>N-acetylglucosamine</td>
<td>-</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>-</td>
</tr>
<tr>
<td>Ribose</td>
<td>+</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>-</td>
</tr>
<tr>
<td>Xylitol</td>
<td>-</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>++</td>
</tr>
<tr>
<td><strong>Di/trisaccharides</strong></td>
<td></td>
</tr>
<tr>
<td>Maltose</td>
<td>++</td>
</tr>
<tr>
<td>Melibiose</td>
<td>++</td>
</tr>
<tr>
<td>Salicine</td>
<td>-</td>
</tr>
<tr>
<td>Sucrose</td>
<td>++</td>
</tr>
<tr>
<td>Trehalose</td>
<td>-</td>
</tr>
<tr>
<td>Lactose</td>
<td>+++</td>
</tr>
<tr>
<td>Raffinose</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Oligosaccharides</strong></td>
<td></td>
</tr>
<tr>
<td>Arabinogalactan</td>
<td>+</td>
</tr>
<tr>
<td>Raftilose (oligofructose)*</td>
<td>++</td>
</tr>
<tr>
<td>Starch</td>
<td>-</td>
</tr>
</tbody>
</table>

Growth capacity was scored as: slow growth (+), moderate growth (++) and fast growth (+++)

* Composition of raftilose was 93.2 % oligofructose and up to 6.8 % glucose, fructose and sucrose.

### 1.3.3.2 Optimal growth temperature and pH

Being bifidobacteria intestinal bacteria of warm blooded animals, their optimal growth temperature ranges between 37 and 41°C and the optimum pH at the beginning of the growth is between 6.5 and 7.0 (Biavati and Mattarelli 2001). Most species do not grow below 20°C and above 46°C and pH lower than 4.5 or higher than 8.5 with the exception of *B. thermacidophilum*, which is able to grow at pH 4.0 and at 49°C (Dong et al., 2000).
1.3.3 Oxygen sensitivity

Bifidobacteria are strict-anaerobic microorganisms and sensitive to oxygen. However, sensitivity to oxygen differs between species and strains (Biavati and Mattarelli 2001). Some Bifidobacterium strains have been described to grow in liquid media in the presence of oxygen. For example, B. lactis tolerates 10% oxygen in the headspace atmosphere above a reduced liquid media (Meile et al. 1997). Maxwell et al. (2004) showed that B. boum DSM 20432T grew to a similar optical density in air compared to anaerobic conditions. B. thermophilum was shown to be tolerant to exposure to air and grew under reduced oxygen atmosphere (Beerens et al. 2000; von Ah et al. 2007) and also a recently isolated Bifidobacterium, B. psychraerophilum, was found to be aerotolerant (Simpson et al. 2004). This species was even able to grow on agar plates under aerobic conditions. Bifidobacteria are sensitive to oxygen due to several reasons: some strains require a high redox potential or harbor enzymes (e.g. phosphoketolase) that are sensitive to hydrogen peroxide (Biavati and Mattarelli 2001). Hydrogen peroxide is a reactive oxygen species and it accumulates in bifidobacteria exposed to oxygen (Kawasaki et al. 2006). Although, bifidobacteria are all catalase-negative when grown under anaerobic conditions, two species tolerant to air, B. indicum and B. asteroides, become catalase-positive when grown in the presence of air, with or without addition of hemin, respectively (Scardovi and Trovatelli 1969).

1.3.4 Bifidobacteria as probiotics

The use of bifidobacteria as probiotics is to ascribe to their natural habitat in the intestine during their complete life cycle. Their presence in the GIT has been associated with a number of beneficial health effects as e.g. reduction of diarrhoea, relief of lactose intolerance, resistance to microbial infections, prevention of cancer, treatment of inflammatory bowel disease, alleviation of constipation, elevated immune function, and reduction of serum cholesterol (Leahy et al. 2005). However, mechanisms of action for the majority of these effects are not yet elucidated.
1.3.4.1 Reduction of diarrhoea

Acute diarrhoea in children is often caused by rotavirus. Beneficial effects of different probiotics, including bifidobacteria, such as decreased frequency of infections, shortening of the duration of episodes have been observed (de Vrese and Schrezenmeir 2008; Goldin and Gorbach 2008).

A broad range of microbial pathogens causes traveller’s diarrhoea. These include *Escherichia coli*, *Salmonella*, *Campylobacter* and *Shigella* strains as well as viruses. Even though *in vitro* and animal studies confirmed that some probiotic strains inhibit growth and metabolic activity as well as the adhesion to intestinal cells of enteropathogenic bacteria like *Salmonella*, *Shigella* or *Vibrio cholerae*, few studies have been published demonstrating positive effects in humans (Leahy et al. 2005; de Vrese and Schrezenmeir 2008).

The frequency and/or duration of episodes of antibiotic-associated diarrhea and the severity of symptoms may be reduced by administration of probiotics, including *Bifidobacterium* strains, before and during antibiotic treatment (de Vrese and Schrezenmeir 2008).

1.3.4.2 Relief of lactose intolerance

Individuals with lactose maldigestion tolerate lactose in yogourt clearly better than in milk. A possible mechanism for this is that yogourt-bacteria release high amount of lactase (β-galactosidase) when lysed by bile salts in the gastrointestinal tract (Goldin and Gorbach 2008). Also bifidobacteria have been shown to reduce abdominal bloating, pain, flatulence and nausea in individuals with lactose intolerance (Leahy et al. 2005). Delivery of lactase to the intestine via the consumption of lactase-producing probiotics is a practical approach for treatment of lactose intolerance.
1.3.4.3 *Inhibition of Helicobacter pylori*

*H. pylori* infection is treated by antibiotic therapy and probiotics administration is most probably not sufficient to eradicate *H. pylori*. Administration of probiotics concomitantly to antibiotic treatment may resolve problems associated with side effects (Vasijevic and Shah 2008). Several mechanisms regarding the effect of probiotics on *H. pylori* have been suggested including production of organic acids, hydrogen peroxide or bacteriocins, enhanced gut barrier function and competition for adhesion sites; however, the relative importance of these mechanisms is still unclear (Leahy et al. 2005).

1.3.4.4 *Prevention of inflammatory bowel disease*

Inflammatory bowel disease (IBD) comprises a spectrum of disorders characterized by inflammation, ulceration and abnormal narrowing of the gastrointestinal tract resulting in abdominal pain, diarrhoea and gastrointestinal bleeding (Vasijevic and Shah 2008). Probiotics, including bifidobacteria, with anti-inflammatory properties and a demonstrated positive impact on the intestinal flora have shown promising effects against IBD in studies with experimental animals (de Vrese and Schrezenmeir 2008).

1.3.4.5 *Alleviation of constipation*

Clinical studies showed that administration of certain probiotic, including bifidobacteria reduced gastrointestinal transit time (de Vrese and Schrezenmeir 2008). Nevertheless, more controlled clinical studies with sufficient numbers of participants are necessary to confirm these results and the mechanisms of action need to be elucidated.

1.3.4.6 *Prevention of colorectal cancer*

Data for the efficacy of probiotics against colorectal cancer is derived from the use of animal models. Probiotics, including some *Bifidobacterium* strains were shown to indirectly lower carcinogenicity by decreasing bacterial enzymes that activate carcinogenesis and by producing short-chain fatty acids, which reduce activities of enzymes involved in the generation of carcinogenic products (Vasijevic and Shah 2008).
1.3.5 Pathogenesis of bifidobacteria

Many positive effects have been assigned to bifidobacteria, however since most bifidobacteria are common members of the human intestinal microbiota, they may behave as opportunistic pathogens, like other commensal bacteria (Saarela et al. 2002). In fact, bifodobacteria have been isolated in cases of infections and dental caries. *B. longum* was isolated from blood after a sepsis (Ha et al. 1999). Other *Bifidobacterium* sp. have been found in pulmonary infections, bacteremia, abscesses and bloodstream infections (Green 1978; Gasser 1994; Saarela et al. 2002). *B. dentium, B. subtile, and B. breve* have been isolated from human dental caries (Modesto et al. 2006; Mantzourani et al. 2009). However, until now, probiotic bifidobacterial strains have never been recovered in infections following ingestion of dairy products and can therefore be considered no health hazards (Meile et al. 2008). More problematic in probiotic strains are antibiotic resistance genes since the potential of a transfer to intestinal bacteria, including pathogenic ones, is highly probable (Meile et al. 2008). Kastner et al. (2006) detected the tetracycline resistance gene *tet(W)* in *B. lactis* and suggested that strains used for probiotic or starter cultures should be tested for the presence of transferable resistance genes prior to commercial use.
1.4 Stress

Bifidobacteria have to survive a number of harsh conditions to reach the GIT in sufficient amounts to be effective as probiotic (Figure 3). Some of these conditions are already encountered during fermentation. The composition of the growth medium and production of toxic by-products may have an influence on the viability of probiotic strains. Moreover, industrial processes during starter handling, downstream processing, and storage, including freeze drying, freezing and spray drying, cause mechanical, and physiological stresses (De Dea Lindner et al. 2007; Lacroix and Yildirim 2007). Additionally, after ingestion with the functional food, conditions like acidic pH in the stomach and bile salts in the small intestine influence the number of living bifidobacteria at their target site (Sanchez et al. 2008). Survival of bifidobacteria during these steps, and thus their functionality as probiotic, depends on their capacity of adaptation and resistance to these different adverse conditions.

Figure 3 Main factors affecting the viability of probiotics from production to the gastrointestinal tract (Lacroix and Yildirim 2007).
1.4.1 Stress response

Various reviews about stress responses in bifidobacteria have been published in the last years (Ventura et al. 2006; De Dea Lindner et al. 2007; Sanchez et al. 2008). Additionally, analysis of the *B. longum* complete genome sequence has revealed the presence of several different regulatory mechanisms by which the cells protect themselves against various stresses (Klijn 2005b).

A common regulatory mechanism in stress response at the level of transcription of genes depends on sigma factors. Sigma factors are relatively small proteins that direct binding of the RNA polymerase to the promoter region. The sigma factor greatly reduces the affinity of the RNA polymerase for nonspecific DNA, while increasing specificity for certain promoter regions. Most bacteria possess more than one sigma factor, each with its own promoter sequence, allowing regulation of gene expression. The presence of a specific sigma factor allows the RNA polymerase enzyme to bind at a specific promoter sequence on the chromosome, and thereby starting transcription of downstream genes (Abee and Wouters 1999). One sigma factor (σ^A^) is known as the “house-keeping” sigma factor and it regulates transcription of the majority of genes. However, under stress conditions, alternative sigma factors with different promoter specificities are induced (Chung et al. 2006), leading to the higher expression of genes involved in stress response. The number of alternative sigma factors can range from 0 to over 50 (Helmann 2002; Pridmore et al. 2004). The common regulatory mechanism of sigma factors themselves includes an anti-sigma factor that prevents the sigma factor from binding to the RNA polymerase. When the anti-sigma factor receives a stimulus from the environment, it releases the sigma factor thereby activating differential gene expression (Helmann 2002). In most cases the sigma and anti-sigma factors are autoregulated as an operon. However in *B. longum* NCC2705, BL1357 (a possible alternative sigma factor) and BL1358 (a possible anti-sigma factor) are regulated differently. BL1358 was over-expressed under stress conditions, whereas BL1357 was constitutively transcribed (Klijn 2005).
Most stresses cause denaturation, misfolding and aggregation of proteins. Molecular chaperones bind and stabilize polypeptides generated by protein synthesis or by denaturation of proteins, thereby promoting protein proper assembly, initial folding, and refolding (Hartl 1996). Proteins that are irreparably damaged are degraded by proteases. Bifidobacteria possess a smaller set of chaperones/proteases than found in other bacteria (De Dea Lindner et al. 2007), which include representatives of Hsp100 (Heat shock proteins 100), Hsp70, Hsp60 and small Hsps. In bifidobacteria, the members of the Hsp100 protein family, ClpB, ClpC and the ClpP protein are present, which in the Clp complex exerts proteolytic activity on misfolded proteins (De Dea Lindner et al. 2007). Also proteins belonging to the Hsp70 (DnaK) and Hsp60 (GroEL) families are present. These molecular chaperones interact with co-chaperone molecules (e.g. DnaJ and GrpE in the case of DnaK, or GroES in the case of GroEL) and form the DnaK and GroEL complexes (Bukau and Horwich 1998). The genes encoding for the DnaK complex are located within the dnaK operon, encompassing the dnaK, grpE, dnaJ1 and hspR genes (De Dea Lindner et al. 2007). The genes groESL are generally organised in an operon (Segal and Ron 1996), but this is not the case in bifidobacteria.

Small heat shock proteins (sHSPs) are characterized by a relatively low molecular mass, ranging between 14 and 27 kDa. They prevent irreversible protein denaturation of heat-damaged proteins (Narberhaus 1999). The sHSPs act as ATP-independent chaperones by binding to denaturated proteins and maintain them in a folding-competent state (Lee et al. 1997). One representative (hsp20) of the small heat shock proteins (sHSPs) was found in the genome of B. breve UCC2003 and B. longum NCC2705 (De Dea Lindner et al. 2007; Ventura et al. 2007).

During aminoacid starvation and also after stress with sufficient physiological impact to prevent proper charging of tRNA with amino acids bacteria activate the stringent response. RelA, a protein situated in the membrane, activates (p)ppGpp synthesis. The unusual guanidine nucleotide (p)ppGpp binds to the RNA polymerase thereby repressing transcription. Further it can also induce specific sigma factor (Potrykus and Cashel 2008). However, in B. longum NCC2705, BL1439, similar to relA, was not induced under stress (oxidative stress, starvation and heat shock) (Klijn 2005a).
General stress genes are thought to provide non-specific protection to the cell in the event of adverse conditions, whereas additional genes can be transcribed in response to a specific type of stress. Expression profiles from a range of different environmental stresses (oxidative stress, starvation and heat shock) were used to identify genes implicated in the general stress response of \textit{B. longum} NCC2705 (Klijn et al. 2005a). The \textit{dnaK} operon (\textit{dnaK}, \textit{grpE}, \textit{dnaJ}, \textit{hspR}), known to participate in the common stress response in other bacteria (Narberhaus 1999), was higher expressed during all three stresses; as were several regulatory genes such as \textit{hspR}, \textit{hrcA} and the putative anti-sigma factor. The most rapidly and highly induced gene in the three stress situations was the small heat shock protein encoded by BL0576 (\textit{hsp20}) (Klijn et al. 2005a).

1.4.2 Heat stress response

The production of bifidobacteria for incorporation into food requires survival of the microbes during processes that involve heat shock treatments such as spray drying. Therefore, understanding the genetic basis of heat stress response might be crucial for selecting new strains with probiotic properties (De Dea Lindner et al. 2007). High temperatures induce principally protein denaturation but membranes and nucleic acids have also been identified as cellular sites of heat injury. When cells are exposed to heat stress, responses occur via increased synthesis of chaperones, which promote the correct folding of nascent polypeptides, assembly of protein complexes, degradation and translocation of proteins (De Angelis and Gobbetti 2004).

Genome sequence analysis revealed that the strain \textit{B. breve} UCC2003 possesses two groups of chaperones, one related to the response to moderate increase of temperature, up-shift of 5 °C, (\textit{groEL}, \textit{groES}, \textit{clpC} and \textit{clpP1P2}) and the other involved in the response to more severe increases, up-shift of 13 °C, (\textit{dnaK}, \textit{grpE}, \textit{dnaJ1} and \textit{clpB}) (Ventura et al. 2006).

Global transcriptome analysis of the heat shock response of \textit{B. longum} NCC2705 highlighted a profound modification of gene expression upon exposure to 50°C, resulting in a slow-down of the general metabolic activity and activation of the classical heat shock stimulon. The \textit{dnaK} operon (BL0516–BL0520), which includes genes encoding the major
molecular chaperones DnaK, GrpE, DnaJ as well as the transcriptional repressor HspR, was among the highest and most rapid induced operons. The genes encoding the second major molecular chaperone system, groEL (BL0002) and groES (BL1558) and the corresponding transcriptional regulator HrcA (BL0718), were also induced but at lower levels (Rezzonico et al. 2007). Using protein labeling in combination with two dimensional gel electrophoresis an accumulation of the molecular chaperones GrpE, ClpA/B, HtrA, DnaK and GroEL in the cytoplasm of B. longum was detected during heat stress (Savijoki et al. 2005). Additionally, it has been shown in bifidobacteria that gene hsp20, coding for a protein member of the small heat shock protein family, is strongly induced in response to severe heat shock regimens, by oxidative shock and starvation (Klijn et al. 2005a; Rezzonico et al. 2007; Ventura et al. 2007).
1.4.3 Osmotic stress response

Increasing osmotic pressure in the environment provokes the activation of osmoregulation systems to prevent shrinkage and eventual plasmolysis of the cell (Chung et al. 2006). Accumulation of non-toxic low molecular weight compounds including sugars, polyols, amino acids and amine derivatives allow the cell to retain positive turgor pressure, enhance enzyme stability at low aw, and maintain the integrity of the cellular membrane during desiccation (Girgis et al. 2002). Additionally, osmotic stress, comparably to heat stress leads to the induction of synthesis of proteins. In bifidobacteria regulation of different chaperone proteins was observed. Induction of the *dnaK* gene after heat, osmotic (NaCl), and bile treatments was observed in *B. adolescentis* (Schmidt and Zink 2000). In *B. breve* the homologous gene *dnaK* and also *dnaJ1*, *grpE* and *clpB*, were induced during osmotic and severe heat treatment, suggesting the existence of an overlapping regulatory network controlling both osmotic and (severe) heat induced genes (De Dea Lindner et al. 2007). However, in *B. longum dnaK* was induced only after heat treatment (Schmidt and Zink 2000). In *B. breve* HspR and HrcA were induced upon osmotic treatment (De Dea Lindner et al. 2007). The *hsp20* gene is induced after heat and also after osmotic stress, which appears to be a unique expression pattern because in other bacteria small HSP-encoding genes are not induced upon exposure of the cells to osmotic stress (Ventura et al. 2007). In the intestine, bifidobacteria are not exposed to significant temperature changes, whereas they may encounter frequent changes in osmotic conditions as a consequence of diet composition. It is therefore possible that HSP20 in bifidobacteria acquired an osmotic functionality as a consequence of the adaptation to the human intestinal ecological niche (Ventura et al. 2007). All the studies mentioned in this paragraph have been performed using NaCl, which causes osmotic stress but may also affect the the ionic balance of the cells.
1.4.4 Bile stress response

The ability to resist to bile salt stress is extremely variable among bifidobacteria (De Dea Lindner et al. 2007). Bile salts are detergent-like biological substances that disrupt the lipid bilayer structure of cellular membranes and cause oxidative damages to the cells (Payne et al. 1998). Enteric bacteria defend themselves from bile salt stress with the production of chaperones proteins that prevent or repair protein misfolding and repair oxidative damaged DNA (Bernstein et al. 1999).

Proteome analysis in *Bifidobacterium* revealed an increase in the chaperone synthesis as a response to, or adaptation to, bile salts (Sanchez et al. 2005; Sanchez et al. 2007b). For example, a strain of *B. animalis* subsp. *lactis* resistant to bile salts showed a constitutive over-expression of the chaperones HtrA and ClpB. Furthermore, the adaptation to bile salts by *B. animalis* subsp. *lactis* also resulted in the constitutive overexpression of several enzymes involved directly or indirectly in redox reactions, especially those that catalyse the last two steps of methionine biosynthesis, whose sulphur group is susceptible to oxidization (Sanchez et al. 2008).

Another way of protection against bile salts was found in *B. longum* where a cholate efflux system was involved in resistance to bile salts, chloramphenicol and erytromycin (Price et al. 2006). Detoxification of bile salt by hydrolase (BSH) could have an influence on bile salt resistance since the deconjugated form of bile salt has a strong effect on cell viability. However, to date there is no solid evidence of a relationship between this enzyme and the resistance level to bile salts (Grill et al. 2000).

