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The Study of Probiotic Bacteria in Human Gastrointestinal Tract Simulator

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Declaration:

Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any academic degree.

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ABSTRACT

Functional foods including probiotic foods are nowadays an established dietary trend. One of the main preconditions for a bacterial strain to be an effective probiotic is its high survival in the gastrointestinal environment. There are lots of studies on the viability of bacteria in the GI tract, mostly on resistance to low pH and bile. As *in vivo* studies are too complex to be used in initial screening experiments, there is a clear need for reliable *in vitro* tests preceding *in vivo* experiments. Models of the gastrointestinal tract designed to study the viability of probiotics *in vitro* are typically multi-compartmental simulators which are complicated and difficult to operate. In this study a realtively simple one-vessel GIT simulator was developed to evaluate the probiotic potential of food.

The survival (probiotic potential) of well known probiotic strains in model foods and commercial products was tested to evaluate our GIT model. Viability of *Lactobacillus acidophilus* La-5, *Lactobacillus johnsonii* NCC 533, *Lactobacillus casei* strain Shirota, and *Lactobacillus rhamnosus* GG were studied as a function of their physiological state. The effect of physiological state of the bacterial culture on survival during gastrointestinal transit have been shown to be strain (and species) specific. *L. acidophilus* and *L. johnsonii* survived in the GITS better when introduced at an early stationary or exponential phase compared to being previously stored for 2 weeks at 4 °C. These two species were more resistant to bile salts and survived better than *L. casei* and *L. rhamnosus* GG, the numbers for which decreased about 6 log units independent of the growth state. The protective effect of the food matrix against GIT barriers was demonstrated with *L. rhamnosus* GG that showed a significantly higher survival in GITS experiments if incorporated into a milk based environment compared to a laboratory growth medium.

The possible technological implications of stress pretreatment of probiotics was also investigated in order to increase their viability during gastrointestinal transit. The cultures of *L. acidophilus* La-5, *L. rhamnosus* GG, and *L. fermentum* ME-3 were pregrown in a pH-auxostat, subjected to temperature, acid, or bile stress treatment, fast freezing in liquid nitrogen (LN_2), and tested for survival in the GITS. A statistically relevant positive effect of the stress pretreatments was not observed in our experiments, although there are reports about a positive effect of stress pretreatments on the survival of probiotics in otherwise lethal conditions.

Furthermore, the dynamics of the natural microbial population of semi-hard cheeses in the GITS was studied. The aim of this study was to isolate acid and bile resistant, potentially probiotic lactic acid bacteria (LAB) from cheeses made in two dairies. Our study showed significant differences between LAB species from semi-hard cheese and between strains within one species (*L. casei/paracasei*) on their survival in conditions of the GI-tract. The results obtained revealed several *L. casei* strains as the dominating NSLAB in cheeses

from both dairies. These strains can be further characterized and, if suitable, included into the cheese starter cultures to improve product quality and consumer health.

Our studies confirm that the single bioreactor GI tract simulator provides a good platform for the simulation of environmental conditions of the human GIT to study probiotic food.

PUBLICATIONS

This thesis is based on the following papers referred to by their Roman numerals in the text.

I Ingrid Sumeri, Liisa Arike, Signe Adamberg, Kaarel Adamberg, Toomas Paalme (2006) The bioreactor model of human intestinal tract. *In: FinMed 2006: Saariselkä, Lapland, Finland 2006, March 27-31. (Toim.) Sorvari, S.; Toldi, O.. BioBien Innovations (BBi)*, 2006, 52 - 61.

II Ingrid Sumeri, Liisa Arike, Kaarel Adamberg, Toomas Paalme (2008). Single bioreactor gastrointestinal tract simulator for study of survival of probiotic bacteria. *Applied Microbiology and Biotechnology*, 80: 317-324.

III Ingrid Sumeri, Liisa Arike, Jelena Stekolštšikova, Riin Uusna, Signe Adamberg, Kaarel Adamberg, Toomas Paalme (2010). Effect of stress pretreatment on survival of probiotic bacteria in gastrointestinal tract simulator *Applied Microbiology and Biotechnology*, 86: 1925–1931

IV Ingrid Sumeri, Signe Adamberg, Inga Sarand, Toomas Paalme. Survival of cheese bacteria in gastrointestinal tract simulator (submitted to *International Dairy Journal*)

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INTRODUCTION

The human gastrointestinal tract harbors a complex collection of microorganisms, which form a specific individual microbiota for each person. The intestinal microbiota provides important contact with the environment for the host and a barrier against harmful food components and pathogenic bacteria. Many diseases and their prevention can be linked to intestinal microbiota. Thus, the flora of the intestinal tract is very important for health. Poor eating habits, stress, diseases and the use of antibiotics can disturb the microbial balance in the gastrointestinal tract by destroying good bacteria and allowing undesirable bacteria to multiply. Research has shown that probiotics can help re-populate the gastrointestinal tract with healthy bacteria and are beneficial for improving the balance of the intestinal flora.

The probiotic research conducted over the past 20 years has resulted in a valuable source of data related to the beneficial health effects of probiotics. The development and production of probiotic foods is one of the fastest growing industries worldwide. A common problem in the use of probiotic microorganisms is that the number of viable cells given to each individual is important for exerting their beneficial effect and thus, the viability is generally considered a prerequisite for optimal probiotic functionality.

To demonstrate the health effects of probiotic bacteria *in vivo*, especially in humans is very complicated. Therefore, the *in vitro* methods are often used as evidence of efficacy. Models of the gastrointestinal tract designed to study the viability of probiotics *in vitro* are typically multi-compartmental simulators which are complicated and difficult to operate.

The single bioreactor gastrointestinal tract simulator (GITS) was developed to evaluate the probiotic potential of food. The advantage of this simulator is its relative simplicity compared to multi-vessel systems used in previous studies. This single bioreactor is able to simulate the conditions of ingested bacteria during transit through the human upper GI tract – stomach and small intestine.
The simulator can be applied to study both single cultures and microbial population.

LITERATURE REVIEW

1.2 Probiotic organisms

According to the definition of the World Health Organization, probiotics are "living microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO 2002).

Probiotics have been consumed by human beings for thousands of years (Cross, Stevenson, & Gill, 2001; Kopp-Hoolihan, 2001). It was in 1907 when the Russian scientist Elie Metchnikoff first proposed the concept of probiotics as it is known today. He found out that the consumption of fermented milk products containing lactobacilli prolonged life and gave the first scientific explanation for the beneficial effects of lactic acid bacteria present in fermented milk (Rasic, 2003). Today it is accepted that a daily intake of these probiotics contributes to improving and maintaining a well balanced intestinal flora, and prevents gastrointestinal disorders (Lavermicocca, 2006). Various species of the genera *Lactobacillus* and *Bifidobacterium* mainly, and some other species of micro-organisms have been widely used as probiotics (Boyle & Tang, 2006). Different strains of *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus crispatus, Lactobacillus delbrueckii subsp. Bulgaricus, Lactobacillus fermentum, Lactobacillus gasseri, Lactobacillus johnsonii, Lactobacillus paracasei, Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus rhamnosus, Lactobacillus helveticus, Lactobacillus lactis, Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium longum, Bifidobacterium lactis, Bifidobacterium adolescentis, Bifidobacterium essensis, Bifidobacterium laterosporus* and other species like *Escherichia coli* Nissle, *Saccharomyces boulardii*, *Streptococcus thermophilus, Enterococcus francium, Propionibacterium, Pediococcus and Leuconostoc* could be considered as the main microbial species that have been used as probiotics (Senok, Ismaeel, & Botta, 2005; Shah, 2007).

Lactic acid bacteria (LAB) are the most commonly used organisms in probiotic preparations, because LAB are presumed to impart beneficial effects on the host such as improving intestinal tract health, enhancing the host's immune system and reducing the risk of certain diseases (Kramer et al., 2009). Several aspects, including functional and technological characteristics, have to be taken into consideration while selecting probiotic strains.

1.2.1 Criteria for probiotics

The generally accepted criteria for probiotics may be summarized as follows: human origin, nonpathogenic behavior, resistance to technological processes, resistance to gastric acidity and bile toxicity, adhesion to gut epithelial tissue, an ability to persist within the gastrointestinal tract, a production of antimicrobial substances, an ability to modulate immune responses, and an ability to influence metabolic activities like cholesterol assimilation, lactase activity, and vitamin

production (Fuller, 1991; Salminen et al., 2005). Moreover, each potential probiotic strain should be documented and assessed independently, extrapolation of data from closely related strains is not acceptable and only well-defined strains should be used in trials. Whenever possible, all human studies should be randomized, double-blind, and placebo-controlled; results should be confirmed by independent research groups and preferably, the study should be published in a peer-reviewed journals (Collins et al., 1998). It is also important that viability of the strain and stability of the desirable characteristics of the strain can be maintained during commercial production as well as in the final product.

1.2.2 Health benefit of probiotics

Several studies have been reported using probiotic strains, documenting their health benefits and specific effects (Fujiwara et al., 2001; Yuki et al., 1999). The health benefits of probiotics manifest mainly through maintaining a good balance and composition of the intestinal microflora, helping to increase the body's ability to resist the invasion of pathogens and maintain the host's well being (D'Aimmo, Modesto, and Biavati, 2007). Although some of these benefits have yet to be thoroughly proven it is generally accepted that probiotic help maintain the normal intestinal gut microflora, protect against gastrointestinal pathogens (Lourens-Hattingh and Viljoen, 2001), enhance the immune system, reduce lactose intolerance (Gilliland, 1990), reduce serum cholesterol level and blood pressure (Rasic, 2003), reduce the risk of cancer (Gilliland, 1990; Ouwehand et al., 1999; Rasic, 2003), improve utilization of nutrients and nutritional value of food (Lourens-Hattingh et al., 2001). Probiotics have been widely used in therapeutic applications including the prevention of urogenital diseases (candida vaginitis), an alleviation of constipation, a prevention of traveller's and infantile diarrhoea, a reduction of antibody-induced diarrhoea, a control of inflammatory bowel diseases and irritable bowel syndrome (Brigidi et al., 2001; Nobaek et al., 2000; Saarela et al., 2002; Venturi et al., 1999), a reduction of hypercholesterolemia, a prevention of osteoporosis and a prevention of food allergy and atopic diseases (Lourens-Hattingh and Viljoen, 2001). These potential benefits may result from the growth and action of probiotics during the manufacturing of cultured foods, while some may result from the growth and action of certain species of probiotics in the intestinal tract following ingestion of foods containing them (Lourens-Hattingh and Viljoen, 2001).

1.2.3 Mechanisms of probiotic action

The effects of probiotics may be classified by three modes of action. All three modes of probiotic action are involved in infection defence, prevention of cancer and in stabilising the physiological balance of the intestinal microbiota and its host (Oelschlaeger, 2009). Firstly, probiotics might be able to modulate the host's defences including the innate as well as the acquired immune system. It is obvious that these nonpathogenic probiotic bacteria must interact with the

epithelial cells and with the immune cells associated with the gut to start the network of immune signals. The increase in the number of IgA-producing cells was the most remarkable property induced by probiotic microorganisms (Perdigon, 2001). The physiological role of IgA in the mucosal surface is unquestionable. In particular, immunoglobulin A (IgA), the predominant mucosal antibody, is thought to mediate defense functions at different anatomic levels in relation to mucosal epithelium (Mazanec et al., 1993). Maldonado Galdeano et al. (2007) have demonstrated that some probiotic bacteria can act as adjuvants of the mucosal and systemic immune response. Probiotic bacteria induce signals on epithelial and immune cells that evoked different patterns of cytokines in the intestine (Perdigon, 2002, Vinderola, 2005), depending on the dose administered, as has also been shown by Maldonado Galdeano (2007). This mode of action is most likely important for the prevention and therapy of infectious diseases but also for the treatment of (chronic) inflammation of the digestive tract or parts thereof (Oelschlaeger, 2009).

Secondly, probiotics can also have a direct effect on other microorganisms, commensal and/or pathogenic ones. One of the primary benefits associated with probiotic cultures, is the exclusion of pathogenic bacteria in the small and large intestine (Kos, 2008). *In vitro* studies showed inhibition of pathogen replication mediated by low-molecular-weight substances. Top of this list are short chain fatty acids. A similar effect was observed with hydrogen peroxide. Also antimicrobial compounds (i.e. low-molecular-weight bacteriocins (LMWB) and high-molecular-weight bacteriocins) are produced by lactobacilli (Maqueda et al., 2008).

Thirdly, probiotic bacteria can synthesize biogenic compounds. Examples are usually reported during cheese ripening (Mangia et al., 2008; Milesi et al., 2008), in the process of kefir production (Simova et al., 2006) and sourdough fermentations (Gobbetti et al., 2005), through the use of primary starters and/or adjunct cultures. In particular, methionine over-producing strains may deserve some interest as a means of supplying this amino acid, which is usually deficient in an exclusively vegetable diet (Kumar and Gomes, 2005). The over-production of vitamins by lactic acid bacteria provides very attractive approaches for improving the nutritional composition of fermented functional foods (Stanton et al., 2005). Folacin (folic acid and related compounds) is an essential vitamin for growth and reproduction in all vertebrates. It has a preventative role against several disorders, including the development of neural tube defects, risk of coronary heart disease, and certain types of cancers (Finglas et al., 2003). Folic acid is an essential cofactor in bacterial metabolism and many bacteria used in food fermentations possess the biosynthetic capability to produce folate (Hugenholtz et al., 2002; Sybesma et al., 2003). Vitamin B12 is an essential cofactor in fatty acid, amino acid, carbohydrate, and nucleic acid metabolism (Quesada-Chanto et al., 1994). Besides dietary sources, intestinal microorganisms contribute to the vitamin B12 status in humans. Propionic acid

bacteria are well known as efficient producers of this vitamin (Hugenholtz et al., 2002).

The functionality of dairy proteins is further enhanced upon release of bioactive peptides by proteolysis. Proteolysis is carried out by naturally occurring enzymes in milk, starter cultures, and the enzymes of the digestive tract, resulting in the release of many peptides with a variety of biogenic activities (Gobbetti et al., 2002). Antihypertensive, antimicrobial, antioxidative, and antimutagenic are the main bioactivities discovered, depending on the type of process and protein substrate. When compared to other species of lactic acid bacteria, strains of *Lb. helveticus* appeared to be superior, especially with regard to the synthesis of antihypertensive peptides (Shah, 2007; Gobetti, 2010).

However, it has to be stressed that there does not seem to be one probiotic exhibiting all three principles. Which probiotic actions a certain probiotic strain might show depends on the metabolic properties, the kind of surface molecules expressed and components to be secreted. (Oelschlaeger, 2009).

1.2.4 Viability of probiotics

The viability of probiotics is a key parameter for developing probiotic foods. Although the amount of cells required to produce therapeutic benefits is not known and might vary as a function of the strain and the health effect desired, in general a minimum level of more than 10^6 viable probiotic bacteria per millilitre or gram of food product is accepted (Champagne, 2005, Lacroix and Yildirim, 2007).

Good growth and high viability during food manufacture and storage as well as in the gastrointestinal tract, protection against acid, bile and gastrointestinal enzymes, adhesion to intestinal epithelium, antimicrobial properties and antibiotic resistance could be considered as key factors important in maintaining probiotic efficacy. However, the viability of probiotics is arguably the most important factor since it could affect all the other parameters.

One of the main preconditions for a bacterial strain to be called probiotic is its ability to survive in the gastrointestinal environment. The importance of viability for the beneficial effects of probiotics is not absolute since inactivated and also dead cells have shown to possess immunological and health-promoting effects (Lopez et al., 2008, Saxelin, 2010). Still the viability is generally considered a prerequisite for optimal probiotic functionality (Maukonen et al., 2006). Many studies have shown that the viability of bacteria is not a simple question of cells being dead or alive (Dodd et al., 1997; Bloomfield et al., 1998; Colwell, 2000; Nystrom, 2001; Lahtinen et al., 2005). Traditionally, plate counting has been the method of choice for viability determination, but there are obvious disadvantages, including the relatively long times needed for the growth of colonies. The viable plate count method can be frustrated by clumping, inhibition by adjacent cells, and the composition of the growth media used (Breeuwer and Abee, 2000). In addition, many possible probiotic effects of bacteria depend on activity rather than culturability, and even dead cells can

have some probiotic effect, such as immunomodulation (Ouwehand et al., 2000). Therefore, obtaining information about all individual bacteria and their physiological status is relevant (Bunthof and Abee, 2002). Kell et al. (1998) have suggested four terms to describe the different stages of microorganisms: viable (active and readily culturable), dormant (inactive but ultimately culturable), active but nonculturable, and dead (inactive and nonculturable) (Lahtinen et al., 2005). Bunthof and Abee (2002) reported that such dormant population might exist in probiotic products and dairy starters. A similar stage of bifidobacteria occurring after exposure to stress conditions was later demonstrated by Ben Amor et al. (2002). Further studies showed that probiotic bacteria may become dormant in fermented products during prolonged storage (Lahtinen et al., 2005, 2006). Therefore, there is an increasing interest in the development of rapid methods for cell viability determination (Breeuwer and Abee, 2000; Kramer et al., 2009).