Generally it was shown in bifidobacteria that bile salts produce changes in the synthesis of a large number of proteins, involved in transcription and translation, and in the metabolism of amino acids and nucleotides, and several enzymes of glycolysis and in pyruvate catabolism, indicating that such compounds affect the global metabolism of bifidobacteria (Sanchez et al. 2005; Sanchez et al. 2007b). Furthermore it was observed that bifidobacterial strains showing the highest resistance to bile salt exposure were also acid-resistant and therefore it was concluded that in bifidobacteria bile—as well as acidic stresses are inducing the synthesis of a very similar arsenal of molecular players (Noriega et al. 2004).
1.4.5 Gastric conditions and low pH stress response

Humans generate a fasting gastric pH of 1.5, which increases to between pH 3.0 and 5.0 during alimentation. To reach the small intestine in a viable state, bacteria will need to survive these highly acidic conditions. Hence, acid tolerance can be considered a virulence factor in pathogens, whereas it is a desirable property of potentially probiotic strains (Cotter and Hill 2003). Beside, fermentative bacteria, including bifidobacteria, produce large amounts of organic acids, mostly acetic and lactic acids, as end products of sugar metabolism and must therefore cope with acid conditions during growth (De Dea Lindner et al. 2007). Acid conditions have a negative impact on bacteria by reducing the cytoplasmic pH, which can result in loss of activity of the relatively acid-sensitive glycolytic enzymes and to structural damage of the cell membrane and macromolecules such as DNA and proteins. Mechanisms to counteract the effect of low pH include efflux of protons (H⁺), alkanization of the external environment, changes in the composition of the cell envelope, production of general shock proteins and chaperones, expression of transcriptional regulators, and responses to changes in cell density (Cotter and Hill 2003). The F₁F₀-ATPase proton pump is one of the main mechanisms for the survival of Gram positive microorganisms in acidic environments and therefore plays a key role in bifidobacteria as well. In bacteria that do not possess a respiratory chain and thus are not capable of using the F₁F₀ complex for synthesis of ATP via oxidative phosphorylation, the sole function of this complex is the extrusion of protons and consequently the establishment of pH homeostasis. In bifidobacteria the role of the F₁F₀-ATPase complex for survival to low pH has been mentioned in *B. animalis* and *B. longum* (Ventura et al. 2004; Sanchez et al. 2006). Remarkably, acid-adapted *B. longum* cells maintained their intracellular pH close to appropriate physiological values compared to non-adapted cells in acidic conditions (Sanchez et al. 2007a).
1.4.6 Oxygen stress response

Bifidobacteria are strict anaerobes (Biavati and Mattarelli 2001), they possess neither a respiratory chain nor catalase. Hence exposure to oxygen causes accumulation of reactive oxygen species, mainly hydrogen peroxide, which lead to cell death of oxygen sensitive bifidobacteria (Kawasaki et al. 2006). Reactive oxygen species (ROS) include superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical (•OH). Superoxides are formed through spontaneous univalent electron transfer from molecular oxygen and hydrogen peroxide is produced out of the enzymatic or spontaneous dismutation of superoxide. Hydroxyl radicals are generated by the reaction of iron with H$_2$O$_2$ (the fenton reaction) (Kiley and Storz 2004). Enzymatic reactions involved in the reduction of molecular oxygen are catalized by NADH oxidase for the regeneration of NAD$^+$ from NADH. There are two types of NADH oxidases, one catalyzes the reduction of O$_2$ to H$_2$O$_2$, the other one carries out the four-electron reduction of oxygen to water (Condon 1987; Talwalkar and Kailasapathy 2004). Bifidobacteria most probably reduce O$_2$ via the four electron reduction to water. Genome sequence’s analysis of _B. longum_ NCC2705 revealed an open reading frame, BL1266, encoding a putative NADH oxidase, which seems to contain an active site similar to one in _Enterococcus faecalis_ (Mallett and Claiborne 1998; Klijn 2005a). Short lived O$_2^-$ can damage DNA, membrane lipids and proteins, particularly those that contain a (Fe-S)$_4$ cluster. Elimination of this ROS can be achieved by superoxide dismutase, superoxide reductase and intracellular accumulation of manganese. H$_2$O$_2$ causes DNA damage and oxidation of proteins. Catalases, NADH peroxidases and reductases can eliminate this ROS. At last, formation of the extremely reactive hydroxyl radicals may be prevented by quenching H$_2$O$_2$ or sequestering free iron (Imlay 2002; Klijn 2005a). Klijn (2005a) showed that the response to oxidative stress of _B. longum_ NCC2705 includes intracellular accumulation of manganese and up-regulated expression of genes coding for alkylhydroperoxide subunit C, thioredoxin, glutaredoxin and the ferritin-like protein Dps. Additionlly, the specific role of proteins like BL1626 (a class I pyridine nucleotide-disulphide oxidoreductase superfamily), BL0460 (a possible OxyR-type regulator) and BL0409 (a putative manganese transporter) in the oxidative stress response remains to be established.
1.5 Methods to improve cell viability

The most important aspect of the functionality of probiotic cultures is their ability to promote human health at the site of action. Although some studies show that dead probiotic cells may mediate physiological benefits, most of the studies with probiotic cells have been performed with live cells and data on non-viable preparations is often limited (Ouwehand and Salminen 1998). The effects of dead cells as probiotics are probably limited to the release of antimicrobial compounds and stimulation of the immune system (Ouwehand and Salminen 1998; van Baarlen et al. 2009). Additionally, according to the widely accepted definition of probiotic by a joint FAO/WHO working group (2002) probiotics must be alive. Therefore it is crucial that cells survive the acid conditions of the stomach and the exposure to bile salts in the small intestine in sufficient numbers in order to be effective. The efficient delivery of live cultures at their site of action represents a major challenge in probiotic product development. Industrial standards require that products claiming health benefits contain a minimum of $10^6$ viable probiotic bacteria per gram of product when consumed in order to achieve a specific health effect and/or to provide bacterial concentrations that are technologically attainable and cost effective (Lacroix and Yildirim 2007). However, large scale production and subsequent storage of probiotic lactobacilli and bifidobacteria presents a major bottleneck in the realization of their commercial potential, because of high losses in viability due to confrontation with various stresses (Ross et al. 2005). Moreover, most probiotic strains are intestinal isolates and therefore obligate anaerobe and difficult to cultivate. For all these reasons, the ability of probiotics to tolerate heat, osmotic and oxygen stress is an important property for successful incorporation of probiotic bacteria into functional foods.
Probiotic cultures can be protected by encapsulation and by addition of protective compounds. Different encapsulation methods have been applied to probiotics: spray-coating, extrusion, emulsion and gel-particle technologies for protection against various stresses, such as heating, freezing, exposure to gastric and bile solutions and for improving survival during storage (Picot and Lacroix 2004; Doleyres and Lacroix 2005; Champagne 2006). These techniques have been recently reviewed by Champagne and Fustier (2007). Addition of protective compounds during growth or prior to further processing has been investigated mainly in regard to survival to spray and freeze drying. Many compounds, including for example trehalose, sucrose, gum acacia, gum arabic and starch were shown to have protective effects (Desmond et al. 2002; Lian et al. 2002; Saarela et al. 2005; Morgan et al. 2006; Termont et al. 2006). Additives such as carbohydrates, proteins and polymers protects against drying because they promote the formation of a glassy state, thereby inhibiting crystallization and concentration of waste products from the cells (Morgan et al. 2006). Furthermore, the physiological state of the probiotic cultures added to a product can also be a major factor affecting culture viability. Stress tolerance of cells can be induced by exposition to moderate stresses inducing tolerance to homologous or other stresses (cross-protection). In this respect, the induction of stress responses in probiotic strains can have an effect on the ability of cultures to survive during processing (e.g. freeze drying and spray drying) and gastric transit (Ross et al. 2005).
1.5.1 Application of sublethal stresses to exploit stress response mechanisms to improve technological performance of bifidobacteria

Recently, research efforts have focused on understanding the stress response mechanisms of lactobacilli and bifidobacteria in order to improve their capacity to survive and to function under industrial production conditions and after ingestion.

Acid-tolerance of bifidobacteria varies widely, but may be significantly increased by application of sublethal stress. *B. lactis* showed an increased acid-tolerance (at pH 3.5 in synthetic gastric fluid) after the pH was decreased to 5.2 in early stationary phase and cells experienced starvation (Maus and Ingham 2003). However, sublethal stress may also result in decreased cell survival, for example acid and heat pretreatment impaired the acid tolerance of *B. longum* (Saarela et al. 2004).

Adaptation to bile salts as well as cross protection against bile salts were found in some *Bifidobacterium* strains. In *B. longum* and *B. adolescentis* preconditioning with bile salts protected against otherwise lethal bile salts concentrations (Schmidt and Zink 2000). In an other study *B. animalis* cells tolerated more bile when pretreated at 47°C or at pH 3.5 and *B. longum* showed a similar effect, although the improved resistance to bile was not statistically significant in this strain, showing that optimization capability is strain-specific (Saarela et al. 2004).

Microorganisms exposed to a moderate heat treatment can acquire the transient ability to withstand subsequent lethal heat challenges (Girgis et al. 2002) and the control of such resistance of probiotic bacteria has potential practical benefits for industrial processes in which bacteria with high thermotolerance are required (Ross et al. 2005). *B. adolescentis* was able to survive an otherwise lethal temperature when subjected to a heat stress pretreatment or, salt stress pretreatment (Schmidt and Zink 2000). Similarly, *B. longum* stationary phase cells pretreated at 47°C tolerated clearly better 55°C compared to non-sublethally treated cultures (Saarela et al. 2004).
Adaptation to osmotic stress in bifidobacteria or lactic acid bacteria was only investigated to some extent. In *B. adolescentis* salt pretreatment resulted in increased tolerance after freeze–thawing cycles or lethal heat stress, however this was not the case in *B. longum* (Schmidt and Zink 2000). Recently, it was observed that osmotic stress induced by salt increased survival to lyophilization of *Lactobacillus delbrueckii* subsp. *lactis* (Koch et al. 2007).

Another stress that probiotics encounter during preparation and storage of functional food is oxygen stress. Viability of micro-organisms in fermented dairy foods is strongly influenced by oxygen content in the product in addition to oxygen permeation through the package (Talwalkar and Kailasapathy 2004). To date adaptation to oxidative stress has not yet been investigated in bifidobacteria.

1.6 Fermentation basic principles

1.6.1 Batch operation

The most common industrial fermentation is batch fermentation. In this process, a reactor is first loaded with medium, inoculated with the selected microbial strain(s), and fermentation is operated until the required cell density or optimum product concentrations are achieved. The bioreactor content is subsequently discharged and the bioreactor is prepared for a new charge of medium. It is only during cell growth or product production, that the bioreactor is productive. During the period of cell growth no additional material is either added to or removed from the bioreactor, apart from minor adjustments for pH- or foam-control, small additions of essential precursors, the removal of samples and, continuous supply of air in the case of an aerobic fermentation. Since there is no flow in or out of the bioreactor concentrations of biomass, nutrients and products change in time and various constituents are either produced or consumed (Dunn et al. 2003).
1.6.2 Continuous operation

In continuous operation fresh medium is continuously added to the bioreactor, whereas
simultaneously depleted medium is continuously removed at identical rate. The depleted
material contains any products that have been excreted by the cells and, in the case of
suspended-cell culture, also contains effluent cells from the bioreactor. After a transient
period in which conditions within the bioreactor change in time, a steady state is
achieved, characterized by constant conditions, both within the bioreactor and at the
bioreactor outlet (Dunn et al. 2003).

In continuous culture the specific growth rate of an organism, relative to its theoretical
maximum, is governed by the external substrate concentration of a limiting nutrient,
allowing uncoupling of growth from the transient conditions encountered in batch
culture. Addition of fresh medium to the culture would allow growth to proceed at a
given rate indefinitely. Since growth of new cells is balanced by those washed out, the
growth of new biomass is equal to the rate at which the culture is being diluted
(Hoskisson and Hobbs 2005): Therefore, in steady state, the growth rate is a function of
the dilution rate, provided that the growth rate is below the critical dilution rate \( D_c \). \( D_c \) is
the rate at which the steady-state biomass concentration becomes zero. That is, if \( D_c \) is
higher than the maximum specific growth rate \( \mu_{\text{max}} \) of the organism under the given
conditions then all cells wash out of the reactor.

Continuous culturing has some disadvantages over batch culture (Table 4). However, the
recent trend toward global (post-genomic) assessments of microbial processes has led to
the more frequent use of continuous cultures. The advantage of using continuous cultures
in such studies lies in the removal of secondary growth effects, as found in batch cultures,
that may mask subtle physiological changes, revealing real biologically relevant trends,
and giving confidence in the data obtained. Additionally, continuous cultures in steady
state have constant conditions making it possible to produce cells with constant
physiology. The biological significance of continuous culture is occasionally questioned;
however, many natural microbial systems, such as the mouth and the gut, can be
considered as approaching chemostats (Hoskisson and Hobbs 2005).
Table 4 Comparison of batch and continuous cultures (Dunn et al. 2003)

<table>
<thead>
<tr>
<th>Mode of operation</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch</td>
<td>Equipment simple. Suitable for small production.</td>
<td>Downtime for loading and cleaning. Reaction conditions change with time.</td>
</tr>
<tr>
<td>Continuous</td>
<td>Provides high production. Better product quality due to constant conditions. Good for kinetic studies.</td>
<td>Requires flow control. Culture may be unstable over long periods.</td>
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1.6.3 Immobilized cells

The immobilization of cells can be defined as “the physical confinement or localization of intact cells to a certain region of space without loss of desired biological activity” (El-Mansi et al. 2007). Immobilized cell systems can be classified into four categories (El-Mansi et al. 2007):

1) Surface attachment of cells. Absorption to a support material can be achieved naturally (via the Van der Waals forces and ionic interactions) or induced artificially using linking agents (metal oxides or covalent bonding agents such as glutaraldehyde)

2) Entrapement within porous matrices through in situ immobilization in presence of the porous matrix (i.e. gel) or by allowing the cells to move into a preformed porous matrix. Cells incorporated into porous gel spheres or lodged in porous resin, steel mesh, and glass beads.

3) Containment behind a barrier. The barrier can be either preformed (hollow fiber and flat membrane reactors) or formed around the cells to be immobilized (microcapsules and two-phase entrapment).

4) Cell agglomerated together either chemically (glutaraldehyde) or naturally (microbial flocs)

Cell entrapment in food-grade biopolymer gel matrices (e.g. k-carrageenan, alginate and gellan) has been most widely used for food applications (Lacroix et al. 2005). Diverse culturing technologies, including different applications with immobilized cells, for the production of probiotics with high viability and functionality have been reviewed
recently (Lacroix and Yildirim 2007). Immobilized cells have numerous advantages over free-cell cultures: high cell densities, reuse of biocatalysts, higher resistance to contaminations and bacteriophages, improvement of plasmid stability, prevention of washing-out during continuous cultures, and the physical and chemical protection of cells (Lacroix et al. 2005). Cell growth occurring in the peripheral layers of gel beads has been shown to give a high rate of cell release into the culture broth, as a result of high pressure owing to cell expansion, collisions and shearing forces in the bioreactor, leading to an efficient inoculation of the medium (Lamboley et al. 1997).

Cell immobilization in probiotic production was tested with B. longum cells immobilized in gellan gum beads. It allowed production of high cell concentrations in continuous culture and a fourfold increased volumetric productivity compared to free-cell batch cultures at optimal pH (Doleyres et al. 2002). Furthermore it was shown that immobilization of a mixed culture containing a dominant Lactococcus lactis strain and a less competitive B. longum strain allowed stable continuous production of concentrated mixed cultures (Doleyres et al. 2004a).
1.7 Transcriptomics

The transcriptome is the set of all messenger RNA (mRNA) molecules, or "transcripts," produced in one or a population of cells. Because it includes all mRNA transcripts in the cell, the transcriptome reflects the genes that are being actively expressed at any given time, with the exception of mRNA degradation phenomena (Wang et al. 2009). For this reason the transcriptome of a cell contains information on the biological state of the cell. Additionally, since many cellular processes are regulated at the transcriptional level it contains also information on the genes playing a role in a definite situation. Transcriptomics studies the effect of specific conditions on alterations in the expression levels of large numbers of genes. To realize this, the transcripts present in cells obtained from different samples are quantified by microarray analysis (Figure 4). Using transcriptomic it is possible to monitor the whole genome on a single chip so that researchers can have a picture of the interactions among thousands of genes simultaneously. Since the expression levels are determined for all the genes on the microarray, this method is highly unbiased. This is an important advantage over traditional methods, which generally work on a "one gene in one experiment" basis in process driven by hypothesis based on prior knowledge (Brown and Botstein 1999). Therefore transcriptome analysis can give new insights into general cellular mechanisms, for example into how bacteria cope with environmental stresses. Within the genus Bifidobacterium the first genome sequence, that of B. longum NCC2705, became publicly available in 2002 (Schell et al. 2002) and a microarray covering its whole genome is available at Nestlé Research Center in Lausanne, Switzerland.
1.7.1 Microarray analysis

An array is a small glass slide containing thousands of spots in an orderly arrangement. Usually each spot represents one gene of the strain of interest and consists of a gene or gene fragment that has been spotted or printed on the slide. It provides a medium for matching known and unknown DNA samples based on base-pairing or hybridization rules (i.e., A-T and G-C for DNA; A-U and G-C for RNA). There are two major application forms for the DNA microarray technology: 1) Identification of sequence (gene / gene mutation); and 2) Determination of expression level (abundance) of genes. For the second application (i.e. determination of expression level) RNA is isolated from cells grown or incubated under conditions of interest, after which it is converted into labeled cDNA or amplified RNA. The labeled cDNA’s are hybridized to the microarray. In a certain type of microarrays (e.g. Affymetrix) labeled cDNA from a single sample is hybridized onto one microarray, in others (e.g. Agilent 2-color microarrays) two samples, usually labeled with fluorescent Cy3 and Cy5, are co-hybridized on one microarray (Figure 4). Since the labeled cDNA’s will hybridize to the equivalent spots on the microarray, the total amount of signal from each spot is a measure for the level at which the corresponding genes were expressed (Pieterse 2006).
Figure 4 Main steps of 2-color DNA microarray analysis
1.7.2 Signal quantification and data filtering

After hybridization, the fluorescent signals and background signals from the different labels in the different spots on the array are quantified by confocal laser scanning. After the quantification of the signal the data are filtered according to their quality (signal to background ratio, homogeneity and saturation of the spots). Data from the unreliable spot, or in extreme cases from the complete microarray, may be removed from the dataset.

1.7.3 Normalization

The next step in the data handling is normalization of the data. There are a number of reasons why data must be normalized, including unequal quantities of starting RNA, differences in labeling or detection efficiencies between the fluorescent dyes used, and systematic biases in the measured expression levels (Quackenbush 2002). The most frequently used normalization approach, is the LOWESS normalization (Cleveland 1979). LOWESS is a locally weighed scattered plot smoother. It detects systematic deviations in the ratio to intensity plot and corrects them by carrying out a local weighted linear regression as a function of the log_{10} (intensity) and subtracting the calculated best-fit average log_{2} (ratio) from the experimentally observed ratio for each data point (Quackenbush 2002).

1.7.4 Data analysis

After data normalization, a large set of data containing significant expression and fold-change of expression values for each gene need to be interpreted. Mainly, there are two approaches to analyze these data, an unsupervised and supervised approach (Gollub and Sherlock 2006). The unsupervised approach consists in cluster analyses and is independent of biological knowledge. Biological interpretation of these data is based on the assumption that similar expression patterns indicate related biological function (Brazma and Vilo 2001). Example of unsupervised approaches often used in microarray analyses are hierarchical clustering, K-means clustering and selforganizing- maps
(SOM), and principle component analysis (PCA) (Ehrenreich 2006). These methods may generate significantly different results because the underlying mathematical methodology differs. In contrast to the unsupervised, the supervised approach compares the data with existing knowledge. For example, microarray data can be plotted on genomic maps using softwares as e.g. the microbial genome viewer (Kerkhoven et al. 2004). Alternatively, microarray data can be plotted on metabolic maps using the online database encyclopaedia of genes and genomes KEGG (http://www.genome.ad.jp/kegg/) (Kanehisa 1997; Stevens 2008).


1.8 Thesis outline

Central issues in the application of probiotics as food additives are their fastidious production and their sensitivity to environmental stresses. Furthermore, it is essential that probiotic bacteria are alive and present at high concentrations in the final product for most of their beneficial health effects on the host.

One possibility to produce more robust probiotics against typical stresses encountered during food production and storage is to expose the cells to sublethal stresses during production. Cell responses are strain-dependent and vary with applied stresses (Maus and Ingham 2003; Saarela et al. 2004; Simpson et al. 2005). However, the optimization procedure for sublethal stress application is generally performed using batch cultures, which can be very time consuming and can only be used for testing a limited number of conditions. Continuous cultures could be a suitable and more efficient method to test different stress factors on one culture instead of repeating several batch cultures (Lacroix and Yildirim 2007), but this strategy has never been investigated. The prerequisite for using continuous cultures for stress screening purposes during the same culture experiment is the physiological stability of continuously produced cells.

Another possibility to produce more robust probiotics is by selection of resistant strains after growth under selective pressure. Resistance to oxidative stresses, such as hydrogen peroxide, is of particular interest in bifidobacteria, because oxidative stresses can severely compromise production at industrial scale of sensitive strains as well as their incorporation into food products.

*Bifidobacterium longum* NCC2705 is an ideal model organism for both approaches. Its genome was completely sequenced (Schell et al. 2002), and an oligo-based microarray was developed by Nestlé. Using this microarray, the transcriptional response of *B. longum* NCC2705 has been well characterized, particularly into processes such as stress response, metabolic control, and regulatory mechanisms (Klijn 2005a). This knowledge was applied for the screening and application of stresses in this dissertation.
All this considered, the general objective of this work was thus to produce stress adapted cells of *B. longum* NCC2705 with continuous and batch cultures. More specifically, the objectives were:

1) Develop and validate a new screening method for sublethal stresses using continuous culture

2) Identify effective conditions for sublethal stress application

3) Isolate stress resistant strains using continuous culture and immobilized cell technology

Hence, the dissertation was organized as follows: in **chapter 2**, the question of physiological stability of *B. longum* NCC2705 under continuous culture conditions was addressed using physiological tests combined with transcriptomic analysis. In **chapter 3**, continuous culture was used for controlled application and efficient screening of sublethal stress conditions. This new approach was validated in a two stage continuous culture with *B. longum* NCC2705 cells and results were compared with classical batch cultures. Finally in **chapter 4**, continuous culture in combination with immobilized cells was applied for producing probiotic bacteria with enhanced technological and physiological characteristics. More specifically, a new method for selection of resistant bifidobacteria cells to hydrogen peroxide using continuous culture and immobilized cell technology was evaluated.
2 Physiological stability of *Bifidobacterium longum* NCC2705 under continuous culture conditions

2.1 Abstract

The biological stability of a continuous culture of *Bifidobacterium longum* NCC2705 was investigated to assess the potential of this culture mode for screening of sub-lethal stresses promoting stability of probiotic bacterial strains. A continuous culture was maintained for 211 h at a dilution rate of 0.1 h\(^{-1}\), mimicking a late-exponential growth phase culture. Biological stability over time was assessed by measurement of physiological parameters (i.e. biomass production, sugar metabolism, resistance to different stress factors) and by genome-wide transcriptional profiling. Stable viable cell counts were measured over the 211 h culture period, decreasing only moderately from 8.8 to 8.6 log\(_{10}\) CFU ml\(^{-1}\). A slight shift in production of metabolites, characterized by increased lactate and decreased acetate, formate and ethanol concentrations, was also observed. Measurements of different antibiotic susceptibilities and stress conditions showed either stable (cefotaxim, ampicillin, ceftazidime) or only moderate changes (simulated gastric juices, heat, bile salts, tetracycline, chloramphenicol, penicillin, vancomycin and neomycin) over culturing time. Comparison of gene transcription profiles between samples collected after 31 h of continuous culture and samples collected after 134 and 211 h revealed only limited changes in expression of 1.0 and 3.8 % of total genes, respectively. Based on these results we propose that continuous culture can be used to produce bacterial cells displaying stable physiological properties suitable for fast and efficient screening of sub-lethal stress conditions.

2.2 Introduction

Probiotics are defined as “living micro-organisms which, when administered in adequate amounts (as part of food), confer a health benefit to the host” (FAO/WHO 2002). The most commonly used probiotic strains in functional foods belong to the genera of
Lactobacillus and Bifidobacterium. Members of these genera, such as Bifidobacterium longum, are major colonizers of the human gastro-intestinal tract (Vaughan et al. 2002). A central issue in the use of probiotics in food and food supplements is their sensitivity to many environmental stress factors. It has been recommended that probiotic food products should contain at least $10^6-10^7$ viable cells per gram or ml at the time of consumption in order to achieve a specific health effect and/or to provide bacterial concentrations that are technologically attainable and cost effective (Champagne et al. 2005; Lacroix and Yildirim 2007). Probiotic bacteria must also survive the harsh environment of the gastrointestinal tract. In this context, a better understanding of underlying survival mechanisms and stress responses of probiotic cells, e.g., encountered during production, storage, ingestion and passage through the gastrointestinal tract is fundamental in production process resolutions leading to improved probiotic stability.