The general scientific consensus is that probiotics should be alive to exert their beneficial effect in the human gastrointestinal (GI) tract (Boyle and Tang, 2006). Consequently, probiotics should remain alive in the product, so that the daily effective dose per serving is still present at the end of the shelf life (Holzapfel, 1998). Food matrices, production processes, or product usages that involve heating can affect the viability of probiotics (Ross, 2005). Typically, probiotic microorganisms are selected in consideration of their resistance to passage through the upper GI tract and ability to transiently colonize the gut (Sanders 2003). Human GI tract survival of probiotics should lead to the shedding of live cells in fecal samples. GI tract survival is, however, dependent on both the strain and the food matrix involved (Saxelin 1993).

1.2.5 Factors affecting the viability of probiotic bacteria

The microbial growth is a self-limiting process and is often suboptimal in nature compared to controlled conditions in the laboratory. Natural stresses like acidity and sometimes shortage of nutrients (starvation) are generated by cell growth itself, while other stresses (e.g. temperature, osmotic shock or oxygen) are induced by the environment (Shah, 2000). All these environmental stresses may affect the physiological status and properties of the cells. It is essential to know not only which conditions are favorable or detrimental for the life of microbes but also which mechanisms permit their survival and metabolic activities under stress conditions (De Angelis, 2004).

Bacteria spend most of time in late stationary phase. Growth arrest and entry into the stationary phase can be provoked by numerous stress conditions like cold, heat, osmotic, oxidative or acid stress, or starvation. Among these stresses, nutrient starvation is one of the most frequent and bacterial growth itself contributes to nutrient exhaustion and subsequent starvation of one or several compounds. Moreover, some extreme environmental stress conditions may provoke a deprivation of one or several components, apart from their direct effects on the cells constituents. For example, extreme acidic conditions can

decrease the activity of some transporters, thereby diminishing the availability of essential substrates. Therefore, stress conditions can indirectly provoke starvation or energy depletion, irrespective of the extracellular amount of the substrate (Konings et al. 1997). These conditions of energy or essential elements depletion could be deleterious for long-term cell viability.

In a confined environment the production of lactic acid causes a progressive acidification of the culture medium. The organic acid lactate is the predominant fermentation product of many probiotic lactobacilli. The undissociated form of lactate is a strong growth inhibitor of the organism. Different theories have been postulated to explain the inhibitory effects of lactic acid: (i) toxicity arising from the dissipation of the membrane potential, (ii) acidification of the cytosol, or (iii) intracellular anion accumulation. In general, organic acid stresses are complex to study, since their toxicity is highly dependent on their degree of dissociation and thus on the pH (Pieterse et al., 2005). Considering their optimal growth temperatures, lactic acid bacteria are divided into mesophilic (optimum around 30°C) and thermophilic (40°C). During industrial processes, like frozen storage (freezing) of starter cultures, low temperature fermentation of cheese ripening and refrigerated storage of fermented products, LAB are exposed to temperatures far below their optimal growth temperature. The survival of LAB during freezing and growth at low temperatures contributes to the industrial performance of the strains. A better understanding of the responses to low temperatures and freezing may contribute to the optimization of the fermentation processes, the storage of the products and their conservation conditions. When living cells are exposed to a temperature downshift they undergo important physiological changes such as a decrease in membrane fluidity and a stabilization of the secondary structures of RNA and DNA resulting in a reduced efficiency of translation, transcription and DNA replication (van de Guchte et al, 2002).

Exhaustion of an essential nutrient and/or accumulation of an inhibitory fermentation end product (e.g., lactic acid) limit the length of exponential growth and cause the termination of growth. Entry into the stationary phase is also caused by heat, cold, osmotic and oxidative stresses.

Bile is one of the most toxic agents for microorganisms in the gastrointestinal tract. Bile is a complex digestive secrete that plays a role in the dispersion and absorption of fats. Bile acids (also often referred to as bile salts), the major constituents of bile, are derivatives of cholic acid (CA) that is itself synthesized from cholesterol. Some of the bile acids are conjugated to either glycine or taurine in the liver (Russell et al. 1995). Although the toxicity of bile acids for bacterial cells is not completely understood, bile acids are surface active, amphipatic molecules with potent antimicrobial activity since they act as detergents and disassemble biological membranes.

1.2.6 Technologies for improving probiotic viability

The technological suitability of probiotic strains is critical to their exploitation in the development of effective functional foods. Studies on the adaptive physiology of lactic acid bacteria facilitate efforts to improve their efficacy in industrial applications. It is well established that lactic acid bacteria (LAB), like other bacteria, evolve stress-sensing systems and defenses against stress, which allow them to withstand harsh conditions and sudden environmental changes (van de Guchte et al., 2002). Thus, the induction of a stress response via exposure of the growing culture to a sublethal stress before use in food processing or gastric transit has been suggested to enhance viability of probiotics in the GIT (Corcoran et al., 2008). Stress responses of lactobacilli and bifidobacteria have attracted increasing research interest (Saarela et al., 2004) especially due to their extensive use as probiotics in the food industry. For example, it was found that the heat resistance of *Lactobacillus rhamnosus* GG is affected by mild pressure treatments before exposure to lethal temperatures during spray-drying (Ananta et al., 2004). Strain-specific sublethal treatments have been developed for enhancing the viability of stationary-phase probiotic cells both at the laboratory and fermenter scale. These results hold promise for the production of probiotic cultures with improved viability (Saarela et al., 2004). A better understanding of the mechanisms of stress resistance should allow an understanding of the basis for adaptive responses and cross protection, and to rationalize their exploitation in preparing probiotic products (van de Guchte et al., 2002).

The growth and viability optimization of probiotics, notably on a large scale, is an important area where most developments are made in industrial settings. A wide range of physical and chemical protection systems have been developed to increase viability and health benefits of probiotics. These include immobilization, microencapsulation, and other physical approaches that have been reviewed (Prakash and Martoni, 2006). Among the numerous technologies of immobilization inclusion in calcium alginate is the most described (Leverrier, 2004).

An alternative approach is to enhance the strain's ability to cope with stresses at genetic level (Sleator and Hill, 2007). Engineered probiotic cultures (e.g., *Lactobacillus salivarius* UCC118 and *Bifidobacterium breve* UCC2003) were obtained using stress adaptation genes isolated from more physiologically versatile pathogenic strains (Sheehan et al., 2006; 2007). A recombinant derivative of *Lactobacillus paracasei* NFBC338, which over-produces GroES and GroEL chaperones under the control of the nisin promoter (*Pnis*), exhibited improved thermal tolerance and acquired solvent tolerance (Desmond et al., 2004). These data convince the potential of enhancing the technological performance of probiotic strains through genetic manipulation (Gobetti et al., 2010).

Dairy products are most commonly used to deliver probiotics and, thus, growth in milk or milk-derived media (milk based environments) is essential. However,

using advanced physiological and post-genomics approaches, new insights are being gained on other media. As the addition of cryoprotectants (Saarela et al., 2005) and compatible solutes are conventional methods to stabilize microbial cultures, the use of dedicated substrates that stimulate the growth and activity of probiotic bacteria following intestinal delivery has attracted attention. This has led to the development of the synbiotic concept, exploiting prebiotics and probiotics (de Vos et al., 2006)**.**

1.2.7 The food matrix

Foods are main carriers for the delivery of probiotics to the human body. Food, particularly dairy products are considered as an ideal vehicle for delivering probiotic bacteria to the human gastrointestinal tract. Foods help to buffer the probiotic through the gastrointestinal tract, regulate their colonization and contain other functional ingredients, such as bioactive components, which may interact with probiotics to alter their functionality and efficacy. The growth and survival of probiotics during gastric transit is affected by the physico-chemical properties of food carriers. Fat content, concentration and type of proteins, sugars and pH of the product are some factors that could affect probiotic growth and survival in food. Survival of probiotic in the presence of simulated small intestinal juice varied depending on the carrier material (Sumeri et al., 2008). Different cryoprotectants used in freeze drying further altered probiotic stability in food products due to inhibition of intracellular and/or extracellular ice crystal formation by binding to water (Capela et al., 2006). Therefore, not only the chemical composition of foods but their physical structures are important for the efficacy of the probiotic they are bearing (Lavermicocca et al., 2005; Lavermicocca, 2006; Valerio et al., 2006). Same probiotic strains could vary in functional and technological properties in the presence of different food ingredients. Thus, the product formulation can help to increase their efficacy.

Ingredients in certain food products may naturally contain prebiotics (nondigestible polysaccharides) which help to improve the functional efficacy of probiotics as substrates. By increasing the amount of prebiotics in the diet, it is possible to increase and maintain healthy bacterial gut flora in the host (Sanders, 1998). Many other foods such as dairy and meat products, cereals, beverages and infant formulas can be fortified with prebiotics (during the manufacturing process) to increase probiotic efficacy (Gibson et al., 2004). Food formulations with appropriate pH ranges and high buffering capacity would increase the pH of the gastric tract and thereby enhance the stability of probiotics (Kailasapathy and Chin, 2000). Since combinations of probiotics with synergistically acting components and properties such as pH from the food seems to be one of the best ways of improving probiotic efficacy, careful selection of the food matrix is an important factor that should be considered in developing probiotic products (Ranadheera et al., 2010).

Yogurt and fermented milks are considered as the main vehicle for probiotic delivery. Usually yogurt is prepared by allowing milk to ferment by specific

pure cultures of lactic acid bacteria (*S. thermophilus* and *L. bulgaricus* cultures). Different yogurts have demonstrated different viability levels of probiotics over shelf lives. Fruit mixtures or any other ingredients that contribute to a lowering of pH in yogurt may reduce the viability of probiotics. (Dave and Shah, 1997). Positive effects of adding prebiotics, for example inulin and fructooligosaccharides, on the viability of *L. acidophilus, L. casei, Lactobacillus rhamnosus and Bifidobacterium spp.* were observed by Capela, Hay, and Shah (2006) in yogurts. These prebiotics could improve metabolic activities of probiotics and resulting in higher functional efficacy. The authors reported fructooligosaccharides as the most effective prebiotic in helping to retain the viability of probiotics.

Ice cream, frozen fermented dairy desserts, and freeze-dried yogurt have been used as delivery vehicles for probiotic microorganisms by Capela et al. (2006). Due to the presence of air bubbles in ice cream-type products and injury due to freezing and freeze drying, many of the above products failed to successfully deliver the required level of viable probiotics (Lankaputhra and Shah, 1997). It is also found that viability of frozen probiotic depends on their sugar levels (Akin et al., 2007).

Compared to fermented milk, cheese as a carrier of probiotics has a number of advantages as a higher pH, higher buffering capacity, a solid consistency, and relatively higher protein and fat content. These characteristics offer protection to probiotic bacteria during storage and passage through the gastrointestinal tract. Different cheeses have been shown to support relatively high cell densities of probiotics (Gardiner et al., 2002, Madureira et al., 2005, Gobbetti et al., 1998). Cheeses have been also used to deliver different probiotic bacteria (Corbo et al., 2001; Gardiner et al., 2002; Gobbetti et al., 1998; Stanton et al., 1998; Vinderola et al., 2000, Bergamini et al. 2005).

Although most of the current probiotic foods are mainly dairy based, there is a growing interest in the development of non-dairy probiotic products due to problems such as lactose intolerance in many people and the unfavourable cholesterol content of fermented dairy products. This has led to the development of probiotic products from various food matrices including fruits, vegetables and cereal products (Lavermicocca, 2006; Yoon et al., 2006; Helland, Wicklund, and Narvhus, 2004).

When considered together the above mentioned evidences reinforce the fact that food can influence growth, viability and survival, acid and bile tolerance, and different functionalities of probiotics that determine their efficacy in gastrointestinal tract. Thus, careful investigation of the interaction of different probiotics and food components should be considered in developing probiotic foods. More research is needed to determine the effect of food substrate on adhesion to intestinal epithelium, antimicrobial properties, antibiotic resistance and other metabolic activities of probiotics.

2.1. The human gastrointestinal tract

The digestive process begins in the mouth. Food is partly broken down by the process of chewing and by the chemical action of salivary enzymes. After being chewed and swallowed, the food enters the esophagus. The esophagus is a long tube that runs from the mouth to the stomach. The stomach is a large, sack-like organ that churns the food. Chemical compounds present in the stomach system arise from both the diet and the metabolism. The acid present in the stomach is hydrochloric acid. Hydrochloric acid is produced by a complex series of operations involving an ion separation process operated by the cell membrane involving hydrogen, bicarbonate and chloride ions (Yao et al., 2003). This acid is important for activation of pepsinogen and inactivation of ingested microorganisms such as bacteria. After being in the stomach, food enters the duodenum, the first part of the small intestine. It then enters the jejunum and then the ileum (the final part of the small intestine). In the small intestine, bile, pancreatic enzymes, and other digestive enzymes produced by the inner wall of the small intestine help in the breakdown of food. Bile is a yellow-green aqueous solution whose major constituents include bile acids, cholesterol, phospholipids, and the pigment biliverdin. It is synthesized in the pericentral hepatocytes of the liver, stored and concentrated in the gallbladder, and released into the duodenum after food intake. Bile functions as a biological detergent that emulsifies and solubilizes lipids, thereby playing an essential role in fat digestion. This detergent property of bile also confers potent antimicrobial activity, primarily through the dissolution of bacterial membranes (Begley et al., 2005). Most digested molecules of food, as well as water and minerals, are absorbed through the small intestine. The waste products of this process include undigested parts of the food, known as fiber, and older cells that have been shed from the mucosa. These materials are pushed into the colon, where they remain until the feces are expelled by a bowel movement.

2.1.1 Gut microflora

The human microbiota comprises of several hundred different bacterial species, many of which have a beneficial effect on the host. For example, they are involved in preventing colonization of the gut by pathogens and maintaining gut mucosal immunity (Tancrede, 1992). The gut microbiota is more abundant in the large intestine of mammals, with densities rising to over 10^{11} organisms/g intestinal content (Tani et al., 1998). The number of bacterial cells in the entire gut exceeds the number of eukaryotic cells in the host, but under normal circumstance they coexist without any adverse effect on the host. The influence of the resident microflora on mucosal immune function and gut health has become an area of scientific and clinical importance (Fuller, 1991). There is an active dialogue between the commensal microorganisms and the host mucosal immune system (Dogi and Perdigon, 2006). This cross talk elicits different host responses to commensal and pathogenic bacteria (Maldonado Galdeano 2007).

2.2 In vitro gastrointestinal models

There are lot of studies conducted on the viability of bacteria in the GI tract, resistance to low pH and bile. *In vivo* studies are too complex to be used in initial screening experiments. The need for reliable *in vitro* tests preceding *in vivo* experiments is clear. The *in vitro* tests can be divided into "static" and "dynamic" simulators of the GI tract. In so called static models bacteria are incubated either in an acidic medium at pH values varying between 1.0 and 3.0 or in a medium containing 0.1 – 0.4 % of bile or bile salts (Charteris et al., 1998; Prasad et al., 1998). The static models do not simulate adequately the dynamic changes in the GI tract. Firstly, bacteria are ingested with a meal that buffers the pH in stomach. Therefore not all bacteria are subjected to the pH characteristic to empty stomach that is 1.5-2.0. Secondly, the bile salt concentration in the gut is not static but changes over time. Several dynamic *in vitro* human gastrointestinal models were developed to better simulate *in vivo* conditions.

2.2.1. SHIME

The simulator of the human intestinal microbial ecosystem (SHIME) (Molly et al., 1993) consisted of five reactors representing the duodenum/jejunum, ileum, caecum and ascending colon, transverse colon and descending colon. A sixth reactor was later added to simulate the stomach. pH of this model was controlled by adding 0.1 M HCl or 0.1 M NaOH. The model meal consisted of 20 g of food mixed in 80 ml physiological salt solution and 200 ml of polysaccharide solution (pH 2). Model food was pumped into first reactor and stayed there for 2-3 hours. Then it was pumped into the second reactor (duodenum) where pancreatic juice and bile were added. The food stayed in the second reactor for 4 hours (pH 2.5-7.5) as with the third reactor (ileum, pH 6.5-7.0). The last three reactors simulated the large intestine and its microflora. These were inoculated with human fecal material to establish a microbial population of the human gastrointestinal tract.

The SHIME has been used to investigate the interactions of probiotic bacteria with the human intestinal microflora (Molly et al., 1996; Nollet et al., 1997), the effects of probiotic bacteria, commercially-available plant polysaccharide supplements, and symbiotic products on the human gastrointestinal microbiota (Kontula et al., 1998; Alander et al., 1999; Gmeiner et al., 2000; Marzorati et al., 2010).

Van den Abbeele et al (2010) investigated the microbial colonization process in two identical simulators of the human intestinal microbial ecosystem (SHIME), simultaneously inoculated with the same human fecal microbiota with a high-resolution phylogenetic microarray: the human intestinal tract chip (HITChip). Following inoculation of the in vitro colon compartments, microbial community composition reached steady state after 2 weeks, whereas 3 weeks were required to reach functional stability. This dynamic colonization process

was reproducible in both SHIME units and resulted in highly diverse microbial communities which were colon region specific.