Several approaches have been recently proposed for enhancing probiotic cell viability under adverse conditions (Ross et al. 2005). It was reported that the resistance of cells to a lethal stress can be improved by application of homologous or heterologous sub-lethal stress during culture (Doleyres and Lacroix 2005). Culture conditions must be determined for each strain tested because cell responses are strain dependant and vary with applied stresses (Maus and Ingham 2003; Saarela et al. 2004; Simpson et al. 2005). This screening procedure is generally performed using batch cultures. Continuous cultures could be a suitable and more efficient method testing different stress factors on one culture instead of repeating several batch cultures (Lacroix and Yildirim 2007). A possible design for this system could be a two-stage culture process in which sub-lethal stressing conditions are applied in the second stage, continuously fed by cells from the first reactor which can be used as non adapted control cells. This system can stabilize rapidly after changing conditions in the second reactor, allowing screening of several sub-lethal stresses in one culture experiment and a short time frame (Lacroix and Yildirim 2007). In addition, bacterial growth rate can be accurately controlled in continuous culture by setting the dilution rate (Hoskisson and Hobbs 2005). However, before testing stresses using continuous cultures, the physiological stability of continuously produced cells over a considered time period must be first evaluated.
In this study, the effect of continuous culture time on cells of *B. longum* NCC2705, a probiotic model strain whose genome has been sequenced (Schell et al. 2002) was evaluated by monitoring physiological parameters including biomass production, sugar metabolism and resistance to stresses and combined with genome wide expression analysis.

2.3 Material and methods

2.3.1 Strain and growth conditions

*B. longum* NCC2705 was obtained from the Nestlé Culture Collection (NCC) and cultivated in MRS (De Man et al. 1960) medium (Biolife, Milano, Italy) supplemented with filter sterilized cysteine at a final concentration of 0.5 g l\(^{-1}\) (C-MRS). Two consecutive pre-cultures were performed at 37°C for 16 h under anaerobic conditions (AnaeroGen, Oxoid, Basingstoke, United Kingdom).

2.3.2 Continuous culture experiment

A continuous culture was carried out in a 2.5-l reactor (Bioengineering AG, Wald, Switzerland), equipped with a Biospectra control system (Biospectra AG, Schlieren, Switzerland) and containing 2 l of C-MRS inoculated with 2 % (v/v) preculture. The temperature was maintained at 37°C and the pH at 6.0 by addition of 5 M NaOH. The culture was stirred constantly at 250 rpm using two rushton type propellers. Anaerobic conditions were maintained by flushing the headspace of the reactor with CO\(_2\). After 8 h in batch mode the culture was run in continuous mode at a dilution rate of 0.1 h\(^{-1}\). Fresh medium was added using a peristaltic pump (Alitea, Bioengineering AG, Wald, Switzerland), and fermented broth harvested with a second peristaltic pump (Alitea, Bioengineering AG, Wald, Switzerland) set at a slightly higher flow rate. A stabilization period of 90 h (corresponding to nine reactor volume changes) was operated prior culture monitoring (t=0). Samples were collected three times a day for cell counts, viable cell percentages, metabolites production and cell resistance to stresses. Cells for stress tests were prepared by centrifuging 20 ml samples at 6,000 g, 4°C for 15 min. The pellet was...
resuspended to its initial volume in fresh C-MRS supplemented with 10 % (w/v) glycerol, aliquoted in 2 ml, snap frozen in liquid nitrogen, and stored at -80°C for stress test experiments. Aliquots of 2 ml taken at t=31, 134 and 211 h were centrifuged (4,000 g, 1 min, room temperature) for transcriptomic analysis. Supernatants were discarded and cell pellets snap frozen in liquid nitrogen and stored at -80°C until RNA-extraction. Samples were centrifuged at 4,000 g for 1 min at room temperature and the supernatants were frozen at -80°C for HPLC analysis of glucose, organic acids and ethanol.

2.3.3 Viable cell enumeration and viability assessment

For viable cell counts, fresh samples were diluted in phosphate buffer saline 1x (pH 7.7) supplemented with 0.05 % cysteine (C-PBS), appropriate dilutions were plated in duplicate on C-MRS agar and incubated anaerobically at 37°C for 48 h. Cell viability was also assessed using flow cytometry. To determine total and non viable cells, culture samples (2 ml) were centrifuged 1 min at 4,000 g at room temperature, washed with C-PBS, centrifuged again, resuspended and diluted 1:1000 in C-PBS. To 500 μl of the cell suspension, 0.5 μl SYBR Green I (10,000X, Molecular Probes, Invitrogen, Basel, Switzerland) and 20 μl 7AAD 0.0005 % w/v (Viability Dye, Beckman Coulter, Nyon, Switzerland) were added. The mixtures were incubated for 15 min in the dark and then analyzed using a Cytomics FC 500 flow cytometer (Beckman Coulter International SA; Nyon, Switzerland) equipped with an air-cooled argon ion laser emitting 20 mW at 488 nm combined with a 635 nm red-diode laser and the standard filter setup. The forward and side scatter signals were used as trigger signal in the 488 nm band-pass filter. The green fluorescence of SYBR Green I was detected through a 525 nm band-pass filter (FL 1 channel) and the red fluorescence of 7AAD collected in the FL 4 channel through a 675 nm band-pass filter. The rate settings were adjusted to keep the count to approximately 3,000 events s⁻¹. Data were collected on a logarithmic scale and analyzed using the CXP software (Beckman Coulter International SA, Switzerland). Discrimination between viable and non-viable cells was carried out according to fluorescence intensities of both dyes.
2.3.4 HPLC analysis

Concentrations of glucose, organic acids (lactate, acetate and formate) and ethanol in the broth samples were determined by HPLC. One ml of supernatant sample was diluted 1:5 in MilliQ water and filtered through a 0.45 µm nylon membrane filter (Infochroma, Zug, Switzerland). Separation of compounds was carried out using an Aminex HPX-87-H column (Bio-Rad Laboratories, Reinach, Switzerland) with 10 mM H2SO4 as eluent at a flow rate of 0.6 ml min⁻¹ and a temperature of 40°C. Compounds were detected using a refractive index detector (Merck Hitachi, Darmstadt, Germany). Reported data are means of duplicate analysis.

2.3.5 Survival tests

The parameters for survival tests were adjusted according to literature and results from preliminary experiments in order to obtain losses in cell concentrations between 1 and 3 log10 units during the tests.

Bile salts

Bile salts tolerance was assessed according to Saarela et al. (2004) with slight modifications. Porcine bile extract (Sigma-Aldrich, Buchs, Switzerland) was used to simulate human bile (Mallory et al. 1973). A 2 ml bacterial culture sample was thawed at room temperature for 30 min and subsequently centrifuged at 4,000 g for 2 min at room temperature. The pellet was washed with C-PBS, centrifuged again, resuspended in 2 ml C-PBS and cooled on ice for 30 min. A volume of 30 µl of cell suspension was added to 270 µl of filter sterilized 1.5 % porcine bile extract in C-PBS in a 96-well microtiterplate (Orange Scientific, Braine-l’Alleud, Belgium) and incubated anaerobically (AnaeroGen) at 37°C for 10 min. Cell counts before and after stress application were determined by plating on C-MRS agar appropriate dilutions of cell suspension in C-PBC.
Gastric juice

Cell survival in simulated gastric conditions was determined according to Charteris et al. (1998b). Two ml samples were thawed at room temperature for 30 min and centrifuged (4,000 g, 2 min, room temperature). The pellets were washed, resuspended in 2 ml 0.1 % (w/v) filter sterilized peptone (Becton Dickinson, VWR International AG, Dietikon, Switzerland) and cooled on ice for 30 min. A volume of 30 µl of cell suspension was added to 270 µl of filter sterilized simulated gastric juices containing 0.5 % (w/v) NaCl, 0.3 % (w/v) pepsin (0.7 FIP-U mg⁻¹) from porcine gastric mucosa (Merck, Zug, Switzerland) at pH 2.8 in a 96-well microtiterplate. After addition of the suspension the pH increased to 3.1. Following incubation at 37°C under anaerobic condition (AnaeroGen) for 15 min, the gastric juice-cell mixture was 10-fold serially diluted in C-PBS to neutralize the acidic pH and plated on C-MRS agar. Cell counts were also determined before stress application.

Heat

Heat survival tests were carried out according to Simpson et al. (2005). Two ml samples were centrifuged (4,000 g, 1 min, room temperature), washed and diluted in C-PBS to a final concentration of 10⁶ - 10⁷ cells ml⁻¹. One hundred µl of the diluted sample was filled into a 200 µl PCR tube and kept on ice for 30 min. Heat stress was applied in a thermocycler (Biometra T-personal, Biolabo Scientific Instruments SA, Chatel-St.-Denis, Switzerland) at 56°C for 5 min. After heat stress, samples were immediately cooled down by storing on ice for 15 min. Samples were then diluted five folds in C-PBS and analyzed by flow cytometry. Data are expressed as loss in percentage of live cells.
Antibiotic
Antibiograms were determined as previously described by Doleyres et al. (2004b) using an antimicrobial disc susceptibility assay, with some modifications. Samples were diluted in C-MRS to reach a final concentration of about $10^6$ CFU ml$^{-1}$ and plated with a cotton bud on C-MRS agar plates. After drying the plates, antibiotic discs (0.6 cm diameter) containing neomycin (10 μg), cefotaxime (30 μg), ampicillin (10 μg), penicillin G (10 μg), chloramphenicol (30 μg), vancomycin (30 μg), ceftazidime (30 μg) or tetracycline (30 μg) were added onto the surface. The inhibition zone was measured after incubation under anaerobic conditions at 37°C for 48 h.

2.3.6 Microarrays
Microarray design and RNA extraction
DNA based arrays, produced by Agilent Technologies (www.agilent.com), were obtained by in situ synthesis of 60 mer oligonucleotides on glass slides (Wolber et al. 2006). For each gene, 3 to 6 different probes were randomly distributed on the array. Total RNA was extracted with the Macaloïd method and purified as previously described (Parche et al. 2006).

Array hybridization
For each hybridization, cDNA was synthesized starting from 4 μg of total RNA and subsequently labeled using the Array 900MPX Genisphere kit (Genisphere Inc., Hatfield, PE, USA), following the protocol provided by the supplier. Luciferase and kanamycin control mRNA (Promega, Zurich, Switzerland) at 1 and 10 ng, respectively, were mixed with total RNA before labeling to allow balancing of the two channels during scanning. After the hybridization procedure, array slides were scanned at 10 μm using a Scanarray 4000 (Packard Biochip Technologies, Billerica, MA, USA). Parameters (laser power and photomultiplier tube gain) were set in order to prevent saturation of any spot, except the probes corresponding to rRNA.
**Array analysis**

Data extracted with Imagene 5.6 (Biodiscovery, El Segundo, CA, USA) were treated with homemade scripts in Python language (www.python.org) and a local installation of the ArrayPipe web server (Hokamp et al. 2004). Probes showing a signal smaller than twice the standard deviation of the local background were considered without signal. Probes showing no signal or saturated signals in both channels were discarded from the analysis. Assuming an intensity-dependent variation in dye signal, (limma) loess global normalization was applied on signal ratios. To calculate average gene expression values, data from different probes and hybridization duplicates (with dye swap) were combined as follows. Within each hybridization data set, gene fold changes were calculated from the median of the corresponding probes values. The expression value of a gene was retained if a signal was detected in at least 50% of its probes. The average gene expression values were then calculated by combining data from two independent hybridizations. Genes were considered to be differentially expressed if their log₂-transformed signal ratios were higher than 1.5 or smaller than -1.5. The data have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number **GSE14896**.

**Classification of B. longum genes into functional categories and operon prediction**

*B. longum* NCC2705 genes were classified into clusters of orthologous groups (COG) according to Rezzonico et al. (2007). Briefly, percentages of differentially expressed genes in each COG functional category were calculated by dividing the number of induced/repressed genes in each category by the total number of retained genes in the corresponding category. *B. longum* genes organized in operons were identified using the method published by Price et al. (2005) (http://www.microbesonline.org/operons/OperonList.html). Statistical analysis of the differential expression observed in each COG category was based on hyper-geometric distribution. The p-values assigned to the different COG categories express the significance of the difference observed between the number of differentially expressed genes measured in a given COG category and the overall level of differential expression.
2.3.7 Statistical analysis

The effect of culture time on cell numbers, metabolite concentrations and survival rate to stresses was tested with regression analyses using SigmaPlot 9.0 (Systat Software Inc., USA) and t-tests. The slope of the regression curve over time was tested for its difference from 0 using a $t$-statistic test. The tested parameters were considered to change over time when the regression slope had a p-value smaller than 0.05. A t-test was performed for each parameter between the first 3 samples collected during the first 30 h of fermentation and the last 3 samples collected during the last 30 h of fermentation. Data with p-values smaller than 0.05 were considered to be significantly different.
2.4 Results

2.4.1 Growth parameters and metabolism

Cell production and sugar metabolism of *B. longum* NCC2705 during 211 h continuous culture are presented in Figures 5 and 6, respectively. During culture, viable cells counts and viability percentages decreased from 8.8 to 8.6 log_{10} CFU ml^{-1} (p=0.0020) and from 95.1 to 92.1 % (p=0.0050), respectively (Figure 5). Glucose, initially present at 14.0 ± 0.2 g l^{-1} in the fresh medium, was totally consumed and not detected by HPLC in the fermented broth (detection limit of 0.2 g l^{-1}). The concentration of the main metabolite, acetic acid, decreased from 9.2 to 8.4 g l^{-1} (p=0.0223) between 0 and 211 h of continuous culture, while lactic acid production increased from 1.3 to about 2.8 g l^{-1} during the first 130 h (p <0.0001) and then decreased slightly but significantly (p=0.0052) to 2.2 g l^{-1} at 211 h (Figure 6). During the same period formic acid and ethanol concentrations also slightly but significantly decreased from 2.5 to 2.2 g l^{-1} (p=0.0003) and from 1.0 to 0.8 g l^{-1} (p=0.0013), respectively.
Figure 5 Viable cells counts of *B. longum* NCC2705 measured with plate counts (○) and cell viability measured with flow cytometry (percentages of viable (■) and non-viable (□) cells) during 211 h continuous culture. Time effects were tested by linear regression analyses. Sampling times (i.e. 31, 134 and 211 h) for cell transcriptomic analyses are indicated by vertical lines (---). Time 0 on the graph corresponds to time after 90 h stabilization of continuous culture.

Figure 6 Concentrations of lactic (○), formic (●) and acetic (△) acids and ethanol (◆) during continuous culture of *B. longum* NCC2705 and time effects tested by linear regression analyses. Sampling times (i.e. 31, 134 and 211 h) for cell transcriptomic analyses are indicated by vertical lines (---). Time 0 on the graph corresponds to time after 90 h stabilization of continuous culture.
2.4.2 Cell survival during stress tests

Cells collected at different time points were exposed to various stress conditions including simulated gastric juices, heat, bile salts, and to different antibiotics to analyze the physiological stability of the culture over time. A slight but significant increase of bacterial susceptibility to gastric (p=0.0340) and heat (p=0.0266) stresses was measured with increasing culture time (Figure 7). In contrast, the resistance to bile salts stress increased (p=0.0193) over time, characterized by a reduction of bile salts-induced loss of viability from 4.0 to 2.9 log_{10} CFU ml^{-1} between 0 and 211 h of culture (Figure 7).

The bacterial susceptibility to the β-lactam antibiotics cefotaxim, ampicillin and ceftazidime remained constant over culture time, with inhibition zones of 3.8 ± 0.3, 3.9 ± 0.2 and 4.1 ± 0.2 cm, respectively. A slight but significant increase in the susceptibility to tetracycline (inhibition zones increasing from 4.1 to 4.6 cm, p<0.0001), chloramphenicol (from 3.9 to 4.4 cm, p=0.0006), penicillin (from 3.9 to 4.3 cm, p=0.0020), and vancomycin (from 3.4 to 3.6 cm, p=0.0368) was observed over culture time (Figure 8). In contrast, the tolerance of B. longum NCC2705 cells to neomycin increased with culture time, illustrated by a decrease of the inhibition zone from 1.0 to 0.6 cm (p=0.0001) (Figure 8).
Figure 7  A) Viability loss after gastric (Δ) and bile salts (●) tests of *B. longum* NCC2705 cells produced during continuous culture. Values are given as loss of $\log_{10}$ (CFU ml$^{-1}$). B) Viability loss after heat test (●) is given in percentage of loss in viable cells measured by flow cytometry. Time effects are assessed by linear regression analyses. Sampling times (i.e. 31, 134 and 211 h) for cell transcriptomic analyses are indicated by vertical lines (---). Time 0 on the graph corresponds to time after 90 h stabilization of continuous culture.
2.4.3 Transcriptomic analysis

To gain insight in the physiological response of cells during continuous cultivation, genome-wide microarray-based gene expression analysis was performed on continuously produced cells collected at different time points (31, 134 and 211 h of cultivation). The sample collected after 31 h was used as a common reference. Overall, 1610 and 1282 genes (i.e. about 93 and 74 % of all genes covered by the microarray were expressed in cells collected at 134 and 211 h, respectively. The number of differentially expressed genes exhibiting a log₂-transformed average signal ratio greater than 1.5 or smaller than -1.5 increased with culture time from 18 (134 h vs. 31 h) to 65 (211 h vs. 31 h), corresponding to 1.0, respectively, 3.8 % of the total number of genes (Table 5). Only functional COG categories L (replication, recombination and repair) and G (carbohydrate
transport and metabolism) showed significant differences in percentages of up-regulated, respectively down-regulated, genes (Figure 9).

**Genes involved in replication, recombination and repair (COG L)**
The percentage of under-expressed genes involved in replication, recombination and repair increased from 3 to 11 % (corresponding to 2 to 6 genes, respectively) between 134 and 211 h (Figure 9). The genes BL1147 and BL0127, coding for a DNA primase and a DNA polymerase, respectively, and four genes, organized in two operons (BL1196-BL1197 and BL1750-BL1751) coding for nuclease, became increasingly repressed with time (Table 5).

**Carbohydrate transport and metabolism (COG G)**
In this category the number of induced genes increased from 4 to 10, corresponding to 3 and 10 %, respectively, of total genes expressed at 134 and 211 h in this category (Figure 9). Compared to the sample collected at 31 h, most of the differentially expressed genes with culture time were involved in sugar transport systems. In particular, an operon composed of three genes (BL1694-BL1696) coding for a multiple sugar transport system was over-expressed with a gene expression ratio of approximately 10 and 20 at 134 and 211 h, respectively (Table 5). The operon BL0411-BL0412 coding for proteins involved in a phosphotranspherase system (PTS) and the genes BL0143, BL0144 (in the same operon) and BL0524, coding for permeases of ABC transporter were over-expressed with a gene expression ratio of approximately 4-6 at 211 h (Table 5).

The number of repressed genes in this COG category increased from 0 to 5, corresponding to 0 and 5 % of total genes expressed in COG G at 134 and 211 h, respectively (Figure 9). At 211 h, four genes (BL0033-BL0036) organized in operon and coding for proteins involved in ribose transport (Parche et al. 2007) were down regulated, as well as lacZ, encoding for β-galactosidase.
Table 5 Overview of expression ratios of genes which were differentially expressed with increasing time during continuous culture of *B. longum* NCC2705.

<table>
<thead>
<tr>
<th>Genes</th>
<th>134 h</th>
<th>211 h</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Posttranslational modification, protein turnover and chaperones (COG O)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL0519</td>
<td>1.2</td>
<td>2.8</td>
<td>GrpE protein (HSP70 cofactor)</td>
</tr>
<tr>
<td>BL1558</td>
<td>2.8</td>
<td>1.7</td>
<td>10 kDa chaperonin (GroES)</td>
</tr>
<tr>
<td>BL1620</td>
<td>-2.3</td>
<td>-4.0</td>
<td>Alkylation damage repair protein</td>
</tr>
<tr>
<td><strong>DNA replication, recombination, repair (COG L)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL0127</td>
<td>-1.7</td>
<td>-2.8</td>
<td>DNA polymerase III subunit alpha (DnaE)</td>
</tr>
<tr>
<td>BL1147</td>
<td>-2.1</td>
<td>-2.8</td>
<td>DNA primase (DnaG)</td>
</tr>
<tr>
<td>BL1196</td>
<td>-7.0</td>
<td>-17.1</td>
<td>Widely conserved ATP-dependent DNA helicase; Rep-like protein</td>
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<tr>
<td>BL1197</td>
<td>-4.6</td>
<td>-6.5</td>
<td>Large hypothetical protein</td>
</tr>
<tr>
<td>BL1750</td>
<td>-2.3</td>
<td>-4.6</td>
<td>Exonuclease VII small subunit (XseB)</td>
</tr>
<tr>
<td>BL1751</td>
<td>-1.9</td>
<td>-3.5</td>
<td>Exodeoxyribonuclease VII large subunit (XseA)</td>
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<td><strong>Carbohydrate transport and metabolism (COG G)</strong></td>
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<td></td>
</tr>
<tr>
<td>BL0033</td>
<td>-1.2</td>
<td>-3.7</td>
<td>Probable solute binding protein of ABC transporter system possibly for sugars</td>
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<tr>
<td>BL0034</td>
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<td>-4.6</td>
<td>ATP binding protein of ABC transporter</td>
</tr>
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<td>1.1</td>
<td>-4.6</td>
<td>Probable ABC transport system permease protein for sugars</td>
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<td>BL0036</td>
<td>1.1</td>
<td>-4.3</td>
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<td>BL0143</td>
<td>1.6</td>
<td>5.6</td>
<td>Permease of ABC transporter possibly for oligosaccharides</td>
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<td>BL0144</td>
<td>1.9</td>
<td>4.0</td>
<td>Permease of ABC transporter possibly for oligosaccharides</td>
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<td>3.7</td>
<td>Phosphoenolpyruvate-protein kinase (PtsI)</td>
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<td>BL0412</td>
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<td>5.6</td>
<td>Histidine-containing phosphocarrier protein (Hpr protein) of Pts transport system</td>
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<td>1.4</td>
<td>5.3</td>
<td>Possible permease protein of ABC transporter system</td>
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<tr>
<td>BL0978</td>
<td>-1.9</td>
<td>-3.5</td>
<td>LacZ</td>
</tr>
<tr>
<td>BL1293</td>
<td>1.3</td>
<td>3.5</td>
<td>Similar to xylulose kinase</td>
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<td>BL1294</td>
<td>1.3</td>
<td>4.6</td>
<td>Possible acylphosphatase (AcyP)</td>
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<td>3.7</td>
<td>2.5</td>
<td>Solute binding protein of ABC transporter for sugars</td>
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<td>BL1694</td>
<td>10.5</td>
<td>19.7</td>
<td>Probable sugar binding protein of ABC transporter for pentoses</td>
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<td>BL1695</td>
<td>8.6</td>
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<td>BL1696</td>
<td>6.5</td>
<td>17.1</td>
<td>Probable ABC transport system permease protein for sugars</td>
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<td><strong>Nucleotide transport and metabolism (COG F)</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>BL0668</td>
<td>4.9</td>
<td>6.5</td>
<td>Glutaredoxin-like protein (NrdH, COG O)</td>
</tr>
<tr>
<td>BL0669</td>
<td>3.5</td>
<td>4.3</td>
<td>Hypothetical protein (NrdI)</td>
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<tr>
<td>BL0670</td>
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<td>3.7</td>
<td>Ribonucleotide-diphosphate reductase alpha subunit (NrdE)</td>
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<tr>
<td>BL0671</td>
<td>3.7</td>
<td>4.0</td>
<td>Ribonucleotide-diphosphate reductase beta subunit (NrdF)</td>
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### Transcription (COG K)

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<td>3.0</td>
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<td>BL1529</td>
<td>3.7</td>
<td>8.0</td>
<td>Possible IclR-type transcriptional regulator</td>
</tr>
<tr>
<td>BL1530</td>
<td>2.8</td>
<td>7.5</td>
<td>Probable IclR-type transcriptional regulator</td>
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<tr>
<td>BL1736</td>
<td>-2.0</td>
<td>-4.0</td>
<td>Possible helicase (COG KLS)</td>
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### Aminoacid transport and metabolism (COG E)

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<tr>
<th>BL0933</th>
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<th>O-acetylhomoserine (thiol)-lyase (CysD)</th>
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<td>BL1559</td>
<td>1.0</td>
<td>-3.5</td>
<td>Possible cystathionine gamma lyase</td>
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<tr>
<td>BL1668</td>
<td>1.0</td>
<td>3.2</td>
<td>Branched-chain amino acid permease (AlzD)</td>
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<td>2.1</td>
<td>8.0</td>
<td>Narrowly conserved hypothetical protein</td>
</tr>
</tbody>
</table>

### Cell wall/membrane biogenesis (COG M)

| BL1672  | 5.6 | 2.8  | Possible cyclopropane-fatty-acyl-phospholipid synthase |

### Others and unknown function

<table>
<thead>
<tr>
<th>BL0126</th>
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<th>-2.6</th>
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Figure 9 Percentages of differentially expressed genes in functional COG categories at different time points (■ 134 h, □ 211 h) of the continuous culture of *B. longum* NCC2705. The percentage of induced and repressed, genes in each category was calculated by dividing the number of significantly induced/repressed genes in each category by the total number of retained genes in the corresponding category. Statistical significance: ○, p-value ≤ 0.1; *, p-value ≤ 0.05; ***, p-value ≤ 0.0005. COG groups: J Translation; A RNA processing and modification; K Transcription; L Replication, recombination and repair; D Cell cycle control, mitosis and meiosis; V Defense mechanisms; T Signal transduction mechanisms; M Cell wall/membrane biogenesis; N Cell motility; U Intracellular trafficking and secretion; O Posttranslational modification, protein turnover, chaperones; C Energy production and conversion; G Carbohydrate transport and metabolism; E Amino acid transport and metabolism; F Nucleotide transport and metabolism; H Coenzyme transport and metabolism; I Lipid transport and metabolism; P Inorganic ion transport and metabolism; Q Secondary metabolites biosynthesis, transport and catabolism; R General function prediction only; S Function unknown.
2.5 Discussion

Resistance of cells to a lethal stress can be enhanced by application of homologous or heterologous sub-lethal stress during culture (Doleyres and Lacroix 2005). It was previously shown that responses of probiotic cells to stress conditions are strain dependant and vary with applied stresses (Maus and Ingham 2003; Saarela et al. 2004; Simpson et al. 2005). In addition, sensitivity of bacterial cells to stress is also dependent on their specific growth rate ($\mu$) as reported for *E. coli* and *Lc. lactis* (Berney et al. 2006; Dressaire et al. 2008). In batch culture, $\mu$ changes with culture time especially during the transition between exponential and stationary growth phases where $\mu$ value decreases from its maximum, $\mu_{\text{max}}$, to 0 h$^{-1}$. During pH controlled batch cultures, this progressive decrease of $\mu$ is due to the exhaustion of one or more substrates and/or the accumulation of toxic products. Therefore during batch cultures, the effect of $\mu$ on cellular response to a stress cannot be specifically described because influences of the specific stress and $\mu$ during this period are superimposed (Dressaire et al. 2008). Moreover, $\mu$ changes rapidly during growth phase transition leading to difficulty to collect cells in the same physiological state over different batch cultures. In contrast, bacterial growth rate can be accurately controlled in continuous cultures by setting the dilution rate.

Traditional probiotic batch cultures are harvested in late-exponential or stationary growth phase to achieve maximum cell yield and increase cell viability during downstream processing (Saarela et al. 2004). To simulate the conditions of late exponential/beginning of stationary growth phase, our continuous culture experiment was done with a low dilution rate of 0.1 h$^{-1}$. Therefore, a low generation number of only 30 was produced during 211 h of the continuous culture. Viable cell counts and percentages of viable cells measured by flow cytometry remained at high level even though a slight decrease over time of 0.2 log$_{10}$ CFU ml$^{-1}$ and 3 %, respectively, was measured.

The continuously produced cells were transcriptionally active as indicated by the high percentage of expressed genes over time of 93 and 74% at 134 and 211 h, respectively. The decrease in percentage of expressed genes observed between these two points may
be explained by adaptation of continuously produced cells to environmental conditions in particular glucose availability in the medium. Percentage of differentially expressed genes increased slightly from 1.0 to 3.8% between 134 and 211 h compared to 31 h. This change tested during the continuous culture was much smaller than those occurring in cells collected during different growth phases when cultivated in batch mode. Indeed, Klijn (2005a) reported that 24 and 46% of genes of *B. longum* NCC2705 were differentially expressed for cells collected in early and mid stationary phase, respectively, compared to exponentially growing cells. During continuous culture of *Lactococcus lactis* about 30% of the genes were shown to be differentially expressed in response to changes in the dilution rate from 0.09 to 0.47 h⁻¹ (Dressaire et al. 2008) which also occur during the growth phase transition of batch cultures. A moderate stress application can also greatly influence gene expression. For example, application of a heat stress of 7 min at 50°C did not affect cell viability of *B. longum* NCC2705 but about 50% of the genes were differentially expressed, in particular genes related to stress response (Rezzonico et al. 2007).

In our study, important genes coding for stress related proteins, such as GroEL, DnaJ, ClpA, ClpB and DnaK, involved in the general stress response of bifidobacteria (De Dea Lindner et al. 2007) were not differentially expressed over time. *GrpE* and *groES* were marginally differentially expressed; however they were not increasingly over-expressed with time (Table 5). Therefore we can conclude that the expression of these stress markers was not increased over culture time. In addition, percentages of differentially expressed genes for all COG categories, except L (replication, recombination and repair) and G (carbohydrate transport and metabolism) did not change significantly during continuous culture. A majority of differentially expressed genes from COG L were increasingly repressed with time, whereas in COG G an increase over time of percentage of induced genes was observed. These changes suggest an adaptive response of cells to environmental conditions created in the reactor, in particular due to low carbohydrates availability. Although changes in percentage of differentially expressed genes were detected during continuous culture (at 134 and 211 h compared to 31 h of culture), they remained very small as well as for changes in expression ratios, suggesting that
continuous cultures operated at low $\mu$ can be used to produce cells with controlled physiology.

The complete consumption of glucose during continuous culture indicates that carbohydrate source was limiting. Adaptation to carbon limiting condition can explain the increased expression of genes encoding transport systems for carbohydrates other than glucose (BL0143, BL0144, and BL1694-BL1696) after 134 and 211 h compared to 31 h culture. This adaptive response has already been reported for $B.\ longum$ NCC2705 (Klijn 2005a) and $Lactococcus\ lactis$ (Redon et al. 2005) when cells were grown under carbohydrate limitation. The major metabolites produced from carbohydrate metabolism of bifidobacteria are acetate, lactate, formate and ethanol which come from dissimilation of pyruvate (Devries and Stoutham 1968). During continuous culture, a moderate, but significant, shift in pyruvate metabolism towards an increased production of lactate in contrast to acetate, formate and ethanol was observed using regression analysis. Remarkably, no differential expression of pyruvate dissipating enzymes, such as lactate dehydrogenase and pyruvate formate lyase was detected. The regulation of sugar metabolism in lactic acid bacteria and bifidobacteria is still not well understood. Many factors other than the regulation of the production and/or activity of certain enzymes can lead to changes in end product formation, such as the NADH + H$^+$/NAD$^+$ ratio, pool of ADP and ATP and specific sugar consumption rate (Garrigues et al. 1997; Melchioresen et al. 2002; Palmfeldt et al. 2004; Van der Meulen et al. 2006a).

The physiological stability of continuously produced cells of $B.\ longum$ NCC2705 was also assessed according to their tolerance to different stressing conditions that can be encountered during biomass production and ingestion. Small but significant changes of tolerances to different stresses were detected during continuous culture. Cell resistance to bile salts increased significantly with culture time with an increased survival rate of about 1.1 log between cells collected at the beginning and at the end of the continuous culture experiment. In $B.\ animalis$ subsp. $lactis$, the redox status is involved in the response of cells to the oxidative stress caused by bile salts (Sanchez et al. 2007b). Ribonucleotide reductases are known to counteract the deleterious effect of oxidative stress in $E.\ coli$ cells (Monje-Casas et al. 2001). The overexpression of genes BL0668-BL0671, organized in operon as in $E.\ coli$ and coding for the ribonucleotide reductases NrdHIEF,
could explain the increased resistance of continuously produced *B. longum* NCC2705 cells to bile salts.

In several *Bifidobacterium* strains, the resistance to bile salts was shown to be correlated with a higher tolerance of cells to low pH (Noriega et al. 2004). Bile salts and heat stresses induced also similar responses in *B. longum* cells with upregulation of *dnaK* and *groEL* coding for stress proteins and *htrA* coding for a protease (Savijoki et al. 2005). None of these genes were overexpressed in continuously produced cells of *B. longum* NCC2705 which became more sensitive to gastric juice (log loss increased of 0.6 after 211h culture) and heat (increase of loss in percentage of live cells from 6.7 to 13.5%).

Cell sensitivity to the tested β-lactam antibiotics (cefotaxim, ceftazidime and ampicillin) remained constant through the culture, while sensitivity to penicillin G increased. *B. longum* NCC2705 which was tolerant to neomycin, an aminoglycoside antibiotic that binds to the 30S ribosomal subunits and inhibit protein synthesis (Weisblum and Davies 1968; Kohanski et al. 2007) was completely resistant (10 μg disc on agar plate) at the end of the culture. The tolerance of bifidobacteria strains to high concentrations of aminoglycosides has already been reported (Charteris et al. 1998a; Kheadr et al. 2004; Moubareck et al. 2005; Noriega et al. 2005), and seems to be a common characteristic among *Bifidobacterium* species. *B. longum* NCC2705 cells became progressively more sensitive to tetracycline, chloramphenicol and vancomycin with culture time. Tetracycline and chloramphenicol are bacteriostatic antibiotics targeting the 30S and 50S subunit of the ribosome, respectively, while vancomycin has a bactericidal effect and inhibits cell wall synthesis by interaction with peptidoglycan building blocks (Kohanski et al. 2007). At the transcriptomic level no significant change of expression of genes coding for drug efflux system which can affect antibiotic susceptibility (Dessen et al. 2001) was observed during continuous culture.

Although significant changes in cell responses to physiological tests and gene expression levels were detected during continuous culture, using regression analysis, these changes remained small and could be detected due to a highly sensitive analysis, thanks to the high number of time points analyzed during the continuous culture and the good stability of the system. When comparing data of the first and last 3 sampling points of the continuous culture, used as biological replicates, no significant differences were detected.
for viable cell counts, metabolite concentrations (except for lactic acid), survival tests (except for bile salts) and antibiotic susceptibility. Changes detected with regression analyses were all in the range of standard errors reported in literature for similar tests performed on bifidobacteria cells produced during batch cultures (Maus and Ingham 2003; Saarela et al. 2004; Liu et al. 2007; Sanchez et al. 2007a). In these studies, the difficulty to reproduce the physiological state of cells grown in batch cultures and collected at a specific growth phase was emphasized, especially for cells collected during the transition growth phase (end exponential) and at the beginning of stationary growth phase, where important physiological changes occur over a short period of time. In contrast, cells produced in continuous culture are grown at a constant specific and controlled growth rate, and our data with *B. longum* NCC2705 showed that only small changes in physiological state occurred over 211 h culture.

Over culture time, physiological parameters, such as viable cells counts, metabolites production and resistance to stresses and antibiotics remained stable or showed only small and gradual changes. Therefore we propose that continuous cultures operated at a low dilution rate can then be used to produce cells with controlled physiology for testing the effect of stresses on probiotic bacteria. This approach is tested in chapter 3 for fast and efficient screening of a range of sub-lethal stress conditions with a 2-stage continuous culture system, with a first vessel for controlled cell production and a second vessel in series for sublethal stress application.
3 Validation of a new approach for screening of sublethal stress conditions in a two-stage continuous culture with \textit{B. longum} NCC2705 cells

3.1 Abstract

Resistance of probiotic cells to a lethal stress, such as those encountered during processing and digestion can be improved by sublethal stress pretreatments. In this study, continuous culture was developed for fast screening of sublethal stress pretreatments according to their effects on viability and cell survival to subsequent lethal stresses. A 2-stage continuous culture was carried out with \textit{Bifidobacterium longum} NCC2705 for 211 h. The first stage (R1) was operated under fixed conditions at 37°C and pH 6.0, and with a dilution rate of 0.1 h\(^{-1}\) and was used to produce cells with controlled physiology, mimicking cells in end exponential growth phase. Different stress pretreatments, applied for 42 min, were tested in the second stage (R2), with combinations of pH (6.0, 5.0 and 4.0), temperature (37, 45 and 47°C) and NaCl concentration (0, 5 and 10%), using a 3 by 3 factorial design. This 2-stage continuous culture design allowed efficient screening of several stress pretreatments during the same culture experiment, with up to four different conditions tested per day. Of all tested combinations, only those with pH 4.0 significantly decreased cell viability in the second reactor compared to control conditions (37°C, pH 6.0, 0 % NaCl), and thus could not be considered as sublethal stresses. Pretreatments with 5 or 10 % NaCl had negative effects on cell viability after gastric lethal stress. A significant improvement in cell resistance to a heat lethal stress (56°C, 5 min) was observed for cells pretreated at 47°C. In contrast, heat pretreatments negatively affected cell viability after freeze drying and osmotic lethal stresses. Selected stress pretreatments (pH 4.0, 47°C, 10 % NaCl, 47°C+10 % NaCl and pH 4.0+10 % NaCl) were also tested during early stationary phase of batch cultures, with similar effects compared to continuous culture.

Our data showed that the 2-stage continuous culture is an efficient screening method for sublethal stresses, allowing for fast and controlled testing of several stress pretreatments
during the same culture and leading to optimal sublethal stress conditions which can be also applied for producing cells with traditional batch cultures.
3.2 Introduction

Bifidobacteria are natural inhabitants of the human intestine and have very important functions sustaining gut health (Leahy et al. 2005). Bacteria belonging to Bifidobacterium and Lactobacillus genera are commonly used as probiotics. Probiotics are “live microorganisms which, when administered in adequate amounts (as part of food), confer a health benefit on the host” (FAO/WHO 2002). A central issue in the application of probiotics as food additives is their fastidious production and their sensitivity to many environmental stresses. It is generally agreed that probiotic food products should contain at least $10^6$-$10^7$ viable cells per gram or ml at the time of consumption in order to achieve a specific health effect and/or to provide bacterial concentrations that are technologically attainable and cost effective (Champagne et al. 2005; Lacroix and Yildirim 2007). A key industrial challenge is to increase cell numbers and viability during biomass production, downstream processing, and in the final product during storage and consumption. Furthermore, to survive the harsh conditions in the gastrointestinal tract, probiotics have to adapt to a competitive and changing environment.

New approaches for enhancing stability of probiotics to various stresses have been recently reviewed by Lacroix and Yildirim (2007). The importance of inducible cell-protective mechanisms, triggered by application of sublethal stresses, for survival under stress conditions has been demonstrated for a number of lactic acid bacteria (Girgis et al. 2002) and bifidobacteria (Schmidt and Zink 2000; Maus and Ingham 2003; Saarela et al. 2004). Cell responses were shown to be strain-dependent and vary with applied stresses (Maus and Ingham 2003; Saarela et al. 2004; Simpson et al. 2005). Until now, the optimization procedure was performed using batch cultures, which can be very time consuming and also difficult to control due to fast changing physiology of cells. Typically, sublethal stresses are applied for a short time period of 30-60 min in the exponential phase (Schmidt and Zink 2000) or in stationary phase before cultures are stopped and cell harvested (Maus and Ingham 2003; Saarela et al. 2004).

Continuous cultures could be a suitable and more efficient method to test different stress factors on one culture instead of repeating several batch cultures (Lacroix and Yildirim 2007). However, to our knowledge this strategy has never been investigated.
Furthermore, physiological stability of continuously produced cells is a prerequisite to use continuous culture for stress screening purposes during the same culture experiment. In a previous study we showed that continuous culture can be used to produce *B. longum* NCC2705 cells with controlled physiology over a long period of time (Chapter 2). Susceptibility of continuously produced cells (stable viable cell counts decreasing only moderately from $8.8$ to $8.6 \log_{10} \text{CFU ml}^{-1}$ over 211 h) to different stress conditions (antibiotics, heat, gastric juice and bile) was either stable or showed only small changes over the 211-h culture. Comparison of gene transcription profiles of samples collected after 31 h of continuous culture and after 134 and 211 h revealed only limited changes in expression of 1.0 and 3.8 % of total genes, respectively.

In this study, we tested the application of a two-stage continuous culture of *B. longum* NCC2705, with a first stage used to produce cells with controlled growth and physiology, mimicking cells in late exponential growth phase, and a second stage where stress pretreatments were applied under well controlled conditions. The effect of pH, temperature and NaCl concentration stress pretreatments and their combinations were tested on cell viability and resistance to various lethal stresses (heat, osmotic, freeze drying, gastric and bile salts lethal tests) in a 3 by 3 factorial design. Stress pretreatments showing an effect on cell tolerance to lethal stresses were further tested in batch cultures to compare stress adaptation responses in both culture modes, and therefore validate the potential of this new screening approach for optimization of industrial batch cultures.
3.3 Material and Methods

3.3.1 Strain and growth conditions

*B. longum* NCC2705 was obtained from the Nestlé Culture Collection (Lausanne, Switzerland) and cultivated in MRS (De Man et al. 1960) medium (Biolife, Milano, Italy) supplemented with L-cysteine hydrochloride monohydrate (Sigma-Aldrich, Buchs, Switzerland) at a final concentration of 0.05 % (C-MRS). Two successive precultures, inoculated at 1 % from a frozen stock, were performed for 16 h under anaerobic conditions (AnaeroGen, Oxoid, Basingstoke, United Kingdom) at 37°C before use.

3.3.2 Viable cell enumeration

Samples were diluted in 1x phosphate buffered saline (pH 7.7) supplemented with 0.05 % L-cysteine hydrochloride monohydrate (C-PBS). Appropriate dilutions were plated in duplicate on C-MRS agar (DIFCO, Laboratories, Detroit, USA) and incubated anaerobically (AnaeroGen) at 37°C for 48 h.

3.3.3 Continuous culture experiment

A schematic diagram of the two stage continuous culture is presented in Figure 10. The first reactor (R1) (2.5-l bioreactor, Bioengineering AG, Wald, Switzerland), equipped with a Biospectra control system (Biospectra AG, Schlieren, Switzerland) had a working volume of 2 l. The second bioreactor (R2) with a working volume of 140 ml (500-ml Sixfors bioreactor, Infors AG, Basel, Switzerland), operated in series, was continuously inoculated with culture broth from R1. Reactor R1 was inoculated at 2 % (v/v) with preculture to start culture. After 8 h in batch mode, reactor R1 was continuously fed with fresh C-MRS using a peristaltic pump (Alitea, Bioengineering AG, Wald, Switzerland), set at a flow rate of 200 ml h⁻¹ giving a dilution rate of 0.1 h⁻¹. Mean residence times in R1 and R2 were 10 h and 42 min, respectively. Two others peristaltic pumps (Alitea), set at a higher flow rate than the feed pump were used to transfer fermented broth from R1 to R2 and to harvest fermented broth from R2 (Figure 10). The useful volume in R1 and R2
was maintained by positioning the out-flow tubes. Carbon dioxide was flushed into the headspace of the two reactors to maintain anaerobic conditions during the culture. Temperature was maintained at 37°C and pH controlled at 6.0 by addition of 5 M NaOH in both reactors. The culture was stirred at 250 rpm using 2 and 1 Rushton type propellers in R1 and R2, respectively. A stabilization period of 90 h, corresponding to nine reactor volume changes in R1, was allowed before starting application of stress pretreatments in R2.

Figure 10 Schematic diagram of the two-stage continuous culture processes to screen sublethal stresses in *B. longum* NCC2705. A first reactor (R1) is operated in fixed conditions of temperature and pH and used to produce cells with controlled physiology. Stress pretreatments (pH, heat, osmotic) are applied in the second reactor (R2) which is connected in series with R1. Cells from R1, used as control, and R2 after stress pretreatments are collected for further analysis and lethal stresses experiments.
3.3.4 Applications of stress pretreatments in continuous culture

Twenty-seven combinations of temperature (37, 45 and 47°C), NaCl concentration (0, 5 and 10 %) and pH (6.0, 5.0 and 4.0) were tested in R2 using a 3 by 3 factorial design in a randomized order. The combination 37°C, pH 6.0 and 0% NaCl, which corresponds to standard conditions for growth of this strain, was used as control condition. Each pretreatment was applied for 4 h corresponding to at least 6 residence times in R2 before sampling the reactor in steady state period. Heat pretreatment was applied by increasing temperature from 37 to 45 or 47°C using the temperature control system of the reactor, which required approximately 10 min. Acid pretreatment was applied by decreasing pH from 6.0 to 5.0 or 4.0 by addition of 5 M HCl. For fast pH adjustment, within 10 min, and to avoid oscillation of the PID-control, pH-control was turned off and pH adjusted manually before restarting the pH-control. Osmotic pretreatment was started by direct addition into R2 of 28 or 70 ml sterile NaCl solution (30 % w/v) in order to rapidly set the NaCl concentration at 5 or 10 % (w/v) in the reactor, respectively. R2 was then fed with 40 or 100 ml h\(^{-1}\) NaCl solution (30%, w/v) to maintain NaCl concentration at fix levels of 5 or 10%, respectively. Because addition of NaCl increased total medium flow rate and therefore dilution rate, the volume of the reactor was increased to 168 and 210 ml, by positioning the outflow tubes, for 5 and 10 % NaCl, respectively, to maintain a mean residence time of 42 min.