2.2.2. TNO model

The TNO model (Minekus et al., 1995) of the upper gastrointestinal tract contains four chambers to simulate the stomach, duodenum, jejunum and ileum, which are connected by computer-controlled valve pumps. The pH conditions in the gastric and duodenal compartments are monitored with pH-meters connected to the computers. Secretion of either 1 mol of HCl or neutral electrolyte solution into the gastric compartment is dosed via the computer for pH control. The same procedure is applied for secretion of either $NaHCO_3$ or the neutral electrolyte solution into the duodenal compartment. The chyme was gently mixed three or nine times per minute in the gastric or intestinal compartments, respectively, by alternate contractions of the flexible walls. The jejunal and ileal compartments were equipped with hollow fiber devices that permit dialysis of the chyme (Minekus et al., 1995).

The pH curve in the stomach is computer controlled to reproduce the values found in humans after yogurt consumption (Conway et al. 1987): pH 5.0 at initiation, pH 4.1 at 20 min, pH 3.0 at 40 min, pH 2.1 at 60 min, and pH 1.8 at >80 min. In the small bowel compartments, pH was kept at 6.5 ± 0.5. Gastric and ileal emptying in the model were regulated by computer *via* the pump valves to reproduce the gastric and ileal emptying of a nonabsorbable meal marker that was ingested with yogurt by human volunteers (Marteau et al. 1997). The model has been used to study the survival of lactic acid bacteria, absorbtion of minerals, vitamines and food mutagens (Marteau et al. 1997; Larsson et al., 1997; Verwei et al., 2003; Krul et al., 2000). Mitea et al (2008) studied the degradation of gluten by *Aspergillus niger* prolyl endoprotease (AN-PEP), in this dynamic system.

2.2.3. Mainville's model

Mainville et al. (2005) developed the dynamic in vitro human upper GI tract model system. It consisted of two 1-l jacketed glass beakers representing the stomach and the duodenum. A cover was designed to accommodate a Radiometer Copenhagen „Red Rod" pH electrode, a temperature probe, and entry ports for food and HCl delivery into the stomach reactor. For the duodenum reactor, there were three entry ports for stomach digesta, NaOH and Oxgall bile. Peristaltic pumps were used to control the delivery of the products to be added, as well as the emptying rate of the stomach reactor into the duodenum reactor. The emptying rate of the stomach reactor was controlled to approximate the conditions in the stomach following yoghurt consumption in humans (Berrada et al., 1991). HCl addition in the stomach vessel was controlled

to reproduce the pH curve found in humans during and after milk consumption (Minekus et al., 1995). The pH of the duodenum vessel was maintained at pH 6.5.

This model was tested by comparing survival of bacteria isolated from humans, animals and fermented dairy products with their survival determined by conventional methods. The model was shown to better represent the events during upper GI tract transit than the conventional methods.

2.2.4. Wickham's model

Dynamic Gastric Model (DGM) was developed by Wickham et al. (2007) at the Institute of Food Research (Norwich,UK). The DGM is a computer controlled gastric model which incorporates the chemical, biochemical, physical environment and processes of the human stomach. The DGM incorporates the in homogeneous gastric mixing, antral shearing and the rate of delivery to the duodenum with acidification and addition of gastric enzymes in the normal physiological range. The addition of these simulated gastric secretions computer is dynamically controlled with flow rates modified in real time as the gastric environment changes (by both pH and gastric volume). The pH, temperature, mixing, shear, residence time, and gastric emptying rate are also controlled and varied over time within the normal physiological ranges. This model was used by Pitino et al. (2010) to investigate the survival of selected *L. rhamnosus* strains under simulated in vitro dynamic upper gastrointestinal conditions. In addition this model is applicable for studies on food systems, gastric metabolism and stability evaluation, assessment of effect of food on bio-performance. The DGM has been also for study drug release and dissolution in the stomach (Mercuri et al. 2009)

AIMS OF THE STUDY

1. To development of a single fermenter Gastrointestinal Tract Simulator (GITS) to study survival of microorganisms in conditions of human stomach and small intestine.
2. To evaluate and use of the GITS to study:
   - the effect of physiological state of bacterial culture on survival
   - the effect of food matrix on viability of probiotics
   - the effect of sublethal stress pretreatment on survival of probiotics
   - comparative survival of cheese bacteria as complex microbial consortium

MATERIALS AND METHODS

3.1 Microorganisms

The following probiotic lactobacilli were used in this study:
L. acidophilus La-5 (Chr. Hansen, Denmark),
L. casei Shirota isolated from probiotic product Yakult,
L. rhamnosus GG (Valio, Finland) isolated from Gefilus daily dose drink,
L. johnsonii NCC 533 (kindly provided by Nestec, Switzerland), and
L. fermentum ME-3 (DSM 14241) isolated from Hellus kefir (Tere AS, Estonia).

The semi-hard cheeses studied were manufactured using mesophilic aromatic starter cultures CHN-19 (FIN) and DCC-240 (EST) (Chr. Hansen A/S, Denmark) composed of undefined proportions of *Lactococcus (Lc.) lactis subsp. lactis, Lc. lactis subsp. cremoris, Lc. lactis subsp. lactis biovar diacetylactis* and *Leuconostoc (Ln.) mesenteroides subsp. cremoris*.

3.2 Growth media and model foods

De Mann–Rogosa–Sharp (MRS) agar medium (LAB M, UK) was used for isolation and storage of the bacteria (DeMan et al., 1960).

For GITS experiments the bacteria were pre-grown at 37°C on lactose-containing complex culture medium consisting of (g L^{-1}): lactose – 20, tryptone - 5, yeast extract – 2.5, Tween 80 – 1 (Difco Laboratories, USA), Na-citrate – 2.3, K_2HPO_4 – 2 (Scharlau, Spain), $MgSO_4 \cdot 7H_2O$ – 0.2, $MnSO_4 \cdot 5H_2O$ - 0.04.

For the dilution of bile salts in intestinal tract model the medium consisting of (g L^{-1}) lactose – 5, tryptone – 2.5, yeast extract – 1.25, Tween 80 – 0.5, K_2HPO_4 – 1. The pH of the dilution solution was set to 5.0 by 5M HCl. To study the effect of physiological state the cultures in exponential, and stationary phase, or stored at 4°C for 14 days were used as "model food".

The effect of food matrix was studied using probiotic food products containing *L. rhamnosus* GG. Gefilus milk®, Gefilus daily dose drink®, Gefilus Emmental® cheese, and Gefilus Peptidi Tutteli® baby formula (all produced in Valio Ltd., Finland) were tested in GITS. The composition of these food products are listed in Table 1 of Paper II. Milk and daily dose drink were directly injected into GITS; baby food was prepared according to instructions by diluting 28.8 g of baby formula in 200 ml of water, Gefilus cheese (20 g) was homogenized in 200 ml of sterile water directly before testing. Food products inoculated with LN_2 frozen LGG pH-auxostat culture were Tere Cappuccino milk 3.5% (high-temperature pasteurised milk with fat content of 3.5%), pasteurised 100 % apple juice (Põltsamaa, no preservatives added), Hera80 whey protein powder, manufactured by Func Food Finland OY, Tampere, Finland (Ingredients: whey protein concentrate, fat reduced cocoa powder, flavour (fructose, flavour), salt, sweetener (sucralose). Contains soy lecithin.). Samples for testing in the GITS prepared as follows:

80g of LGG frozen milk pearls were added fresh milk up to 200ml, mixed till melting and subjected to the GITS experiment.

200ml apple juice with pH 3,30 was titrated to pH 6,7 with 2M $NAHCO_3$ and 155ml was mixed with 50g of titrated apple juice pearls before the beginning of GITS experiment.

20g of whey protein powder was mixed with 200ml of destilled water (according to manufacturer´s instructions)and stirred using a magnetic stirrer for about 15min (resulting pH 6,46). 155ml of the whey protein powder drink was mixed with 50g of LGG frozen whey protein powder pearls before subjecting to the GITS experiment.

Cheeses subjected to study survival of microbial population were commercial 90-day-old open texture cheeses produced in two cheese factories of Valio Ltd. (Finland) situating in Estonia and Finland. The cheeses were brine salted and had (an average) NaCl content of 1.1–1.4 %. The average fat content in Finnish cheeses was 31.7% and in Estonian cheeses 26.3% (w/w) and dry matter content 60.7 and 58.2%, respectively.

3.3 pH-auxostat cultivation

Bacterial cultures for freezing and GITS experiments were prepared under controlled conditions using pH-auxostat (Adamberg et al. 2003). The cultivation system consisted of an Applikon 1 l fermenter; controlled by an ADI 1030 biocontroller (‘‘Applikon,’’ The Netherlands) and cultivation control program ‘‘BioXpert’’ (‘‘Applikon’’). Anaerobic cultivation was performed at 37°C with setpoint control at pH=6.0. MRS (LAB M, UK) medium adjusted to pH 7.0 with 2N NaOH was used for pH-auxostat cultivation. After passing through at least five culture volumes the gathering of 200 ml culture portions for stress treatment and GITS experiments was started.

3.4 Incubation under sublethal conditions

200 ml of culture was transferred from pH-auxostat into pH controlled batch reactor containing 200 ml of fresh MRS medium for stress treatments. For bile stress (pH=6, T=37°C, 2h) a 20% solution of bile salts (50% cholate : 50% deoxycholate, Sigma Aldrich, USA) was added to achieve final bile salt concentrations of 0.1%, 0.04%, or 0.02%. Temperature stress was carried out at 40°C (pH=6, for 2h) and pH stress at values 3.5 or 4.5 (37°C, 2h). After incubation, stress treated cells were harvested by centrifugation at 4°C, 5,800 rpm for 15 min and resuspended in the 400 ml of fresh MRS medium (pH 6.3±0.1).

The bacterial cell suspensions were slowly dripped into liquid nitrogen using a peristaltic pump. The beads (1–2 mm size) were harvested from liquid nitrogen (LN_2) and stored at −40°C. For each GITS experiment, 100 g of frozen MRS beads were thawed at room temperature and mixed with 100 ml of MRS media.

3.5. Gastro-intestinal tract simulator (GITS)

3.5.1. Description of the device

The GI tract simulator consisted of a 1 l "Biobundle" fermentation vessel (Applikon, The Netherlands), an ADI 1030 biocontroller, and balances (Sartorius, Germany) connected to a PC equipped with the cultivation control program "BioXpert" (Applikon, The Netherlands). The fermenter was equipped with pO_2, pH and temperature sensors, variable and fixed speed pumps (Masterflex USA) to control the flow of HCl, $NaHCO_3$, bile acids, feeding medium, and the culture volume. The temperature in the bioreactor was kept at 37±0.1°C, and anaerobic conditions were maintained by purging with nitrogen.

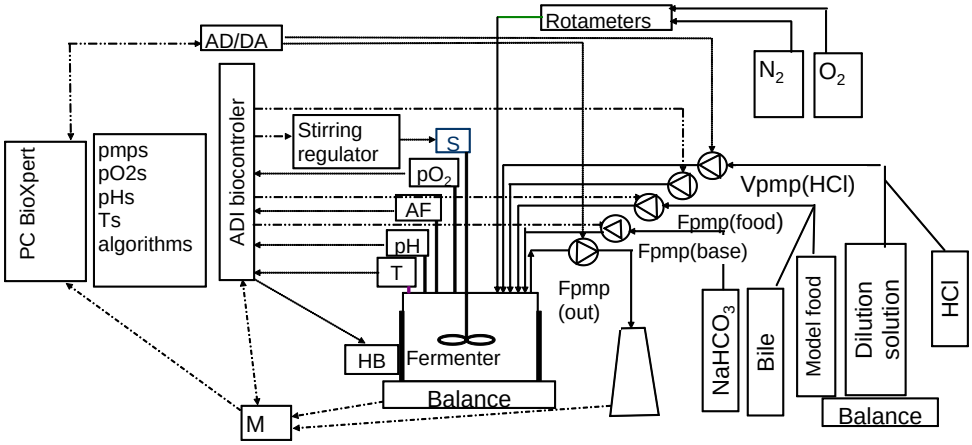

Figure. 1. Technical realization of gastro-intestinal tract simulator: Vpmp – variable rate pump; Fpmp – constant rate pump; AF – antifoam sensor, pH – pH-electrode; pO_2 – oxygen electrode; T – temperature sensor; S – stirrer; HB – heating blanket

3.5.2. The algorithm of gastro-intestinal simulator

In order to simulate the survival conditions of probiotic bacteria during GI transit, 100 ml of 0.01 M HCl (or 0.01 M HCl + pepsin 4 g/l (Sigma)) was added into the vessel to imitate the empty stomach, with pH value near 2. At the beginning of the experiment, 200 ml of food containing 10^7–10^9 cfu/ml of bacteria was pumped into the vessel (Fig. 1), followed by titration of fermenter's content back to 3.0 with 1 M HCl at a rate 20 mmol h^{-1} (reported as the maximum HCl secretion rate for human stomach (Ewe and Karbach, 1990). The content of the bioreactor was then neutralized to pH 6.0 by adding 1 M $NaHCO_3$ at a rate of 4.5 ml min^{-1} to simulate the passing of food from the stomach to the duodenum. Depending on the buffering capacity of the food, the neutralization step took between 3 and 10 min. Thereafter, the concentration of bile salts in the fermenter was adjusted to 0.4% by adding 4% bile salts solution (or bile salts + pancreatine 9 g/l (Sigma)) at rate of 4 ml min^{-1} during a period of 10 min. Finally, the bioreactor content was diluted (D=0.4 h^{-1}) with dilution medium to simulate the absorption of bile acids and food components in the jejunum and ileum. During the dilution phase, the pH of the culture was kept at pH 6.5 by titration with $NaHCO_3$, while the culture volume, V_D was maintained by controlling the weight of the vessel. A dilution rate of D=0.4 h−1 was selected so that at the seventh hour, corresponding to the time food reaches the large intestine, the bile salt concentration was diluted ten times.

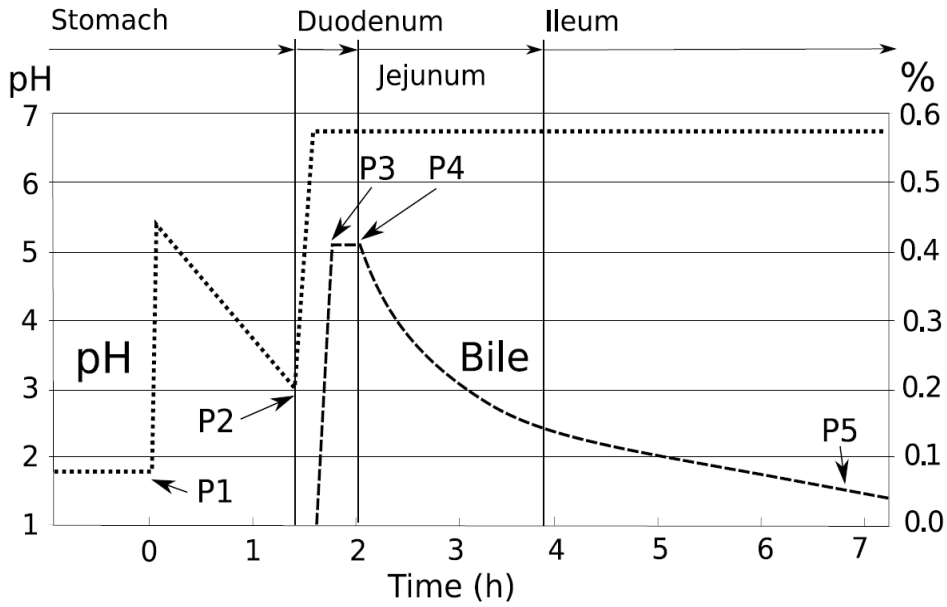

Figure 2. Operation of GITS model. P1, P2, P3, P4 and P5 show the key sampling points corresponding to food before injection, entering into *duodenum*, immediately after bile addition, entering into *jejunum* and in the end of *ileum*

3.6 Viability assessment

Viability of bacteria was assessed by direct microscopic counting and plate counting of bacteria. Microscopic counting was performed using LIVE/DEAD BacLight bacterial viability kit (L-7012) (Molecular Probes, Eugene, OR, USA). The kit comprises two fluorescent nucleic acid stains SYTO9 and propidium iodide. SYTO9 green-fluorescent stain penetrates both healthy (intact) and dead (damaged) bacterial cells whereas red-fluorescent stain propidium iodide penetrates only damaged cell membranes causing a reduction in the SYTO9 stain fluorescence when both dyes are present. Live bacteria stained with appropriate mixture of these dyes fluorescent green and dead bacteria fluorescent red. One milliliter of sample from the bioreactor at different stages of simulating was centrifuged down, suspended in distilled water and incubated with mixture of two dyes in dark for 15 min at room temperature. Then 10 µl of bacterial suspension was placed in improved counting chamber (improved Neubauer chamber with 0.1 mm depth) and examined with oil immersion under Olympus A041 microscope equipped with an HBO 200 w/2 Super Pressure Mercury Lamp (Osram, Germany) and appropriate filters for LIVE/DEAD BacLight bacterial viability kit. The images were taken with Olympus DP70 camera and counted for the live and dead cells (see Figure 3).