Cell samples were collected in R1 (non stressed cells) and R2 for plate counts and lethal stress tests. Two ml samples were directly used for plate count analyses. For lethal stress tests, 20 ml samples were centrifuged (6,000 g, 4°C, 15 min), pellets were resuspended to their initial volume in fresh C-MRS supplemented with 10 % glycerol, aliquoted in 2 ml eppendorf tubes, fast frozen in liquid nitrogen and stored at -80°C for a maximum time of 2 months before lethal stress tests.
3.3.5 Batch cultures

Cells were grown in a 500-ml working volume bioreactor (500-ml Sixfors, Infors AG, Basel, Switzerland) containing C-MRS inoculated at 0.5 % with preculture. Temperature was controlled at 37°C, agitation set at 200 rpm, and pH maintained at 6.0 using 5 M NaOH. Anaerobic conditions were achieved by flushing CO₂ in the reactor headspace.

Four stress pretreatments, selected from the continuous culture were tested in batch cultures: pH 4.0, 47°C, 10 % NaCl, 47°C+10 % NaCl, and pH 4.0+10 % NaCl. They were applied 10 min after beginning of stationary phase, determined by base pump activity as the point when base pump activity dropped for 10 min to lower than 0.0003 ml min⁻¹. This point was reached approximately 10 min after glucose concentration had dropped to 0. Samples for plate counts and lethal stress tests were taken before and after stress pretreatments and prepared as described above. Stress pretreatments were applied for 42 min corresponding to the mean residence time of the cells in R2 of continuous culture. Temperature and pH adjustements of stress pretreatments were done as described above for the continuous culture. Osmotic pretreatment was applied with single addition of 250 ml 30 % (w/v) sterile NaCl solution to reach a final concentration of 10 % NaCl in the reactor. The adjustment of temperature, pH and NaCl concentration was carried out within 10 min after which the stress pretreatment time was calculated. Control cultures without stress application were run at 37°C, pH 6.0 and 0 % NaCl for an additional time of 42 min after reaching stationary phase as described above. All experiments were carried out in duplicate.
3.3.6 Lethal stress tests

Osmotic
Two ml samples were thawed at room temperature for 30 min and subsequently centrifuged 2 min at 4,000 g. The pellet was washed with C-PBS, collected again by centrifugation, resuspended in 2 ml C-PBS and cooled on ice for 30 min. In a microtiter plate 30 µl of cell suspension were added to 270 µl of sterile 30 % (w/v) NaCl solution and incubated anaerobically for 2 h at 37°C. This NaCl concentration, determined from preliminary experiments using overnight cultures (data not shown) induced a loss in viable cells of approximately 2 log\textsubscript{10} units. Viable cell counts before and after osmotic lethal stress were determined by plating in duplicate on C-MRS after appropriate dilution in C-PBS and data were used to calculate viable cell loss in log\textsubscript{10} (log loss) during osmotic lethal stress.

Freeze drying
Freeze drying was performed according to Doleyres et al. (2004b) with some modifications. Three aliquots of 2 ml samples were thawed at room temperature for 30 min and subsequently centrifuged 2 min at 4,000 g. The pellet was washed with C-PBS collected again by centrifugation, resuspended in the same volume of C-PBS and 5 ml cell suspension were pooled together in a sterile bottle for freeze drying and frozen for at least 24 h and maximal 48 h at -80°C. Frozen samples were dried in a Virtis Genesis freeze dryer (MultiTemp Scientific, Kloten, Switzerland) with the following parameters: -40°C for 60 min, -10°C for 300 min with ramp, -10°C for 60 min and 25°C for 600 min with ramp, every step at 75 mTorr. The dried samples were directly resuspended in sterile water. Viable cell counts before and after freeze drying were determined by plating in duplicate and data were used to calculate viable cell loss in log\textsubscript{10} (log loss) during freeze drying.
Heat survival tests were carried out according to Simpson et al. (2005). Two ml samples were centrifuged (4,000 g, 1 min), washed and diluted in C-PBS to a final concentration of $10^6 - 10^7$ cells ml$^{-1}$. One hundred µl of the diluted sample was filled into a 200 µl PCR tube and kept on ice for 30 min. Heat stress was applied in a thermocycler (Biometra T-personal, Biolabo Scientific Instruments SA, Chatel-St.-Denis, Switzerland) at 56°C for 5 min. After heat stress, samples were immediately cooled down on ice for 15 min, diluted five fold in C-PBS and analyzed by flow cytometry as follows. To 500 µl of the cell suspension, 0.5 µl SYBR Green I (10,000X, Molecular Probes, Invitrogen, Basel, Switzerland) and 20 µl 7AAD 0.0005 % w/v (Viability Dye, Beckman Coulter, Nyon, Switzerland) were added. The mixtures were incubated for 15 min in the dark and analyzed in duplicate using a Cytomics FC 500 flow cytometer (Beckman Coulter International SA; Nyon, Switzerland) equipped with an air-cooled argon ion laser emitting 20 mW at 488 nm combined with a 635 nm red-diode laser and the standard filter setup. The forward and side scatter signals were used as trigger signal in the 488 nm band-pass filter. The green fluorescence of SYBR Green I was detected through a 525 nm band-pass filter (FL 1 channel) and the red fluorescence of 7AAD collected in the FL 4 channel through a 675 nm band-pass filter. The rate settings were adjusted to keep the count to approximately 3,000 events s$^{-1}$. Data were collected on a logarithmic scale and analyzed using the CXP software (Beckman Coulter International SA, Switzerland). Discrimination between viable and non-viable cells was carried out according to fluorescence intensities of both dyes. Data were expressed as loss in percentage of live cells.

Simulated gastric conditions
The effects of simulated gastric juices were tested according to Charteris et al. (1998b) with some modifications in order to perform the test in microtiterplates. Cells were washed and resuspended in 0.1 % (w/v) sterile peptone water (Becton Dickinson, VWR International AG, Dietikon, Switzerland), cooled on ice for 30 min, 10-fold diluted in simulated gastric juices containing 0.5 % (w/v) NaCl and 0.3 % (w/v) pepsin (0.7 FIP-U mg$^{-1}$) from porcine gastric mucosa (Merck, Zug, Switzerland) at pH 2.8, and incubated at
37°C under anaerobic conditions (Anaerogen) for 15 min. Cell counts before and after stress application were determined by duplicate plate counts and data were used to calculate viable cell loss in $\log_{10}$ (log loss) during exposition to simulated gastric juices.

**Bile salts**

The effects of bile salts were tested as previously described by Saarela et al. (2004) with some modifications in order to perform the test in microtiterplates. Cells were washed and resuspended in C-PBS, cooled on ice for 30 min, 10-fold diluted in 1.5 % (w/v) porcine bile extract (Sigma-Aldrich, Buchs, Switzerland) in C-PBS and incubated anaerobically (AnaeroGen) at 37°C for 10 min. Cell counts before and after stress application were determined by duplicate plate counts and data were used to calculate viable cell loss in $\log_{10}$ (log loss) during exposition to bile salts.

### 3.3.7 Carbohydrates analysis

Concentrations of glucose, organic acids (lactate, acetate and formate) and ethanol in fermented medium of R1 and R2 over the 211 h of continuous culture and before and after application of sublethal stresses in batch cultures were determined by HPLC. One ml of supernatant sample was diluted 1:5 in MilliQ water and filtered through a 0.45 µm nylon membrane filter (Infochroma, Zug, Switzerland). Separation of compounds was carried out using an Aminex HPX-87-H column (Bio-Rad Laboratories, Reinach, Switzerland) with 10 mM H$_2$SO$_4$ as eluent at a flow rate of 0.6 ml min$^{-1}$ and a temperature of 40°C. Compounds were detected using a refractive index detector (Merck Hitachi, Darmstadt, Germany). Reported data are means of duplicate analysis.
3.3.8 Statistical analysis

Twenty seven different stress pretreatments were tested randomly over time in the second stage of the continuous culture (R2) using a complete 3 by 3 factorial design (Table 6). The effects of 3 factors (pH, temperature and NaCl concentration) at 3 levels (pH 4.0, 5.0 6.0; temperature 37, 45 and 47°C; and NaCl concentration 0, 5 and 10 %) and their interactions on cell viability were tested using analysis of variance (ANOVA) with a significance level of 0.05. The effects of stress pretreatments on cell viability were measured by the difference between cell counts in R1, operated under control conditions (37°C, pH 6.0 and 0 % NaCl), and cell counts in R2 where stress pretreatments were applied. Cell counts were transformed to their base 10 logarithms to stabilize the variance and normalize the residuals. Variables and interactions that showed significant p-values in the ANOVA were further analyzed with Tukey’s HSD (honestly significant difference) post hoc pairwise tests with a significance level of 0.05, in order to compare individual pair of groups. A similar statistical analysis was performed with cell viability data of lethal stresses (expressed in log loss of CFU ml⁻¹ or % increase of dead cells during lethal stress) in order to determine the effects of stress pretreatments on cell robustness. Given the small number of variables, model selection was performed by eliminating interactions with highest non significant p-values. Because continuous culture had to be stopped in advance for technical problems, data for 4 stress combinations were missing. This problem influenced negatively the power of the statistical analysis, but did not bias the estimated parameters, since stress pretreatments were applied randomly. ANOVA was performed in JMP (JMP, Version 7. SAS Institute Inc., Cary, NC, 1989-2007), which omits missing values and uses the available case analysis for dealing with incomplete data sets (Eberle 1999). Measured parameters were expressed as unweighted means with standard error calculated from the ANOVA analysis. For batch cultures, an oneway-ANOVA (significance level of 0.05) was performed after transforming cell counts to their base 10 logarithms to stabilize the variance and normalize the residuals. Two biological replicates were carried out for each stress pretreatment in batch cultures. Statistical analyses were carried out using JMP (JMP, Version 7. SAS Institute Inc., Cary, NC, 1989-2007).
Table 6 Table of treatments for the applied 3 by 3 factorial design.

<table>
<thead>
<tr>
<th>Factor B</th>
<th>Factor C</th>
<th>0</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>000</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>001</td>
<td>101</td>
<td>201</td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>002</td>
<td>102</td>
<td>202</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
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<td>111</td>
<td>211</td>
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<td>112</td>
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<td>220</td>
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<td>021</td>
<td>121</td>
<td>221</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>022</td>
<td>122</td>
<td>222</td>
</tr>
</tbody>
</table>

**Factor A** = Temperature (0 = 37°C, 1 = 45°C, 2 = 47°C)

**Factor B** = pH (0 = pH 6.0, 1 = pH 5.0, 2 = pH 4.0)

**Factor C** = NaCl concentration (0 = 0 % NaCl, 1 = 5 % NaCl, 2 = 10 % NaCl)
3.4 Results

3.4.1 Continuous culture

3.4.1.1 Effect of stress pretreatments on cell viability and carbohydrate metabolism

The effect of stress pretreatments (pH, temperature and NaCl concentration), applied in R2, on cell viability was evaluated by measuring the differences of viable cell counts in medium from R1 and R2. These data were analyzed by ANOVA (Table 8). Stress pretreatments and their interactions showing significant p-values in the ANOVA were further analyzed with Tukey’s HSD post hoc pairwise tests in order to compare individual levels of stress (e.g. pH 4.0, 5.0 and 6.0) (Table 9).

The average cell concentration tested in R1 was of $8.8 \pm 0.5 \log_{10}$ CFU ml$^{-1}$ during 211 h of culture. Cell counts in R2 with control conditions (37°C, pH 6.0 and 0% NaCl) were not significantly different to that in R1, indicating no growth in R2 under these conditions.

Pretreatments of cells in R2 with low pH, osmotic and heat stresses had a significant effect on cell viability, as well as the interactions of acid with osmotic or heat (Table 8). Pretreatments at pH 4.0 combined with 5 and 10 % NaCl lead to significantly lower losses in viable cells ($1.4 \pm 0.1$ and $2.3 \pm 0.1 \log_{10}$, respectively) than for 4.0 combined with 0 % NaCl, which resulted in high loss of $4.5 \pm 0.2 \log_{10}$ (Table 9). No significant effect of temperature increase or NaCl addition was observed on cell viability for pH 5.0 and 6.0 compared to control conditions (37°C, pH 6.0 and 0 % NaCl) (Table 9).

In both reactors glucose was not detectable by HPLC. Mean values for carbohydrates metabolites were not significantly different in R1 and R2 (Table 7).
3.4.1.2 Effect of stress pretreatments on cell robustness after lethal stress application

The effects of heat, low pH and osmotic stress pretreatments applied in R2 on cell robustness to lethal stresses, measured by increase in log loss of CFU ml$^{-1}$ or % of dead cells, were evaluated by ANOVA (Table 8). Stress pretreatments and their interactions with significant effects on cell robustness were further analyzed with Tukey’s HSD post hoc pairwise tests in order to compare individual levels of stress (Table 9). In Table 9, as reference for the interpretation of the post hoc tests the combination or the individual level of stress corresponding to control conditions (37°C, pH 6.0, 0 % NaCl) is highlighted in bold.

Osmotic lethal stress

Osmotic and acid pretreatments as well as their interactions had a significant effect on cell viability after osmotic lethal stress. For pH 5.0 and 6.0 addition of NaCl did not show effects on cell survival to the osmotic lethal stress, with mean viable cell losses of 2.9 ± 0.3 log$_{10}$. Significantly lower losses in viable cells of 1.2 ± 0.2 and 1.5 ± 0.1, were tested for pH 4.0 with 0 and 10 % NaCl, respectively, after the osmotic lethal stress compared to losses of 3.2 ± 0.3 log$_{10}$ at pH 4.0 with 5 % NaCl (Table 9). Pretreatments at 47°C induced higher cell losses after osmotic stress compared to 37 and 45°C, with viability losses of 1.6 ± 0.2 compared to 1.2 ± 0.2 and 1.0 ± 0.2 log$_{10}$, respectively (Table 8 and Table 9).
**Freeze drying**
Survival to freeze drying was significantly affected by low pH, osmotic and heat pretreatments (Table 8). Loss in viable cells after freeze drying increased significantly from $2.6 \pm 0.4$ to $3.7 \pm 0.4 \ log_{10}$ for pH 6.0 and pH 4.0, respectively (Table 9). Heat pretreatments also had a negative effect on cell viability after freeze drying, with losses in viable cell significantly increasing from $3.7 \pm 0.4$ to $4.7 \pm 0.5 \ log_{10}$ for cells pretreated at 37 (reference) and 47°C, respectively (Table 9). In contrast, osmotic pretreatments showed a positive effect on cell resistance to freeze drying, with viability loss after freeze drying decreasing from $3.7\pm 0.4$ to $2.5 \pm 0.4 \ log_{10}$ for pretreatments at 0 and 10 % NaCl, respectively (Table 9).

**Gastric lethal stress**
Cell viability after gastric lethal stress was significantly enhanced by low pH and osmotic pretreatments. In contrast heat pretreatments did not show significant effects (Table 8). Pretreatments at pH 4.0 had a significant positive effect on cell robustness to gastric lethal stress compared to pretreatments at pH 5.0 with viability losses of $0.4 \pm 0.3$ and $1.1 \pm 0.3 \ log_{10}$, respectively (Table 9). Viability loss after gastric lethal stress increased significantly from $0.4 \pm 0.3$ to $1.2 \pm 0.3 \ log_{10}$ for cells pretreated at 0 (reference) and 5 % NaCl, respectively, whereas pretreatment with 10 % NaCl gave intermediate values (Table 9).
Bile salts lethal stress

Cell resistance to bile salts was significantly enhanced by low pH pretreatments (Table 8), with significantly lower viability loss at pH 4.0 compared to pH 5.0 (0.4 ± 0.5 and 2.5 ± 0.5 log_{10}, respectively, which was not different to pH 6.0) (Table 9). Heat and osmotic pretreatments did not have any significant effect (Table 8).

Heat lethal stress

Heat pretreatments showed a significant effect on survival of cells subjected to a heat lethal stress, whereas low pH and osmotic pretreatments did not show effects (Table 8). The percentage of dead cells produced by heat lethal stress progressively decreased significantly from 12± 6 to -3 ± 7 % with heat pretreatment temperature from 37 to 47°C (Table 9).
Table 7 Metabolites concentrations in continuous (reactor 1 and 2) and batch cultures of *B. longum* NCC2705

<table>
<thead>
<tr>
<th></th>
<th>R1 (g l(^{-1}))</th>
<th>R2 (g l(^{-1}))</th>
<th>Batch cultures before stress pretreatments (g l(^{-1}))</th>
<th>Batch cultures after stress pretreatments (g l(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>8.8 ± 0.7</td>
<td>9.1 ± 0.7</td>
<td>6.5 ± 0.2</td>
<td>6.0 ± 0.7</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>2.2 ± 0.5</td>
<td>1.9 ± 0.4</td>
<td>6.5 ± 0.2</td>
<td>6.1 ± 0.4</td>
</tr>
<tr>
<td>Formic acid</td>
<td>2.3 ± 0.2</td>
<td>1.7 ± 0.2</td>
<td>0.4 ± 0.0</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.9 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.2</td>
</tr>
</tbody>
</table>

n.d. = non detectable
Table 8 Results of analyses of variance (ANOVA) for the effect of stress pretreatments in continuous culture of *B. longum* NCC2705 for the loss in viable cells in log₁₀ after stress pretreatments or lethal stresses and % of dead cells after heat lethal stress.

<table>
<thead>
<tr>
<th>Variable</th>
<th>df</th>
<th>Viable cells</th>
<th>Osmotic lethal stress</th>
<th>Freeze drying lethal stress</th>
<th>Gastric lethal stress</th>
<th>Bile salts lethal stress</th>
<th>Heat lethal stress</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SS</td>
<td>p</td>
<td>SS</td>
<td>p</td>
<td>SS</td>
<td>p</td>
</tr>
<tr>
<td>Acid (pH)</td>
<td>2</td>
<td>18.197</td>
<td>&lt;0.001</td>
<td>4.637</td>
<td>&lt;0.001</td>
<td>4.491</td>
<td>0.027</td>
</tr>
<tr>
<td>Osmotic (% NaCl)</td>
<td>2</td>
<td>6.687</td>
<td>0.001</td>
<td>2.669</td>
<td>&lt;0.001</td>
<td>5.458</td>
<td>0.015</td>
</tr>
<tr>
<td>Heat (Temp.)</td>
<td>2</td>
<td>0.521</td>
<td>0.002</td>
<td>1.129</td>
<td>0.005</td>
<td>3.647</td>
<td>0.049</td>
</tr>
<tr>
<td>Osmotic*acid</td>
<td>4</td>
<td>7.372</td>
<td>&lt;0.001</td>
<td>2.376</td>
<td>0.001</td>
<td>n.i.</td>
<td>n.i.</td>
</tr>
<tr>
<td>Osmotic*heat</td>
<td>4</td>
<td>0.153</td>
<td>0.392</td>
<td>n.i.</td>
<td>n.i.</td>
<td>n.i.</td>
<td>n.i.</td>
</tr>
<tr>
<td>Acid*heat</td>
<td>4</td>
<td>0.908</td>
<td>0.024</td>
<td>n.i.</td>
<td>n.i.</td>
<td>n.i.</td>
<td>n.i.</td>
</tr>
<tr>
<td>Error df</td>
<td>5</td>
<td>12</td>
<td>17</td>
<td>15</td>
<td>14</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Model p</td>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.019</td>
<td>0.031</td>
<td>0.019</td>
<td>0.027</td>
</tr>
<tr>
<td>Model R²</td>
<td>0.996</td>
<td>0.935</td>
<td>0.555</td>
<td>0.562</td>
<td>0.619</td>
<td>0.531</td>
<td></td>
</tr>
</tbody>
</table>

n.i., not included in the ANOVA model; df, degree of freedom; SS, sum of squares; p, p-value.
Table 9 Stress pretreatments in continuous culture of *B. longum* NCC2705 significantly affecting viable cells numbers and lethal stress tests. Pretreatments not connected by same letter are significantly different (Tukey’s HSD post hoc test). Pretreatments highlighted in bold corresponds to control conditions, indicating the reference for each post hoc test.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>M E</th>
<th>Pretreatment</th>
<th>M E</th>
<th>Pretreatment</th>
<th>M E</th>
<th>Pretreatment</th>
<th>M E</th>
<th>Pretreatment</th>
<th>M E</th>
</tr>
</thead>
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<tr>
<td>(pH, Temp.)</td>
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<td>(% NaCl, pH)</td>
<td></td>
<td>(% NaCl)</td>
<td></td>
<td>(% NaCl)</td>
<td></td>
<td>(%) NaCl</td>
<td></td>
</tr>
<tr>
<td>4/47°C</td>
<td>A</td>
<td>5.7, 0.2</td>
<td>5%/4</td>
<td>A 3.2, 0.3</td>
<td>0%</td>
<td>A 3.7, 0.4</td>
<td>47°C</td>
<td>A 4.7, 0.5</td>
<td></td>
</tr>
<tr>
<td>4/37°C</td>
<td>A</td>
<td>4.5, 0.1</td>
<td>0%/5</td>
<td>A 3.1, 0.2</td>
<td>5%</td>
<td>A, B 3.4, 0.4</td>
<td>37°C</td>
<td>A 4.3, 0.4</td>
<td></td>
</tr>
<tr>
<td>4/45°C</td>
<td>A</td>
<td>4.3, 0.1</td>
<td>5%/5</td>
<td>A 3.0, 0.1</td>
<td>10%</td>
<td>B 2.5, 0.4</td>
<td>45°C</td>
<td>A, B 3.7, 0.4</td>
<td></td>
</tr>
<tr>
<td>5/47°C</td>
<td>B</td>
<td>0.7, 0.2</td>
<td>0%/6</td>
<td>A 3.0, 0.1</td>
<td>0%</td>
<td>A 1.1, 0.3</td>
<td>6</td>
<td>A, B 0.7, 0.3</td>
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<tr>
<td>5/45°C</td>
<td>B</td>
<td>0.4, 0.1</td>
<td>5%/6</td>
<td>A 2.8, 0.1</td>
<td>5%</td>
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<td>A, B 0.4, 0.3</td>
<td></td>
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<tr>
<td>6/45°C</td>
<td>B</td>
<td>0.4, 0.1</td>
<td>10%/5</td>
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<td>10%</td>
<td>B 0.7, 0.3</td>
<td>4</td>
<td>A 0.1, 0.1</td>
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<tr>
<td>6/37°C</td>
<td>B</td>
<td>0.3, 0.1</td>
<td>10%/6</td>
<td>A, B 2.2, 0.2</td>
<td>0%</td>
<td>A 1.5, 0.1</td>
<td>47°C</td>
<td>A 4.7, 0.5</td>
<td></td>
</tr>
<tr>
<td>5/37°C</td>
<td>B</td>
<td>0.1, 0.1</td>
<td>10%/4</td>
<td>B, C 1.5, 0.1</td>
<td>5%</td>
<td>A 0.3, 0.1</td>
<td>45°C</td>
<td>A, B 4.3, 0.4</td>
<td></td>
</tr>
<tr>
<td>6/37°C</td>
<td>B</td>
<td>-0.2, 0.1</td>
<td>0%/4</td>
<td>C 1.2, 0.2</td>
<td>45°C</td>
<td>B 1.0, 0.2</td>
<td>37°C</td>
<td>B 3.7, 0.4</td>
<td></td>
</tr>
<tr>
<td>(%NaCl, pH)</td>
<td></td>
<td>(Temp.)</td>
<td></td>
<td>(Temp.)</td>
<td></td>
<td>(pH)</td>
<td></td>
<td>(Temp.)</td>
<td></td>
</tr>
<tr>
<td>0%/4</td>
<td>A</td>
<td>4.5, 0.2</td>
<td>47°C</td>
<td>A 1.6, 0.2</td>
<td>37°C</td>
<td>A 4.7, 0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10%/4</td>
<td>B</td>
<td>2.3, 0.1</td>
<td>37°C</td>
<td>A 4.7, 0.5</td>
<td>37°C</td>
<td>B 3.7, 0.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5%/4</td>
<td>B, C</td>
<td>1.4, 0.1</td>
<td>45°C</td>
<td>B 1.0, 0.2</td>
<td>47°C</td>
<td>B -3, 0.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10%/5</td>
<td>C, D</td>
<td>0.6, 0.1</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>10%/6</td>
<td>C, D</td>
<td>0.3, 0.1</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>5%/5</td>
<td>D</td>
<td>0.3, 0.1</td>
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<td></td>
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<tr>
<td>5%/6</td>
<td>D</td>
<td>0.2, 0.1</td>
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<tr>
<td>0%/5</td>
<td>D</td>
<td>0.1, 0.2</td>
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<tr>
<td>0%/6</td>
<td>D</td>
<td>-0.2, 0.1</td>
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</table>

M, unweighted mean and E, standard error for the loss in viable cells in log10 after stress pretreatments or lethal stresses and % of dead cells after heat lethal stress.