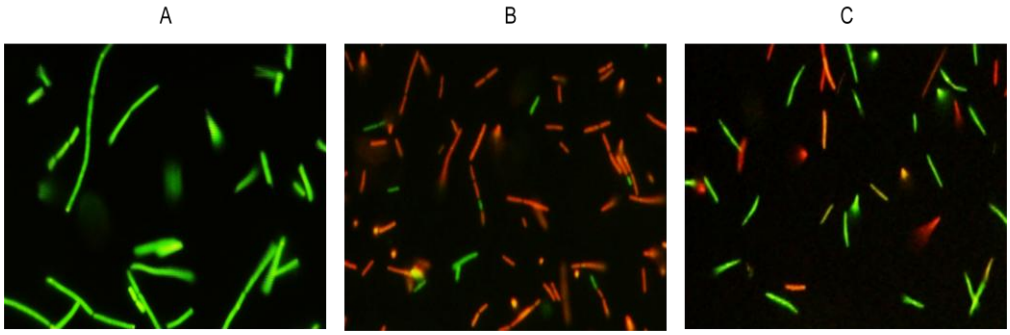

Figure 3. Microscopic counting of *L. acidophilus* La-5. A – from inoculum, B – from sample after bile is added into fermenter, C – from sample taken after 24 hours from beginning of the experiment

The number of colony-forming units (cfu; viable bacteria) ml^{-1} of the sample was determined by counting colonies from serial (decimal) dilutions of the bacterial suspensions plated out on MRS agar media. Anaerobic incubation (AnaeroGen™, Gas Pack System, Oxoid, Inc., Basingstoke, UK) for 72 hours at 30°C was used for growing of cheese bacteria.

In experiments with cheese microflora about 10 – 15 colonies per sample were randomly picked (taken) from MRS agar plates and purified by 2 successive subcultures on the same medium. Culture from a single colony in MRS broth (LAB M, UK) was subjected to DNA extraction and storage culture at -80°C in MRS supplemented with 25% (v/v) of glycerol.

3.7 DNA extraction and PCR analysis

For DNA collection 30 µl of cell cultures were applied to FTA™ Cards (Whatman, Maidstone, UK). Bacterial DNA was extracted from FTA™ Cards according to the manufacturers procedure. For strain typing PCR amplification was performed with $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3′) oligonucleotide primer, using Taq DNA polymerase (Fermentas UAB, Vilnius, Lithuania) (Versalovic et al., 1994). All PCR-reactions were carried out in an Eppendorf Mastercycler thermal cycler (Eppendorf, Hamburg, Germany).

The cycling program comprised: initial denaturation of DNA for 6 min at 95°C; 30 cycles each consisting of 1 min at 94°C, 1 min at 40°C and 8 min s at 65°C; and final extension for 16 min at 65°C.

The PCR products were separated by electrophoresis on 2.0 % agarose gel containing 0.05 µg ml^{-1} ethidium bromide, for 1 h in 1×TAE buffer and 100 V (constant voltage) at room temperature and a 1 kb Gene Ruler ladder (Fermentas) was used as molecular weight marker. Electrophoresis was performed using Bio-Rad Power Basic power supply (Biorad, USA). The buffer 50×TAE prepared from 242 g TRIS BASE, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA pH 8.0 and demineralized water to 1000 ml. The rep-PCR profiles

were visualized under ultraviolet light, followed by digital image capturing using a camera ImageQuant 400 + IQuant Capture 400 (Amersham Biosciences).

3.8 16S rDNA sequence analysis

The 16S rDNA region was amplified with universal primers 27- f – YM1 and 16R-1522 1U (20 pmol both) using Taq DNA polymerase (Fermentas UAB, Vilnius, Lithuania). The cycling program consisted as follows: initial denaturation of DNA for 1 min at 95°C; 34 cycles each consisting of 20 s at 94°C, 1 min at 65°C and 1.5 min at 72°C; and final extension for 5 min at 72°C. The PCR products were purified with MSB Spin PCRapace 50 kit (Invitek GmbH). The isolated PCR products were sequenced using 27- f – YM1 primer and the obtained partial 16S rRNA gene sequences were aligned with those in GenBank using Blast program.

3.9 Calculation of GITS survival

Characterization of survival in GITS The survival of bacteria (SURt) in our Gastro-Intestinal Tract Simulator for the stomach and duodenum phase (up to point P4 in Fig. 1) was calculated as:

$$\mathrm{SUR}_t = \mathrm{CFU}_t \times V_t / (\mathrm{CFU}_\mathrm{F} \times V_\mathrm{F}) \quad (1)$$

where CFUt is the instantaneous concentration of colony forming bacteria (cfu ml−1), Vt is the volume of the culture (ml) in the bioreactor vessel at time point t, CFUF is theconcentration of bacteria in food (cfu ml−1), and VF is the volume of the food (ml) injected into the vessel. For jejunum and ileum (dilution) GITS phases (after point P4 inFig. 1), the CFUt number was corrected by a dilution factor eDt :

$$\mathrm{SUR}'_t = e^{D\tau} \times \mathrm{CFU}_t \times V_t / \mathrm{CFU}_\mathrm{F} \times V_\mathrm{F} \quad (2)$$

where τ is the running time after start of dilution and D is the dilution rate.

RESULTS AND DISCUSSION

4.1. Development of single bioreactor gastrointestinal simulator GITS (Paper I, II)

The main obstacle to building up the model system for simulating the human gastrointestinal tract was a somewhat insufficient knowledge of the digestion process in vivo rather than technical realization. Therefore, selecting the algorithms and input parameters was a critical factor in the simulation of the environmental conditions during food transit through the stomach and small intestine. The input parameters used in our GITS model were drawn up for an initially empty stomach supposing that it contains 100 ml of 0.01 N HCl (Robertson 2005). Certainly, the conditions for the GIT model can be easily changed for better simulation of the individual peculiarities of food consumption habits, gastrointestinal disorders, and effect of food composition on the digestion process. It was supposed, in building up the GIT model, that the food does not enter the duodenum from the stomach before the contents of the stomach has been acidified back to pH 3.0. According to the literature the maximum capacity of acid secretion in the stomach is 20 mmol h^{-1} (Ewe and Karbach 1990). Thus, the model takes into account the buffering capacity of food as well as the physical state of food. The next step is the neutralization of "food" which is somewhat different to the physiological situation in which food continuously enters the duodenum. After neutralization bile salts are added to the food over a period of 10 min to such an extent that it achieved a concentration of 0.4% and was further held at that concentration for 30 min. Not all foods induce bile secretion to a similar extent (Kristoffersen et al. 2007). However, the peculiarities of bile secretion can be easily taken into account in the GITS model. The type of bile is also important. The use of different types of bile in similar studies makes it impossible to directly compare results of the bile tolerance of bacteria even if the same strains are used (Begley et al. 2005). In this study, bile salts (Sigma-Aldrich, a mixture of sodium cholate and sodium deoxycholate) were used. One of the most difficult technical solutions is to simulate the adsorption of food components and bile salts in the jejunum and ileum. We solved this problem by dilution of the fermentor's content. This method is technically simpler, and substrate concentrations can be more accurately controlled by adjusting the composition of the dilution media, resulting in better simulation ability than the use of dialysis. In this work, we simply used modified MRS as the dilution medium supporting the growth of probiotics during whole small intestine phase. Although part of the bacteria are washed out in the dilution phase of the GIT simulation , this does not affect their survival (see equations 1 and 2) as the concentration of bacteria itself has little effect on death or growth at substrate surplus. The factor which has a stronger influence on the survival profile in the small intestine is the adhesion of

probiotics to the epithelium cells of the small intestine, which can decrease the rate of probiotic transit through small intestine compared to food. In principle, the retention of probiotics in the GI tract can be taken into account in our GITS model by changing the dilution profile.

Our GIT simulator is suitable for various applications. It can be used to determine the survival profiles of probiotics in single-strain or mixed-strain cultures, or in different food matrices and provides the ability to take into account individual peculiarities in human GI processes.

4.2. The effect of physiological state of bacteria on their survival in GITS (Paper I, II)

The simulator of the human upper gastro-intestinal tract was validated in series of experiments with probiotic lactobacilli at different physiological states. It is known from literature that the physiological state of bacteria and food composition heavily determines the ability of the bacteria to pass through the stomach, colonize the intestine and grow at the site of action (Kos et al. 2000, Tuomola et al. 2001). The products of fermentation like lactic and acetic acid, cause considerable stress to microorganisms. Whilst passing through the stomach and small intestine the microorganisms are affected by additional stress factors – the low pH, and antimicrobial action of bile salts. The bacteria can also be influenced by digestive enzymes. We studied the effect of the physiological state of probiotic lactobacilli on their survival in the GIT simulator. Strains of *L. acidophilus La-5* (Chr. Hansen, Denmark), *L. casei Shirota* isolated from the probiotic product Yakult, *L. rhamnosus GG* (Valio) isolated from the Gefilus daily dose drink, *and L. johnsonii NCC 533* (kindly provided by Nestec) were used. Bacteria were pre-grown to an exponential or stationary phase in a lactose-containing complex media (see materials and methods). In addition, the effect of storage for two weeks at 4^{o}C on survival of stationary phase bacteria was studied.

In respect of the functionality of lactic acid bacteria in the gastrointestinal tract, the point when they start to grow is very important. If there is no adhesion to epithelial cells the culture will leave the small intestine within 7 hours. Thus, the ability of probiotics to recover and grow during this time is extremely important to exert their positive health effect. To find the time when probiotics start to grow, the specific growth rate of colony forming cells was determined (see Materials and methods).

Specific growth rate is defined as the increase in cell mass per unit time, *e.g.*, grams cells (g) per gram cells (g) per hour ($g \cdot g^{-1} \cdot h^{-1}$). The maximum specific growth rate of a strain is a fundamental characteristic, both physiologically and ecologically. Assessment of the maximum specific growth rate of a strain in its natural or near natural environment is essential for the evaluation of its capacity to exploit this environment (Salvesen et al. 2000). In the GIT the maximum

specific growth rate is probably never achieved due to the bile inhibition and the absorbtion of substrates in the small intestine.

The behaviour of the specific growth rate in digestive tract simulations with *L. acidophilus* LA-5 at different physiological states of bacteria is shown on Fig. 4 of Paper I. In case of not adding bile, growth starts imminently after neutralization (Fig. 4A), in other cases it reaches a positive value after several hours. The positive specific growth rate after bile shock (Fig. 4B) may be more related to an improvement in the colony forming ability of the culture rather than multiplication, in the case of cells in an early exponential phase.

One prerequisite for probiotics is their survival and resistance to human defense mechanisms during gastrointestinal tract transit (Fuller, 1989; Saarela et al., 2000). The results on survival of the four lactobacilli in our gastrointestinal tract simulator irrespective of their physiological state are given in the Table 2 of Paper II. The results show that when using MRS culture as model food *L. acidophilu*s La-5 and *L. johnsonii* NC533 survive in GIT simulator significantly (100–1,000 times) better than *L. casei* strain Shirota and *L. rhamnosus* GG. Although *L. casei* and *L. rhamnosus* GG were somewhat more resistant to the stomach-phase acid treatment than *L. acidophilus* and *L. johnsonii*, the colony-forming cell number promptly dropped by three magnitudes after the addition of bile. The decrease of cfu numbers of *L. acidophilus* and *L. johnsonii* after bile treatment, was about one magnitude.

Surprisingly, the cfu count (as well as Live/Dead cell ratio determined by microscopy of bacteria stained with LIVE/DEAD BacLight bacterial viability kit) increased for *L. casei* and *L. rhamnosus* during bile incubation (duodenum phase) and bile absorption (during jejunum/ileum phase) to an extent that cannot be explained by growth alone. Such an increase was not observed for *L. acidophilus* neither *L. johnsonii*.

Statistically relevant differences in behavior of *L. acidophilus* and *L. johnsonii* as well as for *L. casei* and *L. rhamnosus* were not observed. Therefore, to analyze the effect of the physiological state, the bacteria were grouped into two categories for better statistical analysis. Such a division of lactic acid bacteria can be justified by their phylogenetic tree, according to which the *L. casei* and *L. rhamnosus* species are closely related as are the *L. acidophilus* and *L. johnsonii* species (Siezen et al. 2004). The results on survival of bacteria at different physiological states are shown in Table 3 of Paper II.

All of the bacteria studied were found to be more resistant to low pH stress in GITS when introduced at an early stationary growth phase; however, the observed differences in survival were marginal compared with bacteria introduced while in an exponential growth phase. Compared with stationary phase cells, storage at 4 °C for 2 weeks significantly decreased the ability of bacteria from *L. acidophilus* and *L. johnsonii* strains to resist acid stress in stomach step of GITS. The influence of bile was clearly group-specific as shown above, with *L. acidophilus* La-5/*L. johnsonii* NC533 being more resistant to the bile phase in our GI model than *Shirota/L. rhamnosus* GG group (the difference

in viable counts was over 3 magnitudes). An impact of the physiological state was observed in the case of the *L. johnsonii* NC533/L. *acidophilus* La-5 group; the exponential and stationary phase cells resisted the bile stage significantly better than the cells stored for 2 weeks. The *L. casei Shirota* and *L. rhamnosus* GG group showed a significant decrease in viable count irrespective of their physiological state.

In this study we analyzed the use of a tightly controlled fermentation system for the simulation of survival conditions experienced by probiotic bacteria during food transit through the upper part of the human GI tract. Our results demonstrate that, in respect of probiotic survival, the bile treatment of food is much more important than acid treatment. Also we showed that the physiological state can considerably influence the resistance of some strains to acid and bile.

4.3. The effect of food matrix on viability of probiotics in GITS

(Paper II, and unpublished data)

As a previous study showed the resistance of *L. rhamnosus GG* to bile in MRS matrix was very low. In order to determine the factors that affect performance of LGG in the GITS, we studied *L. rhamnosus* GG included in different commercial probiotic products. Gefilus milk, Gefilus cheese, Gefilus daily dose drink and Gefilus Peptidi Tutteli baby formula were used in this study. The composition of these products is given in Table 1 of Paper II.

Our results using the GIT model demonstrated that the same probiotic strain in different food products behave very differently. We demonstrated that in some commercial food products, *L. rhamnosus* GG showed surprisingly good survival in respect to MRS media (Paper II, Fig. 2).

The GITS survival of LGG in Gefilus cheese and Gefilus milk remained between 10% and 100%, while survival in Tuttely baby formula was comparable to that in MRS broth - below 0.1%. Similarly to MRS model food, acid treatment in stomach phase reduced the cfu number of LGG in all commercial foods tested by tenfold at most. A significant loss of colony formation ability was observed after addition of bile in baby formula and model food; however, the effect of bile on survival was surprisingly low for the daily dose milk. All this demonstrates the great impact on product efficacy of both the food matrix and probably also the technology of incorporating probiotic strains into the product.

We also studied the survival of LGG inoculated into MRS medium, milk, apple juice and sports drink in form of LN_2 frozen beads of pH auxostat culture. An apple juice was titrated with sodium bicarbonate before inoculation with frozen LGG cells in order to retain the viability of bacteria during storage. The mean viable counts of *L. rhamnosus* bacteria in sample from MRS, milk and sports drink before GITS experiment were $4.8 \pm 0.2 * 10^7$ cfu g^{-1}, $4.6 \pm 0.17 * 10^7$ cfu g^{-1}, and $3.0 \pm 0.04 * 10^7$ cfu g^{-1}, respectively. The mean viable counts

in titrated apple juice were 2.8 ± 0.1 * 10^6 cfu g^{-1} . The results of GITS experiments are shown in Figure 4.