Heat lethal stress

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>M E</th>
<th>Pretreatment</th>
<th>M E</th>
<th>Pretreatment</th>
<th>M E</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Temp.)</td>
<td></td>
<td>(pH)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0% 0%/4</td>
<td>5</td>
<td>A 2.5, 0.5</td>
<td>45°C</td>
<td>A, B 5, 0.6</td>
<td></td>
</tr>
<tr>
<td>5% 5%/6</td>
<td>5</td>
<td>A, B 1.8, 0.5</td>
<td>45°C</td>
<td>A, B 5, 0.6</td>
<td></td>
</tr>
<tr>
<td>0% 0%/6</td>
<td>4</td>
<td>B 0.4, 0.5</td>
<td>47°C</td>
<td>B -3, 0.7</td>
<td></td>
</tr>
</tbody>
</table>
3.4.2 Batch culture

Because the traditional production method for probiotics is batch cultures, five selected stress pretreatments (47°C; pH 4.0; 10 % NaCl and combinations 47°C+10 % NaCl and pH 4.0+10 % NaCl) showing enhancing and/or decreasing effects on cell robustness to various lethal stresses were tested during batch cultures of *B. longum* NCC2705 to compare stress adaptation responses in both culture modes. Stress pretreatments at pH 4.0 and pH 4.0+10 % NaCl were chosen because of the protective effect of salt against low pH observed in the continuous culture. Moreover in continuous culture, pretreatments at 47°C were shown to enhance resistance to heat stress and decrease resistance to osmotic and freeze drying, whereas stress pretreatments with 10 % NaCl improved resistance to freeze drying. Batch control cultures without stress application were carried out at 37°C, pH 6.0 and 0 % NaCl for an additional time of 42 min after reaching stationary phase. A comparison of the results observed in continuous and batch cultures is represented in Table 10.

3.4.2.1 Effect of stress pretreatments on cell viability and carbohydrate metabolism

Viable cell counts measured at the beginning of stationary growth phase before sublethal stress applications were similar for all sublethal stress and control culture (8.8 ± 0.3 log CFU ml⁻¹). For control cultures viable cell counts did not change after 42 min in stationary phase (8.6 ± 0.5 log CFU ml⁻¹). Glucose was not detected at the time point of stress pretreatment application (Table 7). Pretreatments at 47°C and 10% NaCl did not significantly affect cell counts compared to control culture (Figure 11). In contrast, cell counts after pretreatments with 10 % NaCl in combination with pH 4.0 or 47°C were significantly lower compared to control culture (Figure 11), and pH 4.0 caused the highest viability loss exceeding 3 log₁₀ CFU ml⁻¹ (data not shown).
Figure 11 Viable cells before and after stress pretreatments in batch cultures of *B. longum* NCC2705 in log_{10} CFU ml^{-1}. Control cultures without stress application were run at 37°C, pH 6.0 and 0 % NaCl. Stress pretreatments of the duration of 42 min included 47°C, pH 4.0, and 10 % NaCl and the combinations 47°C+10 % NaCl and pH 4.0+10 % NaCl. Data are means of two biological replicates ± standard deviation. Pretreatments not connected by same letters are significantly different.
3.4.2.2 Effect of stress pretreatments on cell robustness after lethal stress application

No further lethal stress tests were performed with samples pretreated at pH 4.0 because of high losses in viable cells measured after this pretreatment.

Heat lethal stress

The percentage of dead cells produced by the heat lethal stress in cell samples pretreated at 47°C was significantly lower than for the control, with 15.4 ± 5.7 and 43.3 ± 0.1 %, respectively (Figure 12). The other applied stress pretreatments did not have significant effects on cell resistance to heat.

![Figure 12 Percentages of dead cells after heat lethal stress after stress pretreatments in batch cultures of B. longum NCC2705. Control cultures without stress application were run at 37°C, pH 6.0 and 0 % NaCl. Stress pretreatments of the duration of 42 min included 47°C, 10 % NaCl and the combinations 47°C+10 % NaCl and pH 4.0+10 % NaCl. Data are means of two biological replicates ± standard deviation. Pretreatments not connected by same letters are significantly different.](image-url)
**Osmotic lethal stress**

Control cells showed a cell viability loss of $1.6 \pm 0.2 \log_{10}$ after osmotic lethal stress, which was similar to pretreatment at 47°C (Figure 13). Pretreatment with 10% NaCl and the combination of 47°C + 10% NaCl resulted in significantly higher losses after osmotic lethal stress, with $2.0 \pm 0.3$ and $2.3 \pm 0.3 \log_{10}$, respectively, compared to control cells. On the other hand, pretreatment with pH 4.0 + 10% NaCl showed the lowest viable cells loss of $0.9 \pm 0.2 \log_{10}$ after osmotic lethal stress test (Figure 13).

![Figure 13](image)

Figure 13 Loss in viable cells in $\log_{10}$ CFU ml$^{-1}$ after osmotic lethal test after stress pretreatments in batch cultures of *B. longum* NCC2705. Control cultures without stress application were run at 37°C, pH 6.0 and 0% NaCl. Stress pretreatments of the duration of 42 min included 47°C, 10% NaCl and the combinations 47°C+10% NaCl and pH 4.0+10% NaCl. Data are means of two biological replicates ± standard deviation. Pretreatments not connected by same letters are significantly different.
**Freeze drying lethal stress**

Control cells exhibited a viability loss of $1.8 \pm 0.2 \log_{10}$ after freeze drying (Figure 14), not significantly different with pretreatments at $47^\circ C$, $10 \% \text{ NaCl}$ and the combination of $47^\circ C + 10 \% \text{ NaCl}$. Only pretreatment with pH 4.0 + 10 % NaCl negatively affected survival to freeze drying with high mean viability loss of $2.9 \pm 0.1 \log_{10}$.

![Figure 14](image.png)

*Figure 14* Loss in viable cells in $\log_{10}$ CFU ml$^{-1}$ after freeze drying after stress pretreatments in batch cultures of *B. longum* NCC2705. Control cultures without stress application were run at $37^\circ C$, pH 6.0 and 0 % NaCl. Stress pretreatments of the duration of 42 min included $47^\circ C$, 10 % NaCl and the combinations $47^\circ C+10 \% \text{ NaCl}$ and pH 4.0+10 % NaCl. Data are means of two biological replicates ± standard deviation. Pretreatments not connected by same letters are significantly different.
**Gastric lethal stress**

For the two repeated tests, pretreatment at 47°C showed systematically, although not significantly, lower losses compared to control after simulated gastric conditions (Figure 15). Pretreatment with 10 % NaCl and combination 47°C + 10 % NaCl resulted in higher viability losses than for control cells, with 0.8 ± 0.1 and 1.0 ± 0.3 log<sub>10</sub>, respectively, whereas the combination of pH 4.0 and 10 % NaCl showed intermediate viability losses between control and the other two NaCl pretreatments.

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**Figure 15** Loss in viable cells in log<sub>10</sub> CFU ml<sup>-1</sup> after gastric conditions lethal test after stress pretreatments in batch cultures of *B. longum* NCC2705. Control cultures without stress application were run at 37°C, pH 6.0 and 0 % NaCl. Stress pretreatments of the duration of 42 min included 47°C, 10 % NaCl and the combinations 47°C+10 % NaCl and pH 4.0+10 % NaCl. Data are means of two biological replicates ± standard deviation. Pretreatments not connected by same letters are significantly different.
Bile salts lethal stress

Loss in viable cells after bile salt test was of $1.8 \pm 0.1 \log_{10}$ for control cells (Figure 16). No significant difference between control and cells pretreated at 47°C was found. On the other hand, losses for cells pretreated with 10 % NaCl, 47°C + 10 % NaCl and pH 4.0 + 10 % NaCl exceeded the detection limit of the method (Figure 16).

Figure 16 Loss in viable cells in $\log_{10}$ CFU ml$^{-1}$ after bile salts lethal test after stress pretreatments in batch cultures of *B. longum* NCC2705. Control cultures without stress application were run at 37°C, pH 6.0 and 0 % NaCl. Stress pretreatments of the duration of 42 min included 47°C, 10 % NaCl and the combinations 47°C+10 % NaCl and pH 4.0+10 % NaCl. Data are means of two biological replicates ± standard deviation. Pretreatments not connected by same letters are significantly different.
Table 10 Effect of selected stress pretreatments in continuous and batch cultures of *B. longum* NCC2705

<table>
<thead>
<tr>
<th>Stress pretreatment</th>
<th>Effect in continuous culture</th>
<th>Effect in batch culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>All combinations of temperature and salt concentration with pH 5.0 or pH 6.0</td>
<td>No effect on cell viability (can be considered as sublethal stresses)</td>
<td>No effect on cell viability for 47°C and 10% NaCl, however 47°C+10% NaCl had an effect on cell viability (cannot be considered as sublethal stress)</td>
</tr>
<tr>
<td>All combinations with pH 4.0</td>
<td>Effect on cell viability (no sublethal stress)</td>
<td></td>
</tr>
<tr>
<td>Addition of NaCl at pH 4.0</td>
<td>Lower losses in cell viability compared to without NaCl</td>
<td></td>
</tr>
<tr>
<td>Increase in temperature from 37 to 47°C</td>
<td>Increase in cell resistance against subsequent heat lethal stress</td>
<td></td>
</tr>
<tr>
<td>Increase in temperature from 37 to 47°C</td>
<td>Lower cell resistance against subsequent osmotic and a freeze drying lethal stresses</td>
<td>No effect on osmotic and freeze drying lethal stresses</td>
</tr>
<tr>
<td>Decrease in pH from 6.0 to 4.0</td>
<td>Lower cell resistance against freeze drying</td>
<td></td>
</tr>
</tbody>
</table>
3.5 Discussion

The survival of bifidobacteria to a lethal stress can be improved by pretreatments with sublethal stresses, but strain specific optimization is necessary to obtain notable improvements (Saarela et al. 2004). In this study the potential of a two-stage continuous culture as a fast screening method was investigated to study the effect of stress pretreatments on probiotic cells survival to lethal stresses encountered during production, down-stream processing or in the human gastrointestinal tract. We previously showed as a prerequisite for applying such system that continuous culture allowed the production of \textit{B. longum} NCC2705 cells with constant growth rate and controlled physiology over an extended period of time (Chapter 2). Moreover, the two stage continuous culture design allowed efficient screening of several sublethal stresses during the same culture experiment, because only a short time (corresponding to approximately 6 residence times) is necessary to stabilize the second reactor after changing the conditions (Lacroix and Yildirim 2007). Therefore, up to four different stress pretreatments could be tested per day, with the conditions used in this study (mean residence time of 42 min in R2).

Sublethal injuries occur following exposure of bacterial cells to unfavorable physical and chemical environment (beyond the growth range but not in the lethal range) that cause reversible alterations in the functional and structural organization of the cells (Ray 2004). Pretreatments applied during continuous culture, except for pH 4.0 alone or in combination, could be considered sublethal because they did not affect significantly cell viability compared to control conditions (no stress pretreatment). Pretreatments studied during batch cultures showed similar effects on cell viability as the same pretreatments applied during continuous culture with the exception of pretreatments with combination of 47°C+10\% NaCl, which caused higher losses in batch cultures compared to continuous culture and could not be considered sublethal. In both continuous and batch cultures, reduction of viable cell counts for pretreatments at pH 4.0 was restrained by adding salt, suggesting that addition of salt protects cells against acidic stress. Such protective effect has already been observed in exponential and stationary \textit{E. coli} cells, where addition of salt resulted in an increased cytoplasmic pH and protection against acidic stress (Casey and Condon 2002).
Freeze drying is an industrially important downstream process for probiotic cultures (Lacroix and Yildirim 2007). During freeze drying, cells are subjected to osmotic, freezing and cold stresses. In continuous culture, osmotic pretreatment showed a positive effect on cell survival during freeze drying as already reported for B. adolescentis NCC251 (Schmidt and Zink 2000), whereas in batch culture such effect was not shown. Heat pretreatment improved the survival to lethal temperature for continuously produced B. longum NCC2705 cells. Improved survival to lethal heat stress after heat pretreatment is a general mechanism which has already been reported for many organisms, including B. longum, B. adolescentis and Lactobacillus plantarum (Schmidt and Zink 2000; De Angelis et al. 2004; Saarela et al. 2004). Sublethal heat stress lead to overexpression of the dnaK and groE operons in B. longum (Rezzonico et al. 2007). The DnaK and GroE systems are involved in general stress response and repair of damaged proteins (Hartl 1996). These general stress genes are thought to provide non-specific protection to the cell in the event of adverse conditions. Therefore a positive effect of heat pretreatment against other lethal stresses (cross protection) was expected. However, in our study we did not detect any improvement by heat stress pretreatments against other tested lethal stresses. Cross protection effects, associated to the adaptive response to a given stress, have already been shown to be species specific in lactic acid bacteria (van de Guchte et al. 2002) and bifidobacteria (Schmidt and Zink 2000).

During continuous culture we did not observe any adaptive response to gastric conditions after sublethal stress pretreatments. In contrast, B. lactis, preconditioned at pH 5.2 exhibited increase in survival rate to synthetic gastric fluid (Maus and Ingham 2003). This difference emphasized the species and strain dependence of stress response (Schmidt and Zink 2000; Collado and Sanz 2007). However, it must be considered that differences between studies may be due to the methods used to measure the stress resistance. For instance in our study, because of the large amount of samples, it was necessary to freeze samples before analysis, which could have biased the results in comparison to other studies without freezing.

We also observed that continuously produced cells surviving pretreatment at pH 4.0, which could not be considered a sublethal stress for its high detrimental effect on cell viability, were more resistant to osmotic, gastric and bile lethal stresses. Selection of bile
resistant cells by exposition to acid pH can be explained by the fact that these two stresses induce a very similar molecular response; it was already observed that bifidobacterial strains showing the highest resistance to bile salt exposure were also acid-resistant (Noriega et al. 2004) and vice versa (Collado and Sanz 2007). On the other hand the lethal acid stress can have also selected a resistant subpopulation surviving this stress and exhibiting different characteristics to the original population.

Sublethal stresses can also have negative effects on cell survival to lethal stresses. Survival of B. longum NCC2705 during freeze drying was negatively affected by acidic and heat pretreatments, resistance to osmotic lethal stress was decreased by severe heat (47°C) and resistance to simulated gastric conditions was impaired by addition of NaCl (5 %) in the reactor. Hence heat could improve the viability of probiotic cells for one lethal treatment (heat) but could have a different effect for other lethal stresses (freeze drying and osmotic). This could also be partially explained by the specific responses of cells to heat and cold stress. Heat causes protein and DNA denaturation, whereas cold stress mainly damages the membrane. Response to heat stress is principally given by the activation of chaperone proteins (Rezzonico et al. 2007), whereas bacterial adaptation to low temperatures is an active process resulting in increased fatty acid unsaturation and polypeptide synthesis (Girgis et al. 2002). Additionally we can hypothesize that heat and osmotic stress responses do not overlap in B. longum differently to B. breve UCC2003 (De Dea Lindner et al. 2007). Accordingly, dnaK was not activated after exposition to 1.5 % NaCl in B. longum contrary to B. lactis cultures (Schmidt and Zink 2000).

Because batch cultures are very often used for cell production in the industry we compared the effect of stress pretreatments on B. longum NCC2705 cell viability and stress resistance in continuous and batch cultures. Most pretreatments tested in batch cultures showed similar effects compared to those observed in the continuous culture, with the exception of application of 47°C combined with 10 % NaCl which resulted in significant losses in viable cells in batch in contrast to continuous culture. Additionally, in batch culture, addition of 10 % NaCl had a negative effect against the osmotic lethal tests whereas no effect was observed for continuous culture. These differences between continuous and batch cultures could be due to differences in time of application of stress pretreatments. In continuous culture stress pretreatment was applied for exactly 42 min.
(i.e. the mean residence time of the cells in the well mixed reactor), whereas in batch cultures the time of application was prolonged due to the time needed to reach the chosen set-point in the reactor. Furthermore the physiology of cells produced in continuous and batch cultures was not exactly the same, even though parameters were carefully chosen. The growth rate of cells produced in the first reactor of the continuous culture, set and equal to the dilution rate (0.1 h⁻¹), was selected to mimic end exponential growing cells of batch culture. In contrast when stress pretreatments were applied cell growth was zero during both continuous (R2) and batch cultures due to sugar starvation. Differences in metabolites production were observed, with less lactic acid and more acetic and formic acid and ethanol produced during continuous culture compared to batch cultures.

Data from this study suggest that continuous culture has advantages over batch cultures, allowing fast and efficient screening of sublethal stress conditions for cell adaptation to stress. *B. longum* NCC2705 cells produced during steady state continuous cultures have constant and controlled physiology and can be directly used for sublethal stress screening. Furthermore, cells produced in the first stage reactor can be used as direct control for stressed cells in the second reactor, decreasing experimental time and effort compared to testing in batch cultures. Our data confirmed that adaptive response to sublethal stresses exists in *B. longum*. Moreover, application of sublethal stresses can also have negative effects on cell robustness and therefore must be chosen carefully according to processing and use of the culture to improve cell robustness.
4 Novel method for selection of resistant bifidobacteria cells to hydrogen peroxide using continuous culture and immobilized cell technology

4.1 Abstract

Oxidative stress can severely compromise production at industrial scale of sensitive probiotic strains, such as many bifidobacteria, as well as their incorporation into food products. Exposure to oxygen of bifidobacteria causes accumulation of reactive oxygen species, mainly hydrogen peroxide, leading to cell death. In this study, we tested the suitability of continuous culture under increasing selective pressure combined with immobilized cell technology for the selection of bifidobacteria resistant to hydrogen peroxide. Cells of *B. longum* NCC2705 were immobilized in gellan-xanthan gum gel beads using a two phase dispersion process. These gel beads were used for continuous fermentation of MRS containing increasing concentration of H$_2$O$_2$ from 0 to 130 ppm. Immobilization allowed reaching very high cell densities of $10^{13}$ CFU per litre of bioreactor at the beginning of the culture. The continuous culture gradually adapted to increasing H$_2$O$_2$ concentrations, as shown by the optical density (OD) of culture effluent, which dropped after each increase of H$_2$O$_2$ concentration and then increased again. However, after increasing the H$_2$O$_2$ concentration to 130 ppm the OD of the culture decreased to 0. Full wash out was prevented by the immobilization of the cells in gel matrix. Hence after stopping the stress, it was possible to re-grow the cells that survived the highest lethal dose of H$_2$O$_2$ and to isolate two resistant clones (HPR1 and HPR2). In contrast to HPR1, HPR2 showed stable characteristics over at least 70 generations and exhibited also higher tolerance to O$_2$ than non adapted wild type cells. Preliminary characterization of HPR2 was carried out by global genome expression profile analysis: 2 genes coding for a protein with unknown function and possessing trans-membrane domains and an ABC-type transporter protein were constitutively overexpressed in HPR2 cells compared to wild type cells.
In conclusion, our study showed that continuous culture with cell immobilization is a valid approach for selecting cells adapted to hydrogen peroxide. Elucidation of H$_2$O$_2$ resistance mechanisms in HPR2 could be helpful to develop oxygen resistant bifidobacteria.


4.2 Introduction

According to FAO/WHO (2002), probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host”. One of the crucial points in the production and distribution of probiotic foods is hence to deliver enough live probiotic cells to the consumers. The minimum daily intake of probiotics to obtain a beneficial effect is still under debate. However, a concentration of $10^6$ live cells of probiotic bacteria per gram of product at the time of consumption is generally accepted and selected to provide bacterial concentrations that are attainable and cost-effective for probiotic food products (Sanders et al. 1996; Lacroix and Yildirim 2007). During production and storage of food, microorganisms experience a wide range of stresses, including oxidative stress, which can severely compromise cell viability of sensitive strains as well as their incorporation into food products. Bacterial strains belonging to Bifidobacterium and Lactobacillus genera are the most widely used microorganisms in probiotic food products and supplements (Vasijevic and Shah 2008). With the exception of B. indicum and B. asteroides, bifidobacteria are obligatory anaerobes, that possess neither a respiratory chain nor catalase (Biavati and Mattarelli 2001). Exposure to oxygen induces accumulation of reactive oxygen species, mainly hydrogen peroxide, which cause oxidative damage to DNA, resulting in increased levels of mutagenesis and cell death of sensitive cells (Talwalkar and Kailasapathy 2004; Kawasaki et al. 2006).

Although, oxygen tolerant bifidobacteria can be isolated from the environment (Meile et al. 1997), these isolates do not necessarily exhibit relevant probiotic characteristics. Mutagenesis with UV and NTG (N-methyl-N’-nitro-N-nitrosoguanidine) has been applied to obtain oxygen tolerant bifidobacteria (Shiuann et al. 1998). However mutagenesis can cause random mutations, which may also affect the probiotic characteristics of the mutated strain. Another method to isolate stress resistant bacterial strains consists in culturing cells in presence of a selective agent. Plating and cultivation in batch cultures with varying concentration of a selective agent are fairly simple procedures, but the number of generations over which selection can occur is limited. Therefore repeated cycles of subculturing may be required (Butler et al. 1996). At the
contrary to batch, continuous culture can be performed over an unlimited number of generations under strictly controlled conditions. It has been successfully used in combination with selective pressure for isolating cells resistant to antibiotics (Noack et al. 1988; Butler et al. 1996). Continuous culture can also be used to ensure continuous presence of unstable selective agents, such as hydrogen peroxide, at a constant rate in the medium. In contact with organic matter of rich media such as MRS broth, hydrogen peroxide can break up into nascent oxygen and water (Narendranath et al. 2000). However, the use of continuous culture combined with selective pressure is limited because the resistance level of cells can greatly vary with fermentation time. In addition, an over-dosage of the selective agent leads generally to a wash-out of cells from the bioreactor. This major drawback of continuous cultures can be prevented using immobilized cell technology, which allows retaining cell in reactor even if dilution rate exceed growth rate of the culture (Doleyres and Lacroix 2005; Lacroix and Yildirim 2007).