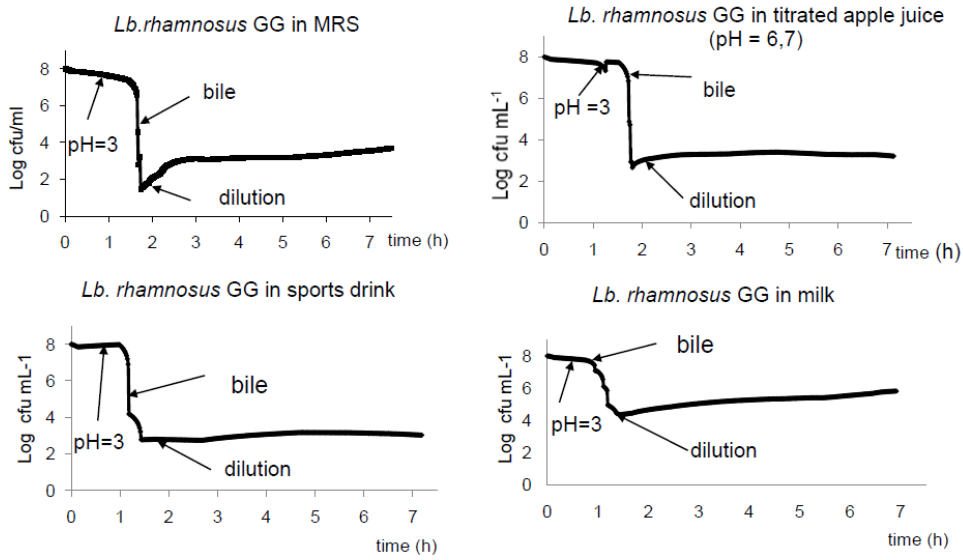

Figure 4. Survival profiles (normalized data) of LN_2 frozen LGG pH auxostat culture in different food matrices during GITS

The results show that acid in stomach phase in our simulator did not influence significantly the survival of fast frozen LGG cells. Colony forming cell numbers dropped less than 0.3 log units in case of any of food matrices. However the bile treatment gave more different results. The viability of *L. rhamnosus* in MRS decrease after incubation with bile over 6 magnitudes. Loss of viable counts of LGG bacteria in titrated apple juice and in sports drink made from whey powder was 4.5 log units. *L. rhamnosus* was most resistant to bile stress in milk (average loss of viable counts up to 4 log units. Also the increase in colony cell number was observed in case of milk and MRS matrix.

4.4. The effect of sublethal stress pretreatment on survival of probiotics in GITS (Paper III)

Several approaches have been investigated to enhance cell viability during downstream processing, storage and eventually digestion. One of them is the sublethal treatment of bacteria to enhance their performance in otherwise lethal conditions.

To investigate the effect of different stress pretreatments on the survival of probiotic lactobacilli under gastrointestinal conditions, we first obtained a steady-state pH-auxostat (pH=6.0) cultures of *L. acidophilus* La-5 (Paper III Fig. 1), *L. rhamnosus* GG, and *L. fermentum* ME-3. The cultures were then treated at

different sublethal conditions, frozen in liquid nitrogen to fix their physiological state and passed through GITS.

The stress treatments of steady-state pH-auxostat cultures were performed at a constant pH (in pH-stat). During stress treatments, significant bacterial growth was observed with the exceptions of pH 4.5 and pH 3.5 induced stress for *L. rhamnosus* GG and pH 3.5 or 0.1% bile stress for *L. fermentum* ME-3. The results of the effect of stress treatment and subsequent freezing on the viability of pH-auxostat-derived cultures are summarized in Table 1 of Paper III. LN2 freezing of stressed cultures of *L. acidophilus* decreased the survival of bacteria significantly compared to that of *L. rhamnosus* GG and *L. fermentum* ME-3. A gradual change in the viable counts of LN2 frozen cells of all studied strains was observed depending on amount of bile or acid applied during stress treatment, i.e., the treatment with 0.02% bile or at pH 4.5 caused smaller decrease in the viability than 0.04% and 0.1% bile or pH 3.5, respectively.

A series of gastrointestinal tract simulator experiments were performed comparatively with stressed and nonstressed LN2 frozen cells of *L. acidophilus* La-5, *L. rhamnosus* GG, and *L. fermentum* ME-3 (Fig. 2 of Paper III).

L. acidophilus La-5 was found to be the most resistant strain to simulated gastrointestinal conditions. The survival profile of frozen nonstressed La-5 cells was comparable with that of cells derived directly from auxostat culture. *L. acidophilus* La-5 showed slightly better acid tolerance after treatment at pH 3.5 compared to nontreated cells in the GITS. In the stomach phase, the average loss of colony forming ability of cells treated with bile, temperatures of 43 °C or pH 4.5 was not found to differ significantly from the survival rate of auxostat cells and that of frozen non-stressed cells. *L. acidophilus* La-5 cells pretreated with 0.02% bile and frozen in LN2 beads improved the tolerance to bile in the GITS compared to cells without freezing and stress pretreatment. Pretreatment with 0.1% bile or pH 4.5 or pH 3.5 only slightly improved, if at all, the survival after the bile phase in the GITS. The viability of *L. rhamnosus GG* auxostat cells decreased after the stomach phase in the GITS to 0.39±0.38 and after bile addition to3.96±1.48 log units. The frozen nonstressed *L. rhamnosus* GG cells showed a similar decrease in viability.

In general, stress treatment of *L. rhamnosus* GG bacteria did not improve their acid tolerance in the GITS model. Only bacteria incubated at pH 3.5 showed better resistance to stomach acid. Treatment with 0.02%, 0.04%, or 0.1% bile solution rather impaired tolerance of *L. rhamnosus* GG to acid in the GIT simulator. The survival of bacteria incubated at pH 4.5 was comparable to frozen nonstressed cells and temperature stressed *L. rhamnosus* GG showed similar results to cells from an auxostat culture. Also the bile pretreatment of *L. rhamnosus* GG did not increase its tolerance to simulated digestive stresses. Only pH 4.5 treatments somewhat improved survival in the GITS compared to untreated cells. Experiments with *L. fermentum* ME-3 also showed a more negative than positive influence of stress pretreatments and freezing on survival in the GITS irrespective of the imposed stress condition. The untreated pH-

auxostat culture showed the highest survival. Sublethal treatment of *L. fermentum* ME-3 did not alter the survival of cells in the stomach phase of the GITS. Also none of these stresses improved the resistance of ME-3 to bile in the GITS. Treatment with 0.1% bile had a less negative effect than other sublethal treatments.

The survival of microorganisms during processing and in the gastrointestinal tract depends on the physiological state of the culture, thus the production and maintenance of the bacteria in the same physiological state is crucial for comparative survival testing.

Although auxostat fermentation may be more difficult to operate under industrial conditions, this technology is worth investigation and could be used to produce cells with different physiologies and to apply stresses under well-controlled conditions (Lacroix and Yildirim, 2007).

Fast freezing in liquid nitrogen fixes the structural properties of cells to resist stress and was considered as a reliable method for the preparation of comparable starting samples to study the effects of stress pretreatment on the survival of lactobacilli in the GIT model. The LN2 frozen MRS beads of *L. acidophilus*, *L. rhamnosus*, and *L. fermentum* were used as model products. The viability of pH-auxostat grown cells in the GITS did not decrease after LN2 freezing and subsequent storage at −40 °C. This was confirmed by similar survival in the GITS model of frozen and unfrozen and non-stress pretreated cells (see Fig. 2 of Paper III). So we consider the conclusions drawn from experiments with LN2 beads to be also valid for the corresponding unfrozen cultures or products. Bile and acid stresses applied to the auxostat cultures of *L. rhamnosus and L. fermentum* before LN2 freezing did not show a statistically relevant positive effect on the survival in the GITS. The resistance to bile stress of L. acidophilus beads was a few log units better than that of *L. rhamnosu*s and *L. fermentum*, and we observed some improvement in bile tolerance of stress pretreated cells in the GITS. This study verifies again that tolerance to bile stress is a critical factor for the survival of probiotic bacteria in the gastrointestinal tract and that bacterial species respond diversely to bile in the GITS: the death rate can be several magnitudes higher. The GIT survival of probiotic strains might not only depend on their number and physiological state but also on food matrix and habits of food consumption which affect bile excretion.

4.5. Survival of natural microbial consortium of cheese in GITS

Cheese involves complex microbial ecosystem, and although inoculated only with few species a very varied microflora develops during ripening in most of cheese varieties (Rantsiou et al., 2004). Several studies have proven that cheese contains also strains with probiotic properties (Kask et al., 2003; Succi et al., 2005; Ugarte et al., 2006, Georgieva et al., 2008).

The aim of this study was to compare survival of different species and strains of lactic acid bacteria of semi-hard cheese and to isolate potential probiotics from

natural microbial population using GITS in combination with molecular identification methods.

Cheeses subjected to study were commercial 90-day-old open texture cheeses produced in two cheese factories of Valio Ltd. (Finland) situating in Estonia and Finland. All cheeses were produced from pasteurized cow's milk with Chy-Max rennet and mesophilic DL starter cultures (Chr. Hansen A/S, Denmark) composed of undefined proportions of *Lactococcus (Lc.) lactis* subsp. *lactis, Lc. lactis* subsp. *cremoris, Lc. lactis* subsp. *lactis* biovar *diacetylactis* and *Leuconostoc (Ln.) mesenteroides* subsp. *cremoris.*

4.5.1. LAB species in semi-hard cheeses and their survival in GITS

The average numbers of colony forming lactic acid bacteria in cheeses from Finnish factory was $8.5 \pm 3.4 \times 10^6$ CFU/g and that of cheeses from Estonian factory $2.4 \pm 1.1 \times 10^7$ CFU/g. Proportions of different species within total LAB before and after GITS are presented in Table 1 of paper IV.

According to 16S rDNA sequence data, totally 8 different species containing 22 different genotypes were detected from FIN cheese (among 315 isolates analyzed) and 9 species containing 13 genotypes were detected from EST cheese (among 284 isolates analyzed). The species *Lactobacillus casei* and *Lactobacillus paracasei* are presented as a combined *L. casei/paracasei* species since we could not differentiate them with the current methods.

The starter lactococci (*Lc. lactis* subsp. *lactis* and biovar. *diacetylactis, Lc. lactis* subsp. *cremoris*) comprised 30.7 % of determined LAB population in cheeses from Finnish factory and 63.6 % in cheeses from Estonian factory (Tab. 1) while *Lc. lactis* biovar *diacetylactis* was found as dominating starter species in cheeses from Finnish factory only. After GITS the percentage of lactococci decreased down to 18.1 and 34.7 % with calculated survival of 2.7-3.1 and 1.6 % for cheeses from Finnish and Estonian dairies, respectively. *Lc.cremoris* did not survive in GITS in detectable amounts.

In the Finnish cheese the NSLAB *L. casei/paracasei* dominated both in the beginning and end of GITS transit comprising 39.1 and 76.5 % of the population respectively (Tab. 1, paper IV) with 9.7% GITS survival clearly exceeding the average for total LAB - 4.9%.Survival of *Ln. mesenteroides, L. plantarum* and *L. rhamnosus* is hard to determine in this study due to very low numbers detected.

In cheeses from Estonian dairy the most dominant NSLAB was *L. casei/paracasei* followed by *Ln. mesenteroides* representing 20% and 12 % of population, respectively. The species *L. curvatus* comprised 2.4%, *Lc. cremoris, Ln. pseudomesenteroides* and *L. diolivorans* altogether 2.6 % from total colony forming units. After GIT simulation the proportion of *L.casei/paracasei* increased up to 34.7% (survival 4.9%). In different from Finnish cheese, *Ln. mesenteroides* showed as good survival (4.9%) constituting 21% of total counts after GIT simulation. The percentage of *L. curvatus* and *Ln. pseudomesenteroides* after GITS increased slightly remaining however

subdominant. Few colonies of *L. rhamnosus* and *Streptococcus thermophilus* were also detected after GITS transit suggesting their survival. Neither *Lc. cremoris* nor *L. diolivorans* survived GIT conditions in detectable amounts.

The average survival of total LAB was somewhat higher for Finnish cheeses compared to that for Estonian cheeses (4.9± 3.6 vs 2.9± 2.3 %). That can be explained with higher content of *L. casei/paracasei* having better survival (in population of Finnish cheese) compared to *Lc. lactis* with relatively lower survival that dominated in Estonian cheese.

4.5.2. Distribution and survival of different genotypes of Lb. casei/paracasei

The most abundant and most resistant NSLAB species during GIT transit was *L. casei/paracasei*. Average extrapolated numbers of *L. casei/paracasei* per gram of cheese before and after GITS were $3.3 \times 10^6 - 3.2 \times 10^4$ cfu/g and $4.8 \times 10^6 - 2.4 \times 10^4$ cfu/g, for Finnish and Estonian factories, respectively.

By rep-PCR analysis 13 different genotypes of *L. casei/paracasei* were detected from Finnish cheese and 6 from Estonian cheese, whereas none of the genotypes were found in both cheeses (Paper IV, Figure 1).

In the Finnish cheese, five genotypes comprised over 85% of the whole *L. casei/paracasei* species and a single genotype (no 8) became dominating after GITS (Paper IV, Figure 1 and 2A). This genotype had the highest GITS survival - 24% comprising 7.8 % of total LAB in the beginning and 38.3 % in the end of GIT simulation. Three genotypes (1, 2 and 13) were detected in low numbers only in the beginning of GITS and one (no. 9) appeared only in the end of GITS. Two genotypes (2 and 6) appeared in low numbers only in the end of GITS.

In the Estonian cheese two dominating genotypes (14 and 17, Fig 2B of Paper IV) comprised more than 80% of the whole *L. casei/paracasei* without considerable change of the proportion during GITS (survival about 5%).

Survival of different genotypes of *L.casei/paracasei* in GITS experiments varied significantly, while isolates from Finnish cheese showed generally better resistance against GIT stresses The effect of the cheese matrix and possibly also physiological state of bacteria was demonstrated in GITS experiments with overnight stationary phase culture of *L. casei/paracasei* genotype no 8 in MRS broth (Paper IV, Figure 3).

The calculated survival of this strain in GITS when applied in cheese matrix was 24.0% that is about 1000 fold higher compared to survival in overnight MRS culture (0.06%).

4.5.3 The effect of digestive enzymes on survival of LAB during GITS transit

The effect of pepsin and pancreatin on the viability of LAB from cheese was studied and compared with the results obtained without enzymes in GITS.

Pepsin (4 g/l) was added in the stomach phase and pancreatin (9 g/l) in small intestine.

Results of these experiments are shown in Figure 4 of Paper IV. Addition of digestive enzymes into GIT model clearly decreased the variability of microbiota of both cheeses. The only species from those detected in cheeses with considerable GITS survival were *Lc. lactis* from starter culture and non-starter *L. casei/ paracasei*.

Proportion of *Lc. lactis* decreased in both cheeses, but remained higher in Estonian cheese. *Lc. cremoris* did not survive neither of the GITS. *L. mesenteroides* in Estonian cheese, which survived well acid and bile treatments, was not detected after GITS complemented with enzymes. Also *L. plantarum*, *L. curvatus* nor *L. rhamnosus* were not detected after GITS with digestive enzymes. The only species which proportion among LAB increased in GITS with enzymes was *L. casei/ paracasei*.

Our study showed significant differences between LAB species from semi-hard cheese and between strains within one species as well as importance of the matrix in which bacteria are ingested on their survival in conditions of GI-tract. We demonstrated that several strains of lactobacilli that grow in ripening cheese can also survive the physiological stresses of GIT.

The gastrointestinal tract simulator can effectively be used for selection of acid, bile and digestive enzyme resistant bacteria from complex food ecosystems and for comparative survival studies of individual probiotic strains in various food matrixes.

These results emphasize the importance of food matrix composition as well as design of the probiotic product on survival of probiotics in gastrointestinal tract.

CONCLUSIONS

The single bioreactor simulator of intestinal tract presented in this work is very effective model for evaluation of probiotic properties of food *in vitro*.

1. The environmental conditions of food transit through the stomach and small intestine can be successfully simulated in a single bioreactor. The single bioreactor gastrointestinal tract simulator developed in this work is an effective tool for the evaluation of potential probiotic properties of food *in vitro*.
2. The factors affecting the survival of probiotic bacteria in gastrointestinal tract are very complex and depend on
    a. the specific properties and physiological state of the strain of the probiotic organism,
    b. the environment of ingestion ie the food matrix (composition) and
    c. the microbial consortium – mutual interactions;
    d. the acid and bile secretion peculiarities of humans (the reaction of humans to food consumption). The effect of bile on the survival of probiotics in the GIT is far greater than that of acid.
    e. There exist significant differences in GITS survival between different LAB species and strains whilst passing semi-hard cheeses through the model.
3. No statistically relevant positive effect of stress pretreatments on survival of LN2 frozen *L. acidophilus* La-5, *L. rhamnosus* GG, and *L. fermentum* ME-3 in the GITS was observed under chosen stress conditions.

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ARTICLE I

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Oral presentations

The bioreactor model of human intestinal tract

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Abstract

To evaluate the viability of probiotic bacteria and potential efficacy of the probiotic food product we developed a single vessel bioreactor model simulating the changes of environmental conditions of human upper gastrointestinal tract. The process was programmed using "BioXpert" (Applikon, The Netherlands) cultivation control program for "BioBundle" bioreactor. The simulated parameters were body temperature, transit time, pH profile and concentration of bile salts. The holding time of food in stomach depends on the composition of the food and pH of stomach content. To simulate the chyme movement into duodenum after pH in stomach has dropped down to 3 the content of bioreactor was neutralized with $NaHCO_3$ followed by addition of bile salts. The decrease of substrate and bile salt concentration in jejunum and ileum due to absorption into blood was simulated by diluting the bioreactor content with dilution medium at constant dilution rate $D=0.4\ h^{-1}$ during 6 hours. The survival of *Lactobacillus acidophilus* La-5 in different physiological states and different "food" composition was studied in the model system. The results showed that acid and bile treatment of bacteria (simulating the stomach and small intestine) reduce the number of viable cells 2 - 300 fold. During the dilution phase (*jejunum and ileum*) the specific growth rate of viable cells rose above zero within 2-6 hours after consumption depending on "food" parameters. Our experiments showed that the proposed model can be used for screening of potential probiotics and probiotic properties of the strain depend significantly on its physiological state and food composition.

1. Introduction

Today about half of fermented milk products - sour milk, yogurt and kefir in Estonia are marketed as probiotic. To demonstrate the health effects of probiotic bacteria *in vivo*, especially in humans is very complicated. Therefore, the *in vitro* methods like culture of bacteria in conditions similar to gastrointestinal tract followed by counting viable cells are often used as evidence of efficacy. Although the viability/activity of bacteria is an important criterion of potential health effect, the physiological state of bacteria and food composition determines a lot the ability of the bacteria to pass stomach, colonize the (large) intestine and grow at site of action (Kos et al. 2000, Tuomola et al. 2001). The fermentation end products lactic and acetic acids provide considerable stress to microorganisms. During passing the stomach and small intestine the microorganisms are affected by additional stress factors – low pH and action of bile salts, which affect bacterial membranes. Also the physiological state of the bacteria as

well as properties of the food matrix effect considerably their survival. Not only survival but also abilities to colonize epithelial cells of intestine and grow/multiply are the important properties of a good probiotic. Passing the intestinal tract the probiotic bacteria should start to grow (multiply) at possibly early stages of passing small intestine, as the availability of substrates decreases further on. The holding time of probiotic bacteria in intestine depends on their adhesion and colonization properties on epithelial cells. Attachment to the mucosa prolongs the time in which probiotics can influence the indigenous microflora of the host and it corresponds to the efficacy of the probiotic strain (Kirjavainen *et al.*, 1998, Pennacchia *et al.* 2006). In result the potential health effects of probiotic bacteria in food depend not only on number of viable cells but also times of consumption, composition of food as well as physiological state of probiotic bacteria consumed. *In vivo* studies are too complex to be used for routine evaluation of probiotic properties of foods. Therefore, reliable *in vitro* tests to study the behaviour of probiotic bacteria in food matrixes are needed.

Numerous studies on the survival of potential probiotic bacteria in simulated upper gastrointestinal (GI) tract conditions have been carried out, but they do not always represent adequately *in vivo* conditions (Morelli, 2000). Most models designed for study the viability of probiotic bacteria are so called static models, which involve growing cells in acidic milieu or in the medium supplemented with Oxgall bile, porcine bile or bile salts. In typical studies (Jakobsen *et al.*, 1999) the resistance to bile salts has been investigated independently of the resistance to gastric juice and in the absence of a protection system. Combined effect of gastric juice followed by the effect of bile salts on the viability of probiotic strains incorporated in a food matrix was studied by Madureira *et al.* (2005).

Several complex simulators of the human intestinal tract have been developed. Best known are SHIME (Molly *et al.*, 1993), which has been used for investigation of the interaction of probiotic bacteria with human intestinal microflora (Nollet *et al.*, 1997).

A dynamic, computer-controlled model (TNO) has been developed to simulate *in vivo* conditions in the stomach and small intestine, such as the kinetics of pH, bile salt concentrations, and transit of the chyme (Minekus *et al.*, 1995). This model has been used for study of survival of different lactic acid bacteria (Marteau *et al.*, 1997).

A dynamic model that simulates the human upper gastrointestinal tract for probiotics (Mainville *et al.*, 2005) was used to test survival of bacteria isolated from humans and dairy products. Nevertheless, the models suitable for wide range evaluation of probiotic potential of foods are still missing.

The main obstacle of building up the model system simulating the human gastrointestinal tract is insufficient knowledge about digestion process *in vivo*. Therefore selecting the algorithms and input parameters in models is more complicated than technical possibilities to simulate the process. According to the literature (Robertson, 2005) empty stomach contains about 100 ml of 0.01 N HCl. The food (200 ml in our model) entering the stomach raises the pH depending on the buffering capacity of the food. It is supposed that food does not enter the *duodenum* before the content of stomach is acidified back to pH 3.0. It takes time as capacity of acid secretion in stomach is limited to 20 mmol h^{-1}. Food entering the *duodenum* is neutralized with $NaHCO_3$ and bile salts up to concentration of 0.4 %. There are total of 3 g of bile acids in the human body that circulate 4 -12 times a day between (through) intestine and liver. Amount of bile salts secreted into *duodenum* depends on composition of food. The main stimulus for bile secretion is ingested lipids: secretion of bile after intake of fatty meal

may increase many times compared to secretion of bile after digestion of fat-free (low-fat) food (Schmidt and Thews, 1990). Most of nutrients suitable for probiotic bacteria as substrates as well as bile acids are absorbed before the food reaches large intestine.

The aim of this study was to design and test an *in vitro* bioreactor model that could be used to evaluate the probiotic potential of food. As we could not see any considerable advantages of complicated multi-vessel systems used in previous studies the single bioreactor model was chosen for process simulation.

2. Materials and methods

2.1 Microorganism

Lactobacillus acidophilus La-5 (Chr. Hansen, Denmark) as commercial probiotic was used in this study.

2.2 Growth media

Bacteria were grown in lactose-containing complex medium: lactose – 20 (g L^{-1}), tryptone - 5, yeast extract – 2.5, Tween 80 – 1 (Difco Laboratories, USA), Na-citrate – 2.3, K_2HPO_4 – 2 (Scharlau, Spain), $MgSO_4 \cdot 7H_2O$ – 0.2, $MnSO_4 \cdot 5H_2O$ - 0.04. For the dilution of bile salts in intestinal tract model the medium consisting of lactose – 5 (g L^{-1}), tryptone – 2.5, yeast extract – 1.25, Tween 80 – 0.5, K_2HPO_4 – 1. The pH of the dilution solution was set to 5.0 by 5M HCl.

2.3 Food

"Food" was prepared by fermenting the lactose containing complex medium (see above) with *Lactobacillus acidophilus* La-5 at 37 °C. The culture was harvested from exponential or early stationary growth phase or in stationary phase after overnight cultivation (Table 1). The "food" (bacterial biomass) was then added into bioreactor imminently after obtaining the desired cell density or was kept at 4 °C for two weeks (simulating the storage of the fermented product) before used in the experiment.

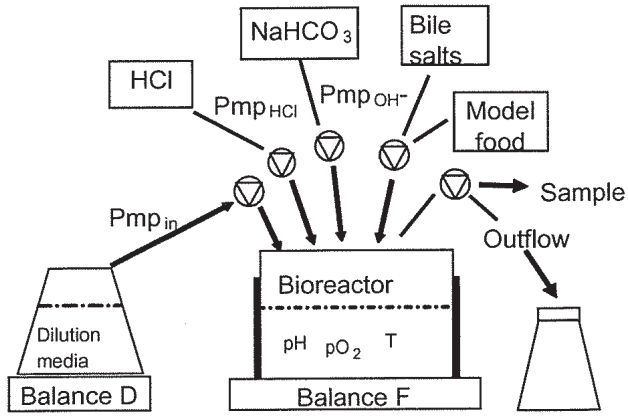

Figure 1. The bioreactor system for simulation of upper intestinal tract.

2.3 The bioreactor and process simulation

The model system (Figure 1) consisted of a 500 ml "Biobundle" fermenter (Applikon, The Netherlands) with a working volume of 300 ml, a biocontroller ADI 1030 and AD/DA interface connected to the PC with a cultivation control program "BioXpert" (Applikon, The Netherlands). A commercial version of the laboratory software „FermXpert" (Vinter *et al.*, 1992) was used. The fermenter was equipped with the pO_2, pH and temperature sensors, an optical density detector (Optek, Germany) and balances to control (measure) the weight of the fermenter and feeding media for precise dilution rate calculation. The variable speed pumps (Cole Parmer, USA) were used to control the flow of HCl, $NaHCO_3$, bile acids, feeding medium and the culture volume.

According to the algorithm (Figure 2) there was 100 ml of 0.01M HCl in the fermenter in order to simulate the conditions in empty stomach. When the temperature reached 37 °C 200 ml of "food" containing $10^7 - 10^8$ cfu/ml *Lactobacillus acidophilus* La-5 was pumped into fermenter. Then the addition of 1M HCl was switched on. The maximum HCl addition rate was adjusted to 20 mmol h^{-1} reported as the maximum HCl secretion rate for human stomach (Schmidt and Thews 1990).

Addition of HCl continued until pH in fermenter reached 3.0. Then, to simulate the passing of food from stomach to *duodenum* the content of fermenter was neutralized with 1 M $NaHCO_3$ during about 10 minutes and then 4 % bile salt solution (bile salts, Sigma-Aldrich Corporation) was added to achieve the final concentration of bile 0.4 %. To simulate the absorption of bile acids and food components in *jejunum* and *ilenum* the feeding with dilution medium at specific dilution rate of D=0.4 h^{-1} was started. The feeding was stopped at 7^{th} hour corresponding to the time the food reaches large intestine.

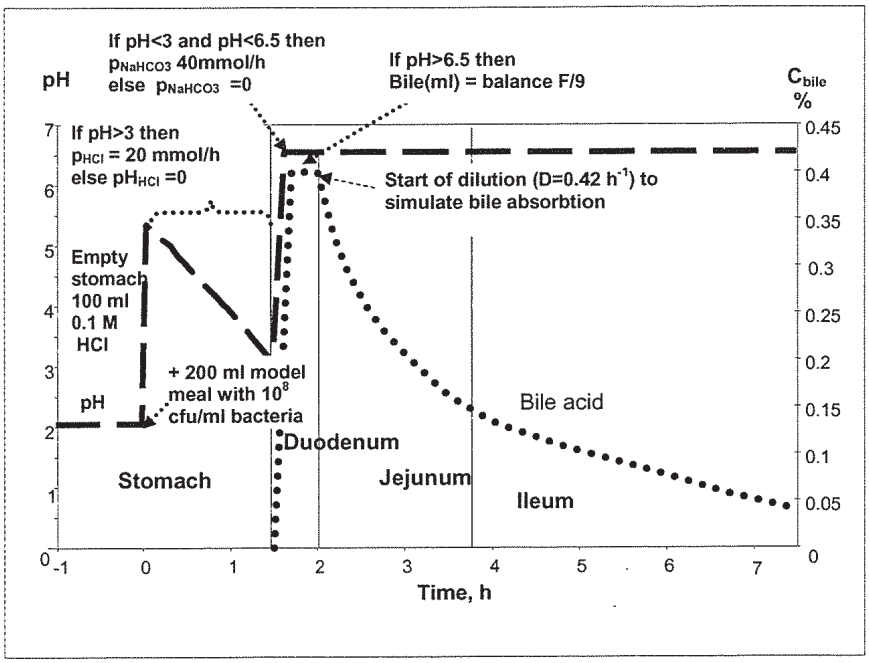

Figure 2. Simulation of environmental conditions in GI tract

2.4 Analytical methods.

2.4.1 Viability assessment

Viability of bacteria was assessed by direct microscopic counts and indirect, plate counts of bacteria. Microscopic counting was performed using LIVE/DEAD BacLight bacterial viability kit (L-7012) (Molecular Probes, Eugene, OR, USA). The kit comprises two fluorescent nucleic acid stains SYTO9 and propidium iodide. SYTO9 green- fluorescent stain penetrates both healthy and damaged bacterial cells whereas red-fluorescent stain propidium iodide penetrates only damaged cell membranes causing a reduction in the SYTO9 stain fluorescence when both dyes are present. Live bacteria stained with appropriate mixture of these dyes fluorescent green and dead bacteria fluorescent red. One milliliter of sample from the bioreactor at different simulating stages was centrifuged, the bacterial biomass suspended in distilled water and incubated with mixture of two dyes in dark at room temperature for 15 minutes. Then 10 µl of bacterial suspension was placed in a counting chamber (improved Neubauer chamber with 0.1 mm depth) and examined with oil immersion under Olympus A041 microscope equipped with an HBO 200 w/2 Super Pressure Mercury Lamp (Osram, Germany) and appropriate filters for LIVE/DEAD BacLight bacterial viability kit. The images were taken with Olympus DP70 camera and counted for the live and dead cells. The numbers of colony-forming units (cfu; viable bacteria) mL^{-1} were determined by counting suitable dilutions of the bacterial suspensions using pour-plate method on MRS.

2.4.2 Determination of sugars, ethanol and organic acids

The concentration of glucose, ethanol, glycerol and organic (lactic, acetic) acids were determined by liquid chromatography (HPLC Waters 600), using isocratic elution with 0.009 N H_2SO_4, flow rate 0.6 mL min^{-1} on an Aminex HPX-87H (BioRad) column (300 x 7.8 mm, particle size of 9 μm) at ambient temperature. Concentrations were monitored with the UV (210 nm) and refractive index detectors, which were applied in parallel, enabling better quantification.

2.4.3 Calculation of culture parameters

The actual dilution rate D_s was calculated as

$$D_s=(pmp_{in})/V \qquad (1)$$

The feeding rate pmp_{in} was calculated as change rate of the weight of feeding vessel (*balanceD*) and the bioreactor volume V from weight difference of the empty and filled bioreactor.

$$pmp_{in}=-\mathrm{d}(balance_D)/\mathrm{d}t \qquad (2)$$

$$V=balance_F-balance_{R0} \qquad (3)$$

The specific growth rate of colony forming cells μ_{cfu} was calculated as

$$\mu_{cfu}=\mathrm{d}cfu/(\mathrm{d}t*cfu)+D \qquad (4)$$

where *cfu* is the concentration of the colony forming cells in reactor.

3. Results and discussion

The bioreactor model of intestinal tract was validated in series of experiments with artificial food containing *Lactobacillus acidophilus* La-5. The food was characterized by plate counts of *Lb. acidophilus*, pH, and lactic acid content. The physiological state of the bacterial culture in moment of entering stomach varied between different experiments (Figure 4) from exponential or early stationary growth phase in some experiments to stationary growth phase in others. In addition the effect of storage of the stationary phase culture at 4 °C was studied. Results of the experiments are given in Table 1.

Table 1. Changes of viability (survival) of *L. acidophilus* La-5 in GI model system added at different physiological states of bacteria

Viable cell count at t=0 (10^6)	pH of model food	Concentration of lactic acid in model food mg/mL	Reduction in cfu counts after addition of HCl	Live/dead cell ratio after addition of HCl	Reduction of cfu counts after addition of bile	Live/dead cell ratio after addition of bile	Reduction of cfu counts at the beginning of dilution	Live/dead cell ratio at the beginning of dilution	$\mu > 0$
Bacteria at stationary growth phase treated only with HCl acid									
116	4.2	16	3x	3			2.5x	9x	2h
Bacteria at exponential growth phase									
3.5	6.1	1	1x	30	7.5x	0.13	2x	0.1	2h
144	5.4	4.5	2x	4	10x	0.3	7x	0.5	5h
9	5.8	3.5	2x	4	2.2x	1.12	6x	1.3	4h
Bacteria at stationary growth phase									
324	4.2	4	2x	6	11x	0.4	8x	1.3	4h
Bacteria stored 2 weeks at 4 °C									
6.5	4.25	6	43x	0.03	70x	0.015	300x	0.4	6h

The results showed that survival of bacteria in model depends on both – physiological state of bacteria as well as food properties (pH, lactic acid content). In all simulations except that of with stored food the decrease of viability after adding HCl was relatively small: reduction of cfu did not exceed 2-3 fold and the live/dead cell ratio remained high. The live/dead cell ratio was especially high in case of cells from exponential growth phase and low concentration of lactic acid.

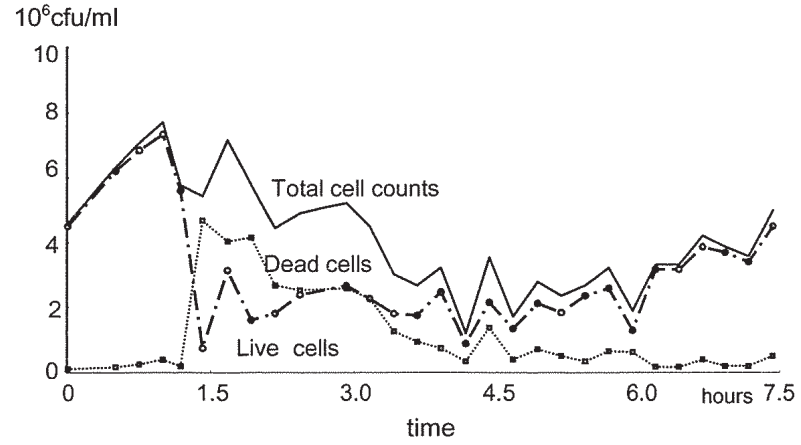

Figure 3. Changes in live and dead cell counts in model system in the experiment where cells from exponential growth phase were used.