The aim of this study was to test the application of continuous culture with immobilized cells for selecting resistant populations of *B. longum* NCC2705. For this purpose, an oxidative stress was applied by increasing concentrations of hydrogen peroxide.
4.3 Material and methods

4.3.1 Strain

*B. longum* NCC2705 was obtained from the Nestlé Culture Collection (Lausanne, Switzerland) and cultivated in MRS (De Man et al. 1960) medium (Biolife, Milano, Italy). Two successive pre-cultures, inoculated at 1 % from a frozen stock at -80°C in MRS with 10 % glycerol (Sigma-Aldrich, Buchs, Switzerland), were performed for 16 h under anaerobic (AnaeroGen, Oxoid, Basingstoke, United Kingdom) conditions at 37°C before use. Part of the second pre-culture, defined as wild type culture, was stored at -80°C in MRS with 10 % glycerol for further analyses.

4.3.2 Cell immobilization

Cell immobilization was based on a two-phase dispersion process as previously described by Cinquin et al. (2004). A mixed gel of 2.5 % (w/v) gelrite gellan gum and 0.25 % (w/v) xanthan gum (both Sigma-Aldrich) was inoculated at 2 % (v/v) with a pre-culture of *B. longum* NCC2705, containing ca. 9.0 log_{10} CFU ml^{-1}. Beads with diameters in the range of 1.0 - 2.0 mm were selected by wet sieving and used for fermentation. The entire process was completed within 1 h.

4.3.3 Continuous culture

Beads (70 ml, measured by volume displacement) with immobilized cells were transferred into a 1-l stirred tank bioreactor (Multifors, Infors-HT, Bottmingen, Switzerland), equipped with a complete control system (Infors-HT), containing MRS (630 ml), corresponding to an inoculation rate of 10 % (v/v). The reactor was stirred at 100 rpm by an inclined blade propeller. Nitrogen (PanGas, Dagmersellen, Switzerland) was aseptically injected into the headspace to maintain anaerobic conditions. Temperature was set at 37°C and pH was maintained at 6.0 by adding 5 M NaOH. Culture was started in batch mode for the first 24 h, followed by 24 h in continuous mode with feeding of MRS, to allow colonization of gel beads. During this colonization step,
immobilized population increased from 7.0 to 11.0 $\log_{10}$ CFU g$^{-1}$ of gel beads. Afterwards, feeding of \( \text{H}_2\text{O}_2 \) solutions was started, using a calibrated peristaltic pump, and the culture was carried out in continuous mode for 23 days. Inflow rate of MRS, initially set to 2.6 ml min$^{-1}$ was decreased to 0.9 ml min$^{-1}$ at day 9. \( \text{H}_2\text{O}_2 \) was continuously added to the reactor at a flow rate 10 fold smaller than those of the MRS and using concentrated solutions ranging from 50 to 1,300 ppm depending on the applied \( \text{H}_2\text{O}_2 \) level. \( \text{H}_2\text{O}_2 \) solutions were prepared using 30 % \( \text{H}_2\text{O}_2 \) (VWR, Dietikon, Switzerland) in sterilized water. Concentrated \( \text{H}_2\text{O}_2 \) solution was kept on ice, protected from light and replaced daily, to avoid \( \text{H}_2\text{O}_2 \) breakdown during the experiment. Bead and effluent samples were taken from the reactor at different time intervals for optical density (600 nm) measurements and cell enumeration using plate counts. Aliquots of 2 ml effluent samples were centrifuged (6,000 g, 2 min) and pellets resuspended in equal volume of fresh MRS containing 10 % glycerol and stored at -80°C for further analyses. Gel beads samples of 1 g were placed into 1 ml of MRS with 10 % glycerol and stored at -80°C.

**4.3.4 Viable cell enumeration in culture effluent and gel beads**

Samples from effluent were serially diluted in 1x phosphate buffered saline (pH 7.7) supplemented with 0.05 % L-cysteine hydrochloride monohydrate (C-PBS). Appropriate dilutions were plated in duplicate on MRS agar (DIFCO, Becton Dickinson AG, Allschwil, Switzerland) and incubated anaerobically at 37°C for 48 h. The immobilized cell population was also monitored by plate counts after adding ca. 0.5 g gel beads to 20 ml 1 % EDTA (Sigma-Aldrich) and treatment in a stomacher for 3 min for bead dissolution before dilution in C-PBS.
4.3.5 $\text{H}_2\text{O}_2$ resistance of cells from culture effluent

Resistance to $\text{H}_2\text{O}_2$ over fermentation time of cells from culture effluent was tested as follows. Frozen samples were subcultured (1% inoculum) 2 times in MRS anaerobically at 37°C for 24 h in order to have similar viable cell numbers in all samples. Aliquot of 1 ml containing ca. $9.0 \pm 0.2 \log_{10} \text{CFU ml}^{-1}$ was centrifuged at 6,000 g for 2 min and the pellet was resuspended in 10 ml 400 ppm $\text{H}_2\text{O}_2$ solution. After 1.5 h incubation at room temperature, the cell suspension was diluted in C-PBS and plated on MRS agar and incubated anaerobically at 37°C for 48 h. Results were expressed as survival rate in percent before and after treatment. The test was performed in duplicate.

4.3.6 Isolation of cells resistant to $\text{H}_2\text{O}_2$

Frozen beads samples (1 g) collected at day 18 of continuous culture, were dissolved in 40 ml 1% EDTA and centrifuged at 6,000 g for 5 min. Cell pellets were resuspended in 10 ml 40 ppm $\text{H}_2\text{O}_2$ and incubated for 60 min at room temperature to recover fractions of populations adapted to $\text{H}_2\text{O}_2$ stress. 1 ml of cell suspension was subsequently plated on MRS agar. Colonies visible within 48 h were sub-cultured 2 times anaerobically for 16 h in MRS broth at 37°C and frozen at -80°C in MRS with 10% glycerol until further analyses.

4.3.7 Characterization of $\text{H}_2\text{O}_2$ resistant isolates

4.3.7.1 $\text{H}_2\text{O}_2$ resistance level of isolates

Frozen samples of wild type and $\text{H}_2\text{O}_2$ resistant isolates cultures containing approximately $9.0 \pm 0.1 \log_{10} \text{CFU ml}^{-1}$ were thawed at room temperature. 1 ml of sample was centrifuged at 6,000 g for 2 min. The pellet was resuspended in 10 ml 200 ppm $\text{H}_2\text{O}_2$ solution. After 2 h incubation at room temperature cell suspensions were diluted in C-PBS and plated on MRS agar and incubated anaerobically at 37°C for 48 h. Results were expressed as survival rate in percent before and after treatment. The test was performed in triplicate.
4.3.7.2 Stability of $\text{H}_2\text{O}_2$ resistant phenotype

Stability of resistant phenotype of isolates was tested by subculturing cells without selective pressure in MRS at 37°C under anaerobic conditions for 24 h. After each subculture, containing ca. $9.2 \pm 0.2 \log_{10} \text{CFU ml}^{-1}$, resistance to $\text{H}_2\text{O}_2$ of cells was tested using 400 ppm $\text{H}_2\text{O}_2$ solution as described above. Tests were performed in duplicate.

4.3.7.3 Growth in presence of oxygen in liquid shaking cultures

Ability of cells to grow in presence of oxygen was tested according to Meile et al. (1997) with some modifications. The headspace of 500 ml serum flasks containing 400 ml MRS, after creating vacuum conditions was flushed with $\text{N}_2$ or $\text{CO}_2$ until recreating normal atmosphere pressure; after which 7.5 or 12.5 % (v/v) sterile oxygen were added. The medium was then inoculated at 2 % with an overnight culture of wild type or $\text{H}_2\text{O}_2$ resistant cells and the flasks were incubated at 37°C in a shaker at 160 rpm (Kühner AG, Basel, Switzerland) for 24 h. Samples were taken every 2 h during the first 12 h of the culture to measure optical density. Two repetitions were carried out.

4.3.7.4 Growth in reactor with and without $\text{H}_2\text{O}_2$

Cells were grown in a 800-ml working volume bioreactor (1000-ml bioreactor, Infors-HT) containing MRS inoculated at 5 % with preculture. Temperature was controlled at 37°C, agitation set at 200 rpm and starting pH adjusted to 6.1 ± 0.1. Anaerobic conditions were maintained by continuously sparging $\text{CO}_2$ or $\text{N}_2$ in the medium, starting overnight prior to inoculation. Cells were cultivated with and without addition of 42 ppm $\text{H}_2\text{O}_2$ in mid-exponential growth phase corresponding to an OD of 0.6. Growth was monitored by measuring OD at 600 nm. Fermentations were performed in triplicate.
4.3.8 Microarray analysis

Collection of samples
Samples for global transcriptional profiling were taken in mid-exponential growth phase after 3-3.5 h of culturing at an OD between 0.7 and 0.8 in reactors sparged with CO$_2$ and without H$_2$O$_2$. Aliquots of 2 ml were centrifuged (4,000 g, 1 min, room temperature), supernatants discarded and cell pellets snap frozen in liquid nitrogen and stored at -80°C until RNA-extraction.

Microarray design and RNA extraction
DNA based arrays for *B. longum* NCC2705, produced by Agilent Technologies (www.agilent.com), were obtained by *in situ* synthesis of 60 mer oligonucleotides on glass slides (Wolber et al. 2006). For each gene, 3 to 6 different probes were randomly distributed on the array. Total RNA was extracted with the Macaloïd method and purified as previously described (Parche et al. 2006).

Array hybridization
For each hybridization, cDNA was synthesized starting from 4 µg of total RNA and subsequently labeled using the Array 900MPX Genisphere kit (Genisphere Inc., Hatfield, PE, USA), following the protocol provided by the supplier. Luciferase and kanamycin control mRNA (Promega, Zurich, Switzerland) at 1 and 10 ng, respectively, were mixed with total RNA before labeling to allow balancing of the two channels during scanning. After the hybridization procedure, array slides were scanned at 10 µm using a Scanarray 4000 (Packard Biochip Technologies, Billerica, MA, USA). Parameters (laser power and photomultiplier tube gain) were set in order to prevent saturation of any spot, except the probes corresponding to rRNA.

Array analysis
Data extracted with Imagene 5.6 (Biodiscovery, El Segundo, CA, USA) were treated with homemade scripts in Python language (www.python.org) and a local installation of the ArrayPipe web server (Hokamp et al. 2004). Probes showing a signal smaller than twice the standard deviation of the local background were considered without signal. Probes
showing no signal or saturated signals in both channels were discarded from the analysis. Assuming an intensity-dependent variation in dye signal, (limma) loess global normalization was applied on signal ratios. To calculate average gene expression values, data from 3 biological replicates were combined as follows. Within each hybridization data set, gene fold changes were calculated from the median of the corresponding probes values. The expression value of a gene was retained if a signal was detected in at least 50% of its probes. Genes were considered to be differentially expressed if their log2-transformed signal ratios were higher than 1.5 or smaller than -1.5. Statistical analysis of the 3 biological array replicates of the hybridization between wild type and H2O2 resistant cells were performed using the statistical software R version 2.6.1 (R Development Core Team 2007). Bayes statistics for differential expression (Smyth 2004) was used to rank genes in order of evidence for differential expression using default parameters. The data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE16039. TMHMM-prediction server v. 2.0 was used to identify transmembrane domains of predicted proteins (Krogh et al. 2001).

4.3.9 Statistical analysis

For H2O2 survival, a t-test assuming equal variances (significance level of 0.05) was performed after transforming cell counts to their base 10 logarithms to stabilize the variance and normalize the residuals. Analysis was carried out using JMP (JMP, Version 7. SAS Institute Inc., Cary, NC, 1989-2007).
4.4 Results

4.4.1 Continuous culture monitoring

The concentration of H$_2$O$_2$ in the continuous culture with immobilized cells of B. longum NCC2705 was increased stepwise from 0 to 130 ppm in order to select for cells resistant to oxidative stress (Figure 17). At day 2, the optical density of the culture effluent reached 8.4, corresponding to 9.1 log$_{10}$ CFU ml$^{-1}$. Subsequently, OD decreased and increased after each increase in H$_2$O$_2$ concentration. Viable cells in effluent samples before changing the level of H$_2$O$_2$ concentration ranged from 8.8 to 9.3 log$_{10}$ CFU ml$^{-1}$ until day 9 (Figure 17). Following H$_2$O$_2$ concentration increase to 130 ppm at day 9, the OD decreased to 0. To enrich potentially hydrogen peroxide resistant survivors after this harsh treatment, the addition of H$_2$O$_2$ was stopped for 2 days and the flow rate of the medium was decreased to 0.9 ml min$^{-1}$, resulting in a new phase of growth with an increase of OD to 3.2 at day 11 in effluent samples, corresponding to 5.8 log$_{10}$ CFU ml$^{-1}$. Concentration of H$_2$O$_2$ was then set again at 100 ppm and OD decreased again to 0 with viable cell counts in the effluent gradually decreasing from 5.6 at day 14 to 4.4 log$_{10}$ CFU ml$^{-1}$ at day 20 (Figure 17). Cell counts were generally 1 to 2 log higher than free cell counts in effluent samples, except the end of culture where both population came very close (Figure 17).

H$_2$O$_2$ resistance of free cells in the culture effluent remained stable during the first 8 days with a survival rate of 0.0001± 0.00004 % before treatment with 130 ppm H$_2$O$_2$. At day 14 and 18 the survival rate was 760 and 16 folds higher than for day 1, respectively (Figure 18) with an apparent time effect.
Figure 17 Optical density of effluent (black line), H$_2$O$_2$ concentration in reactor (grey dashed line) and viable cells counts in gel beads (closed circle) and in the effluent (open circle) during continuous culture with immobilized cells of B. longum NCC2705 and H$_2$O$_2$ selective pressure.

Figure 18 Resistance of culture effluent to H$_2$O$_2$ in % survival (calculated from CFU ml$^{-1}$) after 1.5 h in 400 ppm H$_2$O$_2$ solution. Data are means of two replicates ± standard deviation.
4.4.2 Selection of resistant cells and stability of H$_2$O$_2$ resistance phenotype

Because a large fraction of cell biomass of the system was in the bead matrix (ca. 11.0 log$_{10}$ CFU g$^{-1}$ beads after colonization) at the beginning of the fermentation, a higher number of adapted cells was expected in the beads, which were physically retained in the bioreactor, than in effluent at the end of culture. Cells from beads collected at day 18 (4.8 log$_{10}$ CFU g$^{-1}$) were subjected to an isolation step, and tested for their resistance to H$_2$O$_2$. Two colonies, namely HPR1 and HPR2, were detected after this isolation step. Tolerance to H$_2$O$_2$ of cells from these two isolates were 20 and 30 folds higher, respectively, than that of wild type cells, (Figure 19). A stability test was performed with these two isolates. HPR1 lost its H$_2$O$_2$ resistance phenotype after three successive cultures, showing similar resistance level compared to wild type cells, whereas HPR2 maintained its phenotype over at least 11 subsequent cultures (Figure 20). The survival tests showed large variations depending on the testing day and subculture (Figure 20), however a repetition of this test showed similar results (data not shown).

![Figure 19](image_url)  
**Figure 19** H$_2$O$_2$ resistance level of isolates (HPR1 and HPR2) and wild type *B. longum* NCC2705 (WT) in % survival (calculated from CFU ml$^{-1}$) after 2 h in 200 ppm H$_2$O$_2$ solution. p-values of 0.03 were determined with a t-test.
Figure 20 Stability of H$_2$O$_2$ resistant phenotype in % survival (CFU ml-1) after 2 h in 400 ppm H$_2$O$_2$ solution. Wild type *B. longum* NCC2705 (black bars), HPR1 (dashed bars), and HPR2 (white bars).

### 4.4.3 Growth in presence of oxygen in liquid shaking cultures

HPR2 and wild type cells grew at similar rate of 0.47 ± 0.03 and reached the same optical density of 5.2 ± 0.4 after 24 h culture in presence of 100 % nitrogen or carbon dioxide in the headspace (Figure 21 and Figure 22). When the atmosphere of the headspace was composed of 7.5 and 82.5 % of oxygen and nitrogen, respectively, growth of HPR2 cells was impaired reaching an OD of 0.6 ± 0.2 after 24 h culture and that of wild type cells was negligible (Figure 21). A better growth was observed for both strains in presence of oxygen, even at 12.5%, when nitrogen was replaced by carbon dioxide (Figure 22). Under this atmosphere, growth ability was also higher for HPR2 than wild type strains (Figure 22).
Figure 21 Growth of HPR2 (triangle) and wild type *B. longum* NCC2705 (squares) in presence of 100 % N₂ (closed symbols) and of 7.5 % O₂ - 92.5 % N₂ (open symbols) in liquid shaking cultures. Growth was monitored using optical density at 600 nm.

Figure 22 Growth of HPR2 (triangle) and wild type *B. longum* NCC2705 (squares) in presence of 100 % CO₂ (closed symbols) and of 12.5 % O₂ - 87.5 % CO₂ (open symbols) in liquid shaking cultures. Growth was monitored using optical density at 600 nm.
4.4.4 Growth in presence of H$_2$O$_2$ in reactors

Both wild type and HPR2 cells recovered rapidly and in a similar manner after addition of H$_2$O$_2$ in medium sparged with carbon dioxide, and reached the same OD of 5.9 ± 0.5 than non treated cells after 24 h of culture (Figure 23). In presence of nitrogen, HPR2 strain started to recover earlier and at a higher rate than wild type cells after H$_2$O$_2$ addition reaching an optical density of 1.3 ± 0.03 and 0.7 ± 0.2, respectively, after 8 h culture (Figure 24). Growth of HPR2 and wild type cells, treated and not treated with H$_2$O$_2$, were negatively affected by sparging nitrogen compared to carbon dioxide in the medium (Figure 23 and Figure 24).

4.4.5 Genome-wide transcription analysis

Transcriptome analysis of exponentially growing cells without selective pressure in presence of CO$_2$ showed that the genes BL1404 and BL0931 were significantly overexpressed (p-values of <0.001 and 0.001) in HPR2 compared to wild type cells of B. longum NCC2705. BL1404 was overexpressed with an average log$_2$ ratio of 3.0 and BL0931 with an average log$_2$ ratio of 1.5. All remaining genes had log$_2$ transformed ratios between 1.5 and -1.5.
Figure 23 Growth of HPR2 (triangles) and wild type *B. longum* NCC2705 (squares) in liquid cultures in reactors with CO\(_2\) atmosphere, without addition of H\(_2\)O\(_2\) (closed symbols) and with addition of 42 ppm H\(_2\)O\(_2\) (open symbols). Growth was monitored using optical density at 600 nm.

Figure 24 Growth of HPR2 (triangles) and wild type *B. longum* NCC2705 (squares) in liquid cultures in reactors with N\(_2\) atmosphere, without addition of H\(_2\)O\(_2\) (closed symbols) and with addition of 42 ppm H\(_2\)O\(_2\) (open symbols). Growth was monitored using optical density at 600 nm.
4.5 Discussion

In this study, we tested the suitability of continuous culture under increasing selective pressure combined with immobilized-cells technology for the selection of *B. longum* NCC2705 derivatives resistant to hydrogen peroxide. Continuous culture allows cultivation over an unlimited number of generations under strictly controlled conditions. In addition continuously cultured cells may undergo a number of consecutive mutational events, each contributing to improve adaptation of cells to their environment (Novick and Szilard 1950; Butler et al. 1996). A very high cell concentration was reached in beads (10^{11} CFU g^{-1} beads) leading to a high total population, including immobilized and free cells, of 1 \times 10^{13} CFU per litre of bioreactor similar to that reported by Doleyres et al. (2002) with immobilized *B. longum* cells in gellan gum beads in continuous culture operated at a dilution rate of 0.5 h^{-1}. As a comparison, batch cultures with free cells of *B. longum* NCC2705 generally reach 10^9 CFU ml^{-1}, corresponding to 10^{12} CFU per litre of bioreactor at the end of the culture (data not shown). This high cell density could favor the occurrence of mutations since the rate of appearance of mutational events is proportional to the amount of biomass in the culture (Lane et al. 1999).

The continuous culture gradually adapted to increasing H_2O_2 concentrations, as shown by the culture oscillating OD. However, the *B. longum* population tested in the effluent reached an upper limit of its adaptive capabilities at day 9 with H_2O_2 concentration of 130 ppm. A possible explanation for this lies in the decreasing number of cells: the less biomass, the stronger the effect of the H_2O_2. For a cell biomass of ca 9.0 log_{10} CFU ml^{-1}, 130 ppm is likely to be beyond the resistance threshold. Immobilizing the cells in a gel matrix has prevented full wash-out, which would have very likely occurred with a free cells system. Hence after stopping the stress, it was possible to re-grow the cells that survived this high H_2O_2 concentration. After this enrichment step and under 100 ppm H_2O_2, resistance level of continuously produced cells was higher than those tested at the beginning of the fermentation but decreased to an intermediate level at day 18. Heterogeneity in the immobilized and free populations which can exhibit different resistance level and/or adaptative mechanisms to the selective pressure, as discussed
below for cells isolated from gel beads, could explain this change in resistance to H$_2$O$_2$ with fermentation time and using the same conditions.

HPR1 and HPR2, isolated from gel beads at day 18, showed a 20- and 30-fold higher resistance to H$_2$O$_2$ than wild type cells, respectively. Two different mechanisms can be proposed to explain the adaptative response of these two isolates in regard to the stability of their resistance phenotype. HPR1 isolate lost rapidly its resistance phenotype which could be the result of a transient adaptation caused by the stressing conditions encountered in gel beads and not necessarily by H$_2$O$_2$ selective pressure. Indeed, it was already reported that *B. longum* cells produced with immobilized cell technology, continuously cultivated without specific selective pressure adapt to H$_2$O$_2$ stress (Doleyres et al. 2004b). This phenotype was reversible after subculturing, and could be related to a non specific stress adaptation caused by diffusional limitations of both substrates and inhibitory products in the gel beads (Doleyres et al. 2004b). Resistance phenotype of HPR2 isolate to H$_2$O$_2$ was much more stable (at least over 70 generations) than that of HPR1 indicating a probable stable mutation. To our knowledge, this is the first description of a mutant strain of bifidobacteria resistant to H$_2$O$_2$. HPR2 cells were then further characterized.

HPR2 could tolerate higher O$_2$ level than wild type cells *B. longum* NCC2705 and *B. thermophilum* RBL67 that is considered as moderately oxygen-tolerant (von Ah et al. 2007). The ability of HPR2 isolate to grow in presence of oxygen can be associated with its H$_2$O$_2$ resistance phenotype. Indeed, accumulation of H$_2$O$_2$ during culture of sensitive bifidobacteria in presence of oxygen is generally considered as the primary reason for growth inhibition (Talwalkar and Kailasapathy 2004; Kawasaki et al. 2007; Kawasaki et al. 2009). Additionally, HPR2 and wild type seems to tolerate more O$_2$ in presence of CO$_2$ than with N$_2$ in the headspace of the culture.

The better growth of HPR2 and wild type cells observed in reactor with medium sparged with CO$_2$ instead of N$_2$ are in agreement with others studies reporting the essential role of CO$_2$, even at low level, to stimulate growth of bifidobacteria (Kawasaki et al. 2007; Ninomiya et al. 2009). Presence of residual dissolved CO$_2$ in MRS medium for liquid shaking culture, can also explain the lack of difference between growth of HPR2 and wild
type strains in presence of 100% N\textsubscript{2} or CO\textsubscript{2} in the headspace, in contrast to cultures in reactor where these gases were directly sparged into the medium.

A known mechanism influencing resistance to oxygen in bifidobacteria is the type of NADH oxidase activity. In O\textsubscript{2} sensitive species, NADH oxidase exhibits H\textsubscript{2}O\textsubscript{2} forming activity while H\textsubscript{2}O is produced in microaerophilic species (Kawasaki et al. 2006; Kawasaki et al. 2009). \textit{B. longum} NCC2705 possess a gene (BL1266), which codes for a putative NADH oxidase with an active site for the four electron reduction of O\textsubscript{2} to H\textsubscript{2}O and could therefore be implied in the detoxification of H\textsubscript{2}O\textsubscript{2} (Klijn 2005a). However, BL1266 is not differentially expressed in HPR2 compared to wild type cells. Constitutive overexpression of oxidative stress related proteins can also protect cells to H\textsubscript{2}O\textsubscript{2} as observed in a mutant strain of \textit{Bacteroides fragilis} resistant to H\textsubscript{2}O\textsubscript{2} (Rocha and Smith 1998). In HPR2 another mechanisms seems to be involved in the resistance to H\textsubscript{2}O\textsubscript{2}. Two genes, BL1404 and BL0931, were constitutively overexpressed in HPR2 compared to wild type. These two genes were not differentially expressed in wild type cells exposed to H\textsubscript{2}O\textsubscript{2} (Klijn 2005a). BLAST search (Zhang et al. 2000) showed that BL1404, which is annotated as hypothetical protein (Schell et al. 2002), is only homologous, 99 % on nucleotide level, to a integral membrane protein BLD_0271 of the same species (\textit{B. longum} DJ010A). The BLAST search could not identify homologous genes in other bacteria, indicating that BL1404 encodes a protein specific to bifidobacteria. The BL1404 protein possesses three predicted transmembrane domains and 2 outer and 2 inner domains. BL0931 is annotated as possible ABC-type transport system involved in lipoprotein release. Further experiments are required to confirm the role of these two proteins in H\textsubscript{2}O\textsubscript{2} resistance and to characterize their function.
In conclusion, our study showed that continuous culture with cell immobilization is a valid approach for selecting cells adapted to hydrogen peroxide. Cell immobilization allowed maintaining high cell numbers in the bioreactor, even when high selective pressure was applied. This enabled controlled application of stress at high levels on the culture over a long time. Additionally, characterization of HPR2 revealed the constitutive induction of two genes associated with the cell membrane. However, their function needs further characterization. Elucidation of H$_2$O$_2$ resistance mechanisms in HPR2 could be helpful to improve resistance of bifidobacteria to oxidative stress.
5 General conclusions and perspectives

In this dissertation, two hypotheses were tested. The first hypothesis was that continuous culture in a two stage system can be used as fast and efficient method for screening of sublethal stresses. An important precondition for this is that continuously produced cells show stable physiological properties over culturing time. A second hypothesis was that continuous culture in combination with immobilized cell technology and selective pressure can be used for efficient selection of stress resistant strains. These hypotheses were formulated based on the observation that continuous cultures have been used for producing cells with constant physiology over time (Hoskisson and Hobbs 2005) and also in combination with selective pressure for selection of resistant strains to antibiotics (Noack et al. 1988; Butler et al. 1996). *Bifidobacterium longum* NCC2705 is an ideal model organism for both approaches because some aspects of its stress response, metabolic control, and regulatory mechanisms have been characterized using genome wide expression analyses (Klijn 2005a). This knowledge was applied in this dissertation for screening and application of stresses and for the interpretation of the obtained data.

To test the first hypothesis, a 2-stage continuous culture with *B. longum* NCC2705 was developed and applied for fast and efficient screening of sublethal stresses. To verify the suitability of continuous culture for this purpose, the physiological stability of continuously produced cells was shown during 211 h continuous culture. More precisely, physiological parameters (such as viable cell counts, production of metabolites susceptibility to antibiotic and stress conditions) showed either stable or only moderate changes over culturing time. Comparison of gene transcription profiles between samples collected after 31 h of continuous culture and samples collected after 134 and 211 h revealed only limited changes in expression (1.0 and 3.8 % of total genes, respectively). This stability allowed applying different stress pretreatments with controlled conditions in the second stage of the continuous culture. A large number of stress pretreatments were screened during the same continuous culture in a short time frame using a factorial experimental design. Under the specific conditions used in this study, it was possible to test up to four different stress pretreatments per day, with important advantages relating
to high throughput and control of experimental error by using cells produced in the first stage as control cells. Combinations of pH (6.0, 5.0 and 4.0), temperature (37, 45 and 47°C) and NaCl concentration (0, 5 and 10 %) were tested using a 3 by 3 factorial design. All tested combinations were found to be sublethal apart from conditions with pH 4.0. However, the observed large reduction of viable cell counts for pretreatments at pH 4.0 was restrained by adding salt. Heat pretreatment at 47°C protected the cells from a subsequent heat lethal stress. In contrast, it negatively affected cell viability after freeze drying and osmotic lethal stresses. Generally, selected stress pretreatments (pH 4.0, 47°C, 10 % NaCl, 47°C+10 % NaCl and pH 4.0+10 % NaCl) applied to early stationary phase cells during batch cultures produced comparable effects to continuous culture. Hence, we showed that continuous cultures can be used for fast and efficient screening of a wide range of stress pretreatments, and test conditions that could then be applied for probiotic cells production in classical batch cultures. However, application of sublethal stresses was shown to improve cell resistance to some lethal stresses but also to negatively affect cell robustness to other lethal stresses. Therefore, stress pretreatments must be carefully chosen and adapted to the use of the culture to improve cell robustness. In this work we showed that continuous culture is an efficient approach for screening sublethal stresses to enhance cell robustness in laboratory setting. For future perspective, this technology could be examined, in combination with sublethal stresses, as production method for probiotic cultures. However, additional demonstration for industrial application is needed: the effects of sublethal stresses must be tested in conditions more similar to industrial stresses, such as spray drying. The next steps would then be to scale up the 2-stage continuous culture and assess the stability of this technology to contaminations which is an issue in open systems like continuous cultures. Additionally, combining continuous cultures with immobilized cell technology may allow increasing the productivity and biological stability of the system, but this need further testing.
The second hypothesis of this dissertation was that continuous culture in combination with immobilized cell technology and selective pressure could be used for selection of stress resistant strains. Resistance to oxidative stresses, such as hydrogen peroxide, is of particular interest in bifidobacteria, because oxidative stresses can severely compromise production at industrial scale of sensitive strains as well as their incorporation into food products. To test this second hypothesis, a continuous culture with immobilized cells and selective pressure (hydrogen peroxide) was developed and applied for isolating resistant strains to oxidative stress. Immobilized cell technology allowed maintaining high cell numbers in the bioreactor, even when high selective pressure was applied. This enabled controlled application of stress on the culture over a long time, leading to the successful isolation of a strain (HPR2) with improved and stable resistance to oxidative stress. Compared to wild type \textit{B. longum} NCC2705 the constitutive overexpression of two genes, BL1404 and BL0931, was observed in HPR2. However, these two genes were not differentially regulated in \textit{B. longum} NCC2705 under H$_2$O$_2$ stress and the coded proteins still need to be characterized. Additionally, in order to clarify the molecular mechanisms that protect HPR2 from H$_2$O$_2$ stress its genome-wide response to oxidative stress and the possible mutations present in its genome should be assessed. Identification of H$_2$O$_2$ resistance mechanisms in HPR2 could be helpful to develop oxygen resistant bifidbacteria. The phenotypic characteristics of the HPR2 strain are technologically very promising because this strain can tolerate higher amounts of oxygen and H$_2$O$_2$ as the wild type. However, in order to be able to apply HPR2 in food products its resistance in industrial processes (such as spray and freeze drying) and in food products (as for example yogurt) needs first to be quantified. This study showed that continuous culture with cell immobilization and application of a selective pressure is a valid approach for selecting \textit{B. longum} cells adapted to hydrogen peroxide. In the future, this system could be applied to other microorganisms and to other stresses, as for example heat and acid stresses, which are often encountered in the preparation and application of probiotic as food supplements.

Hence, in this dissertation the benefits of continuous cultures and immobilized cell technology as tools for improving the resistance of probiotic cells to environmental stresses have been demonstrated, opening new doors for technological optimization of
sensitive strains and their utilization in food products. However, additional work with conditions closer to industrial standards is needed.
6 Appendix: Genome wide expression analysis of culture samples produced with different technologies

6.1 Introduction
To characterize the 2-stage continuous culture used in this study for screening of stress pretreatment (Chapter 3) more extensively and to assess if transfer from one stage to the next affected the physiological stability of the cells, a comparison of the transcriptome and of end-fermentation products between the first (reactor 1) and the second stage (reactor 2) was performed. Additionally, to characterize the cellular state of the continuously produced cells, they were compared to exponential and early-stationary phase batch cultured cells. Few studies describe comparison of continuous and batch cultures on the transcriptomic and physiological level and often the comparison is carried out with exponential batch grown cells (Ihssen and Egli 2005; Franchini and Egli 2006). However, the growth phase of continuously produced cells does not necessarily correspond to that of exponential phase cells and because growth phase impacts on stress behavior of cells (Klijn 2005a), the growth rate should be taken into account. In steady state continuous cultures the growth rate corresponds, according to Monod kinetics, to the dilution rate in the reactor and the low dilution rate in this study should correspond therefore to a growth rate comparable to late exponential/early-stationary growth phase.
6.2 **Material and Methods**

6.2.1 **Strain and growth conditions**

*B. longum* NCC2705 was obtained from the Nestlé Culture Collection (NCC) and cultivated in MRS (De Man et al. 1960) medium (Biolife, Milano, Italy) supplemented with filter sterilized cysteine at a final concentration of 0.5 g l\(^{-1}\) (C-MRS). Two consecutive pre-cultures were performed at 37°C for 16 h. Anaerobic conditions were maintained using AnaeroGen (Oxoid, Basingstoke, United Kingdom).

6.2.2 **Batch cultures**

Cells were grown in a 2-l working volume bioreactor (Bioengineering AG, Wald, Switzerland), equipped with a control system (Biospectra AG, Schlieren, Switzerland). The batch fermentations were performed in C-MRS. The bioreactor was thermostated at 37°C and agitation set at 250 rpm. The pH was maintained at 6.0 by addition of 5 M NaOH. To ensure anaerobic conditions the reactor’s head space was flushed with CO\(_2\). The reactor was inoculated at 2 % with pre-culture. Samples for microarrays and HPLC analysis were taken in exponential and early stationary growth phase corresponding to cell concentrations of 8.1 and of 8.8 log\(_{10}\) (CFU ml\(^{-1}\)) and an optical density of 0.9 and 5.8, respectively. These cell numbers were enumerated by diluting the samples in phosphate buffer saline 1x, pH 7.7 (PBS) supplemented with 0.05 % cysteine (C-PBS), and plating appropriate dilutions in duplicate on C-MRS agar. Plates were then incubated anaerobically at 37°C for 48 h.
6.2.3 Continuous culture, substrates, metabolites and transcriptomic analyses

Samples to be analyzed were taken from the 2-stage continuous culture described in Chapter 3. Briefly, the first and second reactor of the 2-stage continuous culture had a working volume of 2 l and 140 ml, respectively. The second reactor, operated in series, was inoculated with culture broth from the first reactor. Medium (C-MRS) was added at a flow rate of 200 ml h\(^{-1}\) corresponding to a dilution rate of 0.1 h\(^{-1}\) in reactor 1 and leading to residence times of 10 h and 42 min in reactor 1 and 2, respectively. Carbon dioxide was injected into the headspace of the two reactors to maintain anaerobic conditions during the culture. The temperature was maintained at 37°C and pH controlled at 6.0 by addition of 5 M NaOH in both reactors.

Substrates and metabolites analyses were performed using HPLC and transcriptomic analysis using microarrays as described in Chapter 3. Genome wide expression analysis between continuously produced cells from reactor 1 and reactor 2 at time 134 h was performed with one biological replicate. Reactor 2 at time 134 h was operated under the same conditions found in reactor 1. Moreover, samples from the continuous culture at 134 h from reactor 2 were hybridized with samples from a batch culture in exponential growth phase and with samples from early-stationary growth phase. These hybridizations were performed with one biological replicate and two technical replicates including a dyes swap, log\(_2\) transformed ratios represent average values (Figure 25).
Figure 25 Hybridization scheme. The experiments were performed in duplicate with dyes swaps. Each arrow represents one hybridization; samples at the base of the arrow were labeled with Cy3 label and samples at the arrowhead with Cy5.
6.3 Results

6.3.1 Comparison of continuously produced cells collected in reactor 1 and 2

In order to further characterize the 2-stage continuous culture described in Chapter 3 and to assess if transfer from one stage to the next affected the physiological stability of the cells produced within this system, metabolite production and genome wide expression of cells from reactor 2 were compared to those of cells from reactor 1. Concentrations of glucose, acetic, lactic and formic acids and ethanol in the 2 reactors of the continuous culture were not different over the whole 211 h of culturing (Table 11). In the full genome based transcription analysis of cells from the first stage compared to cells from the second stage of the continuous culture the relative expression value of 99.6 % of the open reading frames (ORFs) was assessed, 0.6 % (10 ORFs) of which was differentially expressed. Seven genes were higher expressed (log$_2$ ratio up to 2.2) and three lower expressed (log$_2$ ratio up to 1.7) in the second reactor compared to the first (Figure 2). No major differences in metabolites production and gene expression could be detected between cells collected in reactor 1 and 2 of the 2-stage continuous culture (Table 11, Table 12).

Figure 26 Effect of reactor on the global gene expression levels in B. longum NCC2705. Plot displaying the global distribution of gene expression values for cells collected in reactor 2 relatively to cells in reactor 1.
Table 11 Metabolites concentrations in continuous (reactor 1 and 2) and in batch culture (exponential phase and early-stationary phase) of *B. longum* NCC2705

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Reactor 1 (g l^{-1})</th>
<th>Reactor 2 (g l^{-1})</th>
<th>Batch exponential phase (g l^{-1})</th>
<th>Batch early-stationary phase (g l^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>n.d.</td>
<td>n.d.</td>
<td>12.3 ± 0.7</td>
<td>n.d.</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>8.8 ± 0.7</td>
<td>9.1 ± 0.7</td>
<td>1.2 ± 0.3</td>
<td>6.9 ± 0.1</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>2.2 ± 0.5</td>
<td>1.9 ± 0.4</td>
<td>1.1 ± 0.2</td>
<td>6.0 ± 0.2</td>
</tr>
<tr>
<td>Formic acid</td>
<td>2.3 ± 0.2</td>
<td>1.7 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.9 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.3 ± 0.2</td>
<td>0.3 ± 0.2</td>
</tr>
</tbody>
</table>

n.d. = not detectable

Table 12 Percentages of significantly differentially expressed genes in functional COG categories in continuously produced cells collected in reactor 2 relatively to exponential and early stationary phase cells of *B. longum* NCC2705 from batch culture

<table>
<thead>
<tr>
<th>COG</th>
<th>Description</th>
<th>Exponential vs. continuous %</th>
<th>Early stationary vs. continuous %</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>Coenzyme transport and metabolism</td>
<td>+2*</td>
<td>non sig.</td>
</tr>
<tr>
<td>U</td>
<td>Intracellular trafficking and secretion</td>
<td>-13*</td>
<td>non sig.</td>
</tr>
<tr>
<td>C</td>
<td>Energy production and conversion</td>
<td>-11**</td>
<td>non sig.</td>
</tr>
<tr>
<td>E</td>
<td>Amino acid transport and metabolism</td>
<td>+5**</td>
<td>-1***</td>
</tr>
<tr>
<td>M</td>
<td>Cell wall/membrane biogenesis</td>
<td>+3*</td>
<td>non sig.</td>
</tr>
<tr>
<td>J</td>
<td>Translation</td>
<td>+5*</td>
<td>-1**</td>
</tr>
<tr>
<td>K</td>
<td>transcription</td>
<td>+19**</td>
<td>+10*</td>
</tr>
<tr>
<td>G</td>
<td>carbohydrate transport</td>
<td>+26***</td>
<td>-25***</td>
</tr>
<tr>
<td>R</td>
<td>general function prediction only</td>
<td>+6*</td>
<td>non sig.</td>
</tr>
</tbody>
</table>

Percentages of induced (+), respectively repressed (-), genes in each category was calculated by dividing the number of significantly induced/repressed genes in each category by the total number of retained genes in the corresponding category. *, statistical significance (*, p-value \(\leq 0.05\); **, p-value \(\leq 0.005\); *** p-value 0.0005). Functional categories with p-values higher than 0.05 are not shown.

6.3.2 Comparison of continuously produced cells in reactor 2 with exponential and early-stationary phase cells from batch culture

Continuously produced cells from reactor 2 at \(t = 134\) h were compared to exponential and early-stationary phase batch cultured cells in order to characterize their physiological state.

The average concentration of acetic acid, formic acid, and ethanol was higher in the continuous culture, whereas the amount of lactic acid was lower compared to the concentrations in exponential and early-stationary phase batch cultures (Table 11).
DNA micro array analysis between continuously grown cells and exponential phase cells enabled to assess the relative expression value of approximately 95% (1645) of ORFs. In total 220 genes (13 %) were differentially expressed in the comparison, and the log$_2$ transformed ratios minimum was -3.4, the maximum 7.8. A more in detailed analysis of the data revealed induction in continuous culture of 26% of the genes in COG category G (carbohydrate transport and metabolism) and 19% of the genes in COG K (transcription). Repression occured in 13% of the genes in COG U (intracellular trafficking and secretion), and in 11% of the genes in COG C (energy production and conversion) (Table 12). The majority of the regulated genes in the COG category K were transcriptional repressors (MarR type, lacI type and LexA transcriptional regulators).

A relative expression value of approximately 60% (1055 genes) of the ORFs was found in the comparison between continuously growing cells and early stationary phase cells (Figure 27) of which 152 genes (14 %) were differently expressed. The log$_2$ transformed ratios minimum was -3.9, and the maximum 5.0. Main differences between continuous and early-stationary growth phase batch cultures were repression (25 %) of genes in COG category G (carbohydrates transport and metabolism) and induction (10 %) of genes in COG category K (transcription) (Table 12).
Figure 27 Plots displaying the global distribution of gene expression values for cells collected in reactor 2 of the continuous culture of *B. longum* NCC2705 relatively to a) exponential phase and b) early-stationary phase cells of *B. longum* NCC2705 batch cultures.
6.4 Discussion

The comparison of the gene expression patterns and of the organic acid formations between reactor 1 and 2 of the continuous culture showed no major differences between the cells from the two reactors. It is therefore assumable that cells in reactor 1 were in a comparable physiological state as the cells in reactor 2, even though they had to be transferred from one reactor to the next by pumping and that they were harvested 42 min later.

In the continuous culture more acetic acid, formic acid and ethanol and less lactic acid were produced compared to batch cultures. In batch cultures the majority of biomass and metabolites are produced in the exponential phase by fast growing cells, whereas in the continuous culture the cells grow at a slow rate. It was already shown in bifidobacteria grown on different substrates, that slow consumption of the energy source leads to a metabolic shift from lactic acid production to more acetic acid, formic acid, and ethanol produced (Van der Meulen et al. 2006b), and our results are in line with these observations.

The number of differentially expressed genes in continuously grown cells was higher in the comparison to exponential cells than in the comparison to early stationary phase cells. Approximately 60 % of the ORF’s in the hybridization with early stationary cells was expressed. This means that less genes were expressed in stationary phase compared to exponential phase, which parallels previous observations (Klijn (2005a).

The higher expression of genes in involved in carbohydrate transport and metabolism (mainly transporter systems) in continuously grown cells parallels observation in continuous cultures of Escherichia coli with glucose limitation compared to batch cultures in exponential phase (Ihssen and Egli 2005). Here, a large number of up-regulated genes encoding periplasmic binding proteins was observed, indicating that the cells were prepared for high-affinity uptake of all types of carbon sources. On the contrary, we did not observed such elevated expression in the comparison with stationary phase cells. Most probably, entering stationary phase and the absence of glucose (Table 11) trigger a similar or stronger response as the low sugar levels in the continuous culture.
Such a response, due to relief of glucose mediated repression, has been described in *Lactococcus lactis* (Zomer et al. 2007). On the whole, cells produced continuously in reactor 2 compared to exponential growing cells showed up regulation of genes involved in carbohydrates transport and metabolism and repression of genes involved with intracellular trafficking and energy production. A higher expression of genes involved with the carbohydrates metabolism and a general repression of all other functions was previously observed in stationary and early stationary cells compared to exponential cells (Klijn 2005a).

The differences of continuously produced cells to early stationary cells were basically restrained to higher expression of genes involved in carbohydrates metabolism and the transcription machinery, probably due to sugar depletion in the early-stationary phase batch culture and to small differences in growth rate.

Cells of *B. longum* NCC2705 in the two reactors of the 2-stage continuous system described in chapter 3 were in a comparable physiological state. The continuously produced cells grown at a low dilution rate of 0.1 h$^{-1}$ were in a physiological state which was only partially comparable to exponential and early-stationary phase cells. A refined analysis of the distribution of differential expressed genes in functional COG categories revealed that continuously grown cells at low dilution rate shared more similarities with early stationary phase cells than with exponential phase cells, partially supporting the Monod model (Monod 1950) that cells at low dilution rate in continuous culture should be comparable to cells in batch culture at the end of exponential/beginning of stationary growth phase.
7 References


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