Addition of bile (1.5 hours from the start) decreased the plate counts about 10 fold and ratio of viable and dead cells down to 1 in most cases (e.g. Figure 3). Some increase in live to dead cell ratio was observed in the end of the *duodenum* stage (at 2 hours) even before start of decrease in bile acid concentration (start of dilution). Also, in most cases the plate count numbers increased at the same stage. These results suggest that some recovery of the viability of bacteria from shock of the bile acid treatment occurs already 20 minutes after bile addition. The later observed increase of viable cell counts (at 5-6 hours, Figure 3) can be related to the growth and multiplication of the cells and wash out of cells.

In respect of functionality of lactic acid bacteria the starting time of growth is very important. If there is no adhesion to epithelial cells the culture will leave small intestine during 7 hours.

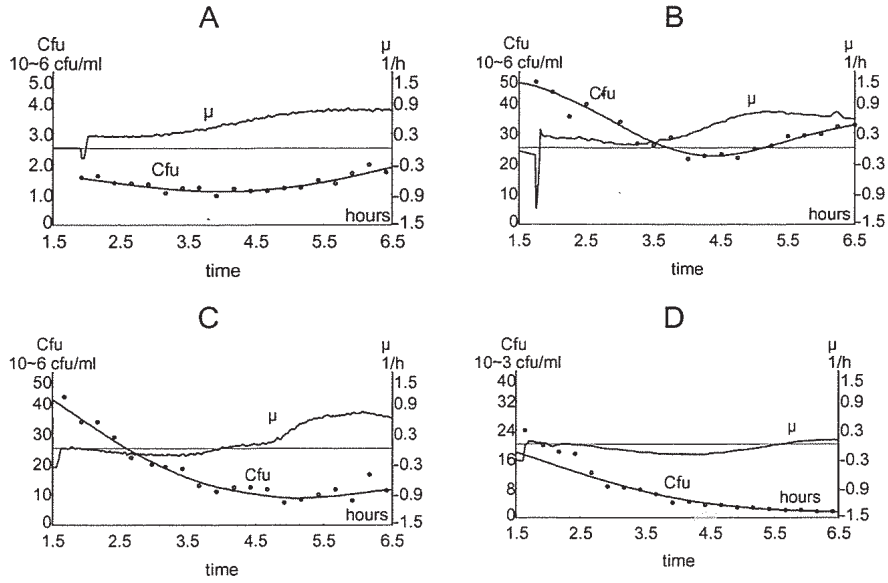

Figure 4. Behaviour of *L. acidophilus* La-5 added into model GI-tract at different physiological states: A – exponential growth phase without bile addition; B – exponential growth phase; C –stationary growth phase; D – bacterial culture stored 2 weeks at 4 °C.

Therefore the recovery time of the probiotic bacteria may be extremely important to have a positive health effect. To find the time probiotics start to grow the specific growth rate of colony forming cells was determined. Behavior of specific growth rate in several simulation experiments is shown in Figure 4. In case of not adding bile the growth starts imminently after acid addition (Figure 4A), in other cases increase of specific growth rate is obtained after several hours. The positive specific growth rate after bile shock in early exponential phase cells (Figure 4B) may be related to improving of colony forming ability of the culture rather than the multiplication.

Another aspect, which is not taken into account in this model yet, is the adhesion properties of the cells after acid/bile treatment. The better the tolerance to bile acid and the stronger the attachment to epithelial cells the better the possibilities of probiotics to colonize the intestine. Thus the determination of adhesion abilities should be further included into the model studies.

4. Conclusions

The single bioreactor model of intestinal tract presented in this work shows a good perspective for evaluation of probiotic properties of bacteria/food *in vitro*. Preliminary studies showed that both the physiological state of the cells as well as properties of the food matrix largely determine the survival of bacteria in gastrointestinal tract.

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ARTICLE II

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Single bioreactor gastrointestinal tract simulator for study of survival of probiotic bacteria

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Abstract The aim of the present study was to design an in vitro model system to evaluate the probiotic potential of food. A single bioreactor system—gastrointestinal tract simulator (GITS) was chosen for process simulation on account of its considerable simplicity compared to multi-vessel systems used in previous studies. The bioreactor was evaluated by studying the viability of four known probiotic bacteria (*Lactobacillus acidophilus* La-5, *Lactobacillus johnsonii* NCC 533, *Lactobacillus casei* strain Shirota, and *Lactobacillus rhamnosus* GG) as a function of their physiological state. *L. acidophilus* and *L. johnsonii* survived in GITS better when introduced at an early stationary or exponential phase compared to being previously stored for 2 weeks at 4 °C. These two species were more resistant to bile salts and survived better than *L. casei* and *L. rhamnosus* GG. The latter two species gave large losses (up to 6 log) in plate counts independent of growth state due to the bile. However, experiments with some commercial probiotic products containing Lb. GG bacteria showed much better survival compared with model food (modified deMan–Rogosa–Sharpe growth medium), thus demonstrating the influence of the food matrix on the viability of bacteria. The study demonstrated that GITS can be successfully used for evaluation of viability of probiotic bacteria and functionality of probiotic food.

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Keywords Probiotics · GIT model · Bile salts · Survival

Introduction

Functional foods including probiotic foods are now an established dietary trend. For example, in the Estonian food market, almost half of fermented milk products contain bacteria marketed as probiotic. Stability, viability in food, and survival in gastrointestinal (GI) tract are becoming main keywords in the probiotic food industry. Probiotic foods should maintain viability of probiotic bacteria not only during preparation and shelf life of products but also during transit through consumers' gastrointestinal tract to exert their beneficial effect. Acid and bile tolerances are two fundamental properties for the survival of a probiotic microorganism (Hyronimus et al. 2000; Prasad et al. 1998). To study the viability of bacteria in GI conditions, both in vivo and in vitro tests have been carried out. In in vivo models, the transit of *Lactobacillus rhamnosus* GG (Goldin et al. 1992), *Lactobacillus casei* Shirota (Spanhaak et al. 1998) and *Lactobacillus acidophilus* (Marteau et al. 1992) showed that with daily intake, 10^{10} to 10^{11} bacteria were detected 10^{6} up to $10^{7.7}$ cfu g^{-1} in the stools and persisted more than 7 days after the intake in the colon. In vivo studies are too complex to be used for routine evaluation of the probiotic properties of foods and to study factors determining bacterial viability during transit through human gut. There are also nomic pre-descriptions to follow when in vivo experiments are conducted. Therefore, there is a clear need for reliable in vitro tests preceding in vivo experiments (Mainville et al. 2005).

Several models designed to study the viability of probiotic bacteria in vitro are termed static models (Marteau et al. 1997), which involve growing or incubating cells at low pH or in a

medium supplemented with Oxgall bile, porcine bile, or bile salts (Jacobsen et al. 1999). They do not usually simulate the sequential stresses that are due to the continuously changing conditions to which ingested microorganisms are exposed during their passage in vivo (Marteau et al. 1997).

In order to obtain more reliable results that simulate human GI tract conditions, several dynamic in vitro gastrointestinal models have been developed. These are typically complex multi-compartmental simulators of which the oldest known is Simulator of the entire Human Gastrointestinal Microbial Ecosystem (SHIME; Molly et al. 1993), which consisted of five reactors simulating the duodenum/jejunum, ileum, caecum and ascending colon, transverse colon, and descending colon. A sixth reactor was later added to simulate the stomach. It has been used mainly for investigation of the interaction of probiotic bacteria with human intestinal microflora (Nollet et al. 1997) and the effects of probiotic bacteria and symbiotic products on the human gastrointestinal microbiota (Kontula et al. 1998; Alander et al. 1999a, b; Gmeiner et al. 2000).

A dynamic, computer-controlled model termed TNO (Netherlands Organisation for Applied Scientific Research) was developed by Minekus in 1995 (Minekus et al. 1995). The model consisted of four chambers to simulate in vivo conditions in the stomach and small intestine, such as the kinetics of pH, bile salt concentrations, and transit of the chyme. Jejunal and ileal compartments were equipped with hollow fiber devices that permit dialysis of the chyme. This model was used to study the survival of a single strain of each of the following species, *Bifidobacterium bifidum*, *L. acidophilus*, *Lactobacillus bulgaricus*, and *Streptococcus thermophilus* (Marteau et al. 1997). The survival of these lactic acid bacteria in the gastrointestinal model was investigated under two different conditions in the small intestine: simulation of physiological secretion of bile and low bile secretion. The survival was significantly different between the bacterial species: *Bifidobacterium* spp. and *L. acidophilus* were more resistant to model conditions than are *L. bulgaricus* and *S. thermophilus*. The dose response effect of bile on survival was found to be more significant than that of acid.

A dynamic model with two 1-l jacketed glass beakers representing the stomach and the *duodenum* (Mainville et al. 2005) was used to test the survival of bacteria (including *L. rhamnosus* GG and *Lactobacillus johnsonii* La-1) isolated from human and dairy products. In this model, bacteria were incorporated into kefir, making the in vivo conditions more realistic while studying the effects of acid and bile salts on bacterial survival. Results showed that, after transit, the stomach phase strain La-1 survived about three magnitudes better than the GG strain. Similarly, Mattila-Sandholm et al. (1999) showed that the TNO and SHIME model did not give promising results with regard to their predictive ability for selecting probiotics. In another study, Charteris et al. (1998) demonstrated limited acid tolerance of *L. paracasei* strains in vitro, but the same strains gave excellent results in vivo (Fonden et al. 2000).

As written above, the in vitro and in vivo experiments are quite controversial, thus necessitating the improvement of in vitro models. The aim of the present study was to design an in vitro single bioreactor model that simulates food transit through the upper part of the GI tract to enable us to evaluate the probiotic potential of food products. The single bioreactor GI model [gastrointestinal tract simulator (GITS)] was chosen for process simulation on account of its relative simplicity compared to multi-vessel systems used in previous studies. This single bioreactor is able to simulate the "culture" conditions of food during transit through the human upper GI tract. The probiotic potential of four well known probiotic strains in model foods and commercial products were evaluated to illustrate the effectiveness of our model.

Materials and methods

Microorganisms and media

Four probiotic strains were used in this study: *L. acidophilus* La-5 (Chr. Hansen, Denmark), *L. casei* Shirota isolated from probiotic product Yakult, *L. rhamnosus* GG (Valio) isolated from Gefilus daily dose drink, and *L. johnsonii* NCC 533 (kindly provided by Nestec). Single-cell colonies from the maintenance cultures on agar plates were used to inoculate the lactose-containing complex medium containing (g l^{-1}): lactose, 20; tryptone, 5; yeast extract, 2.5 (all from LAB M, UK); Tween 80, 1 (Difco Laboratories, USA); Na-citrate, 2.3; K_2HPO_4, 2 (Scharlau, Spain); $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot 5H_2O$, 0.04; pH 6.7± 0.2. The medium for the dilution of bile salts and growth in the intestinal tract reactor consisted of (g l^{-1}) lactose, 5; tryptone, 2.5; yeast extract, 1.25; Tween 80, 0.5; K_2HPO_4, 1. The pH of the dilution solution was set to 5.0 by 5 M HCl.

Viability assessment

Viability of bacteria was assessed by plate counts and microscopic counting. The numbers of colony-forming units (cfu) per milliliter were determined by counting suitable dilutions of the bacterial suspensions using a pour-plate method on deMan–Rogosa–Sharpe (MRS) medium-agar (LAB M, UK; de Man et al. 1960). Direct microscopic counting of bacteria was performed using LIVE/DEAD BacLight bacterial viability kit (L-7012; Molecular Probes, Eugene, OR, USA; Lloyd and Hayes 1995) and fluorescence microscope Olympus AH-2 equipped with Olympus camera DP-70.

Model "foods"

Bacteria were grown in 200 ml lactose-containing complex medium at 37 °C and harvested in exponential or stationary phase culture and used directly as "model food." To study the effect of storage, the stationary phase cultures were kept in a refrigerator at 4 °C for 14 days. Also, a number of probiotic food products containing *L. rhamnosus* GG were tested in GITS: Gefilus milk, Gefilus daily dose drink, Gefilus Emmental cheese, and Gefilus Peptidi Tutteli baby formula. The composition of these food products are listed in Table 1.

Milk or daily dose drink were directly injected into GITS; baby food was prepared according to instructions diluting 28.8 g of baby formula in 200 ml of water; cheese (20 g) was homogenized in 200 ml of sterile water directly before testing.

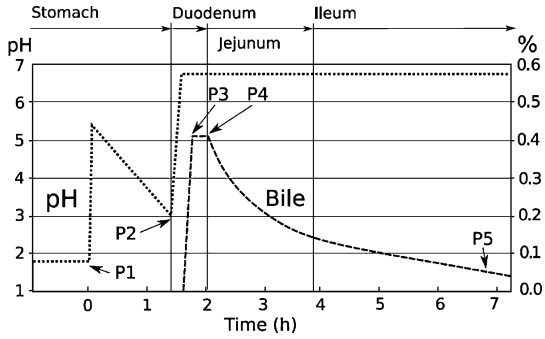

Fig. 1 Operation of GITS model. P1, P2, P3, P4, and P5 show the key sampling points corresponding to food before injection, entering into duodenum, immediately after bile addition, entering into jejunum, and in the end of ileum

GI tract simulator

The *GI tract simulator* consisted of a 1 l "Biobundle" fermentation vessel (Applikon, The Netherlands), an ADI 1030 biocontroller, and balances (Sartorius, Germany) connected to a PC equipped with the cultivation control program "BioXpert" (Applikon, The Netherlands). The fermenter was equipped with pO_2, pH and temperature sensors, variable and fixed speed pumps (Masterflex USA) to control the flow of HCl, $NaHCO_3$, bile acids, feeding medium, and the culture volume. The temperature in the bioreactor was kept at 37±0.1 °C, and anaerobic conditions were maintained by purging with nitrogen.

In order to simulate the survival conditions of probiotic bacteria during GI transit, 100 ml of 0.01 M HCl was added into the vessel to imitate the empty stomach, with pH value near 2. At the beginning of the experiment, 200 ml of food containing 10^7–10^9 cfu/ml of bacteria was pumped into the vessel (Fig. 1), followed by titration of fermenter's content back to 3.0 with 1 M HCl at a rate 20 mmol h^{-1} (reported as the maximum HCl secretion rate for human stomach (Ewe and Karbach 1990). The content of the bioreactor was then neutralized to pH 6.0 by adding 1 M $NaHCO_3$ at a rate of 4.5 ml min^{-1} to simulate the passing of food from the stomach to the duodenum. Depending on the buffering capacity of the food, the neutralization step took between 3 and 10 min. Thereafter, the concentration of bile salts in the fermenter was adjusted to 0.4% by adding 4% bile salts solution (Sigma-Aldrich) at rate of 4 ml min^{-1} during a period of 10 min. Finally, the bioreactor content was diluted (D=0.4 h^{-1}) with dilution medium to simulate the absorption of bile acids and food components in the *jejunum* and *ileum*. During the dilution phase, the pH of the culture was kept at pH 6.5 by $NaHCO_3$ titration, while the culture volume, V_D was maintained by controlling the weight of the vessel. A dilution rate of D=0.4 h^{-1} was selected so that at the seventh hour, corresponding to the time food reaches the large intestine, the bile salt concentration was diluted ten times.

Characterization of survival in GITS

The survival of bacteria (SUR_t) in our Gastro-Intestinal Tract Simulator for the stomach and duodenum phase (up to point P4 in Fig. 1) was calculated as:

$$SUR_t = CFU_t \times V_t/(CFU_F \times V_F) \quad (1)$$

where CFU_t is the instantaneous concentration of colony-forming bacteria (cfu ml^{-1}), V_t is the volume of the culture

Table 1 Composition of commercial products according to label used in this study

Food	Description according to label	Fat g/100 g	Protein g/100 g	Carbohydrate g/100 g
Gefilus milk		1.5	3.2	4.8
Gefilus daily dose drink	Pasteurized milk (72%), water, galactooligosacharides, pectin, vanilla, vanillin, aspartame, acesulfame-K, *Lactobacillus* GG starter	0.1	2.6	7.5
Gefilus Emmental cheese	Milk, starter, salt (0.4%), acidifier	30	26	0
Gefilus Peptidi Tutteli baby formula	Whey protein hydrolysate, palm-, coconut-, rape oil, maltodextrine, modified starch (E1450), minerals, vitamins, acidifier—citric acid	3.5	1.7	7.0

Table 2 Mean decrease (logSUR) and standard deviation (SD) of the survival of probiotic cells at different times in our GITS model

	L. acidophilus La-5 (n=7)		*L. johnsonii* NC533 (n=8)		*L. casei* strain Shirota (n=6)		*L. rhamnosus* GG (n=6)	
	logSUR	SD	logSUR	SD	logSUR	SD	logSUR	SD
Point 1 (food)	0		0		0		0	
Point 2 (after acid treatment)	−0.42	±0.4	−0.30	±0.25	−0.11	±0.08	−0.13	±0.21
Point 3 (0.4% bile)	−0.78	±0.49	−1.05	±1.09	−4.67	±1.28	−5.93	±1.73
Point 4 (start of dilution)	−0.91	±0.67	−1.21	±0.56	−4.12	±1.04	−5.05	±0.92
Point 5 (end)	−0.56	±0.94	−1.12	±0.56	−3.60	±0.80	−4.02	±0.84

n Total number of GITS simulations

(ml) in the bioreactor vessel at time point t, CFU_F is the concentration of bacteria in food (cfu ml^{-1}), and V_F is the volume of the food (ml) injected into the vessel. For jejunum and ileum (dilution) GITS phases (after point P4 in Fig. 1), the CFU_t number was corrected by a dilution factor $e^{D\tau}$:

$$SUR'_t = e^{D\tau} \times CFU_t \times V_t / CFU_F \times V_F \quad (2)$$

where τ is the running time after start of dilution and D is the dilution rate.

Results

Our model of the human upper gastrointestinal tract was validated in a series of experiments on the four above-mentioned strains in which the influence of the physiological state of bacteria on their viability was assessed using "model food." The results are summarized in Tables 2 and 3. Results show that using MRS matrix *L. acidophilus* La-5 and *L. johnsonii* NC533 survive in GITS model significantly (100–1,000 times) better than *L. casei* strain Shirota and *L. rhamnosus* GG. Although *L. casei* and *L. rhamnosus* GG were even somewhat more resistant to the stomach-phase acid treatment than *L. acidophilus* and *L. johnsonii*, the colony-forming cell number imminently dropped by three magnitudes after addition of bile, while in the case of *L. acidophilus* and *L. johnsonii*, the decrease was only about one magnitude at sampling point P3 in Fig. 1. Surprisingly, the cfu count (as well as Life/Dead cell ratio determined by microscopy of bacteria stained with LIVE/DEAD BacLight bacterial viability kit) increased for *L. casei* and *L. rhamnosus* during bile incubation (duodenum phase) and bile absorption (during jejunum/ileum phase) to an extent that cannot be explained by growth alone. Such an increase was not observed for each of *L. acidophilus* and *L. johnsonii*. Statistically relevant differences in behavior of *L. acidophilus* and *L. johnsonii* as well as for *L. casei* and *L. rhamnosus* could not be observed. Therefore, to analyze the effect of physiological state, the bacteria were grouped into two categories for better statistical analysis. Such a division of lactic acid bacteria can be justified by their phylogenetic tree, according to which *L. casei* and *L. rhamnosus* species as well as *L. acidophilus* and *L. johnsonii* species are closely related (Siezen et al. 2004).

All of the bacteria studied were found to be more resistant to low pH stress when introduced at an early stationary growth phase; however, the observed differences in survival were marginal compared with bacteria introduced while in an exponential growth phase. Compared with stationary phase cells, storage at 4 °C for 2 weeks significantly decreased the ability of bacteria from *L.*

Table 3 The effect of the physiological state on survival (logSUR) in our GITS model to the La-5/NC533 and Shirota/GG groups

	La-5/NC533 group		Shirota/GG group	
	logSUR	SD	logSUR	SD
Exponential growth phase		n=6		n=4
Point 1 (food)	0		0	
Point 2 (after acid treatment)	−0.29	±0.30	−0.18	±0.17
Point 3 (0.4% bile)	−0.49	±0.21	−6.10	±1.34
Point 4 (start of dilution)	−0.49	±0.29	−4.88	±1.03
Point 5 (end)	−0.32	±0.57	−3.80	±0.43
Stationary growth phase		n=5		n=4
Point 1 (food)				
Point 2 (after acid treatment)	−0.26	±0.11	−0.03	±0.05
Point 3 (0.4% bile)	−0.74	±0.33	−5.18	±1.99
Point 4 (start of dilution)	−0.90	±0.38	−4.18	±0.77
Point 5 (end)	−0.66	±0.78	−2.88	±0.83
Bacteria stored 2 weeks at 4 °C		n=4		n=4
Point 1 (food)				
Point 2 (after acid treatment)	−0.60	±0.61	−0.15	±0.19
Point 3 (0.4% bile)	−1.60	±1.20	−4.63	±0.96
Point 4 (start of dilution)	−1.88	±0.87	−4.70	±1.35
Point 5 (end)	−1.58	±0.69	−4.15	±0.93

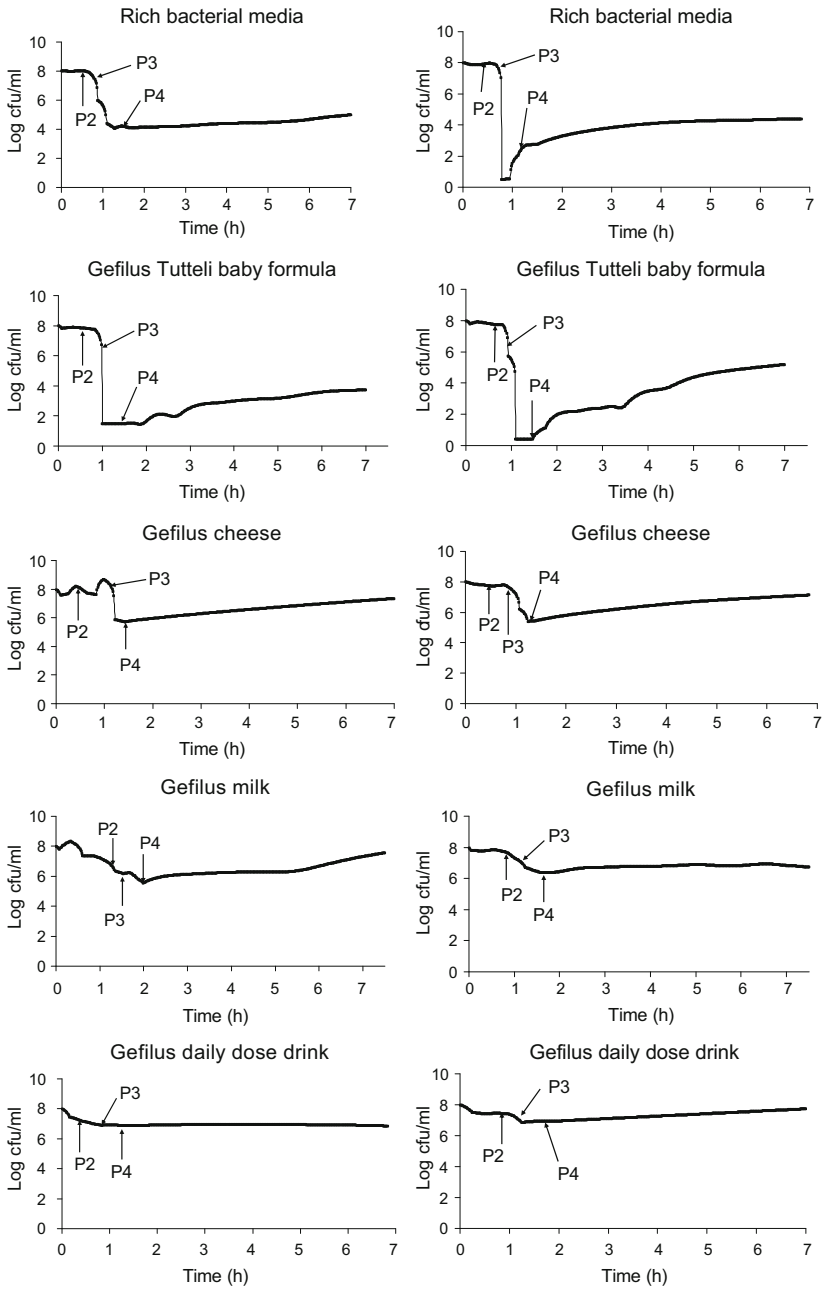

Fig. 2 Survival profiles of *L. rhamnosus* GG in different food matrices in our human GI tract simulator. P2, P3, and P4 designate the same sampling points as in Fig. 1

acidophilus and *L. johnsonii* strains to survive stomach stress. The influence of bile was clearly group-specific as shown above, with *L. acidophilus* La-5/*L. johnsonii* NC533 being over three magnitudes less sensitive to the bile phase in our GI model than Shirota/*L. rhamnosus* GG group.

An impact of physiological state was observed in the case of *L. johnsonii* NC533/*L. acidophilus* La-5 group; the exponential and stationary phase cells resisted the bile stage significantly better than the cells stored for 2 weeks. The *L. casei* Shirota and *L. rhamnosus* GG group showed a

significant decrease in plate count irrespective of their physiological state.

However, in some commercial food products, *L. rhamnosus* GG showed surprisingly good survival (Fig. 2), while in an MRS matrix and Tuttely baby formula, the survival in our GITS model was less than 0.1%, contrasting that of Gefilus cheese and milk where survival remained between 10% and 100%.

Results indicate that, similar to model foods, acid treatment reduced the number of cultivable bacteria in all commercial foods tested by tenfold at most. Significant loss of colony formation ability was observed after addition of bile in baby formula and model food; however, the effect of bile on survival was surprisingly low for the daily dose milk. All this demonstrates the great impact on product efficacy of both the food matrix and probably also the technology of incorporating probiotic strains into the product.

Discussion

Although resistance to human gastric transit of probiotics can be demonstrated in vivo by recording number of probiotics in feces (Alander et al. 1999a, b), the need for a reliable in vitro method which closely simulates in vivo gastric transit is obvious. Satisfactory method has still not been defined (Kos et al 2000). Below, we analyze the use of a tightly controlled fermentation system for simulation of survival conditions met by probiotic bacteria during food transit through the upper part of human GI tract.

The main obstacle of building up the model system for simulating the human gastrointestinal tract was rather insufficient knowledge about the digestion process in vivo than technical realization. Therefore, selecting the algorithms and input parameters was a critical factor in simulation of survival conditions. The input parameters used in GITS model for the present study were drawn up for an initially empty stomach supposing that it contained 100 ml of 0.01 N HCl (Robertson 2005). However, the initial conditions for the GITS model can be easily changed and digestion enzymes (pepsin, trypsin, etc.) added if necessary for better simulation of the individual peculiarities of food consumption habits and effects of food composition.

It was supposed, in building up the GITS model, that the food does not enter the duodenum before the contents of the stomach after injection of food is acidified back to pH 3.0. It takes time as the capacity of acid secretion in the stomach is limited to 20 mmol h^{-1} (Ewe and Karbach 1990). Thus, the model takes into account the buffering capacity of food as well as physical state of food. Solid food can be directly injected into vessel, and by setting up stirrer configuration, the homogenization in GI can be simulated and the effect of solid matrix on probiotic survival studied. The next step is the neutralization, wherein all "food" is simultaneously neutralized. That is somewhat different from the physiological situation in which food continuously enters into the duodenum, and thus the true holding time of food in the stomach is longer than in our model. This step could be better simulated using a separate vessel for the stomach such as has been described by Mainville et al. (2005); however, in light of results obtained in this work, it might not be necessary.

Our results demonstrate that, in respect of probiotic survival, the bile treatment of food is much more important than acid treatment. The bile was added to the food during 10 min to such an extent that it achieved a concentration of 0.4% and was further held at that concentration for 30 min. Not all foods induce the bile excretion to a similar extent (Kristoffersen et al. 2007). In addition, bile secretion depends on the individual and also on age (i.e., bile secretion in babies may remain very low). However, different values of bile secretion can be easily taken into account in the GITS model. The type of bile is also important. The use of different types of bile, broth, etc. in similar studies makes it impossible to directly compare results of the bile tolerance of bacteria even if the same strains are used (Begley et al. 2005). In the present work, bile salts (Sigma-Aldrich, a mixture of sodium cholate and sodium deoxycholate) were used.

One of the biggest problems is to simulate the adsorption of food and bile in the jejunum and ileum. In the small intestine, due to the small number of viable microorgamisms, the adsorption of food by bacteria is negligible, and the main part of nutrients is taken up by epithelial cells. In previous models, the process of digestion/adsorption was simulated by dialysis (Marteau et al. 1997). We used dilution in our GITS model, as this method is technically simpler, and substrate concentrations can be more accurately controlled by adjusting the composition of dilution media, resulting in better simulation than the use of dialysis. In this work, we simply used MRS as the dilution medium supporting the growth of probiotics during whole small intestine transit. Although in the dilution phase of the simulation bacteria in the GITS model are washed out, this does not affect the survival (see equations 1 and 2) as the concentration of bacteria itself has little effect on death or growth at substrate surplus. The factor which has a stronger influence on the survival profile in the small intestine is the adhesion of probiotics on the epithelium cells of the small intestine, which can decrease the rate of probiotic transit through small intestine compared to food. In principle, the retention of probiotics in the GI tract can be taken into account in our GITS model by changing the dilution profile.

Survival profiles were established according to plate count data. However, not all living cells are able to form

colonies. The apparent specific growth rate during dilution of bile was much higher than the maximum for corresponding strains under ideal conditions. Also, while using the LIVE/DEAD BacLight bacterial viability test, it was observed that DEAD (red) cells were turning green soon after bile exposure. Also, adhesion to epithelium cells might improve the colony-forming ability. Therefore, for further experiments, optimization of sample conditioning may be required prior to microbial counting or plating out.

Our results using the model demonstrated that the same probiotics in different food products behave very differently: in an MRS matrix and Tuttely baby formula, the survival of *L. rhamnosus* GG in our GITS model was less than 0.1%, contrasting that of Gefilus cheese and milk, where survival remained between 10% and 100%. This, together with variations in bile excretion of individuals and with the food, can at least partly explain the controversial result of in vivo and in vitro experiments reported in the literature (Charteris et al. 1998; Mattila-Sandholm et al. 1999; Fonden et al. 2000).

Conclusion

This study shows that the single bioreactor GI tract simulator provides a good platform for simulation of survival conditions of probiotics in the human GI. It can be used to determine the survival profiles of probiotics in different food matrices and provides the ability to take into account individual peculiarities in human GI processes.

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ARTICLE III

Sumeri, I., Arike, L., Stekolštšikova, J., Uusna, R., Adamberg, S., Adamberg, K., Paalme, T. (2010).

Effect of stress pretreatment on survival of probiotic bacteria in gastrointestinal tract simulator

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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Effect of stress pretreatment on survival of probiotic bacteria in gastrointestinal tract simulator

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Abstract The effect of stress pretreatment on survival of probiotic *Lactobacillus acidophilus* La-5, *Lactobacillus rhamnosus* GG, and *Lactobacillus fermentum* ME-3 cultures was investigated in the single bioreactor gastrointestinal tract simulator (GITS). The cultures were pregrown in pH-auxostat, subjected to temperature, acid, or bile stress treatment, fast frozen in liquid nitrogen (LN_2), and tested for survival in GITS. After LN_2 freezing the colony forming ability of *L. rhamnosus* GG and *L. fermentum* ME-3 nonstressed and stressed cells was well retained (average survival of 75.4±18.3% and 88.0±7.2%, respectively). *L. acidophilus* La-5 strain showed good survival of auxostat nonstressed cells after fast freezing (94.2±15.0), however the survival of stress pretreated cells was considerably lower (30.8±8.5%). All LN_2 frozen auxostat cultures survived well in the acid phase of the GIT simulation (survival 81±21%); however, after the bile phase, the colony formation ability of *L. acidophilus* La-5, *L. rhamnosus* GG, and *L. fermentum* ME-3 decreased by approximately 1.4±0.2, 3.8±0.3, and 3.5±1.2 logarithmic units, respectively. No statistically relevant positive effect of stress pretreatments on survival of LN_2 frozen *L. acidophilus* La-5, *L. rhamnosus* GG, and *L. fermentum* ME-3 in GITS was observed.

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Keywords Probiotics · pH-auxostat · GIT simulator · Stress response · Survival

Introduction

During food processing, probiotic microorganisms are exposed to multiple physiological stresses including exposure to cold, substrate limitation, low pH, absence or presence of oxygen, suboptimal water activities, etc. The changes of those parameters cause stress and initiate stress response pathways (van de Guchte et al. 2002; De Angelis and Gobbetti 2004). Culture conditions that may be considered "stress-free," are obtained by long-term growth of at least four generations of microorganisms in optimal ("neutral") conditions, for example during exponential growth in batch or auxostat cultures (Kasemets et al. 2003). Processing conditions may also affect their survival during storage and later function in the consumers' gastrointestinal tract.

In some circumstances, stressful conditions before consumption may have a positive effect on the viability and efficacy of probiotics, especially if combined with supportive food matrices.

It is well established that lactic acid bacteria (LAB), like other bacteria, evolve stress-sensing systems and defenses against stress, which allow them to withstand harsh conditions and sudden environmental changes (van de Guchte et al. 2002) similar to those occurring in the gastrointestinal tract. Thus, the induction of a stress response via exposure of the growing culture to a sublethal stress before use in food processing or gastric transit has been suggested to enhance viability (Corcoran et al. 2008). Stress responses of LAB (lactobacilli and bifidobacteria) have recently attracted increasing research interest (Saarela

et al. 2004) especially due to their extensive use as probiotics in food industry.

The technological suitability of probiotic strains is critical to their exploitation in the development of effective functional foods (Corcoran et al. 2008). The ability of microorganisms to survive and grow depends largely on their capacity to adapt to changing environments. Tolerance to the harsh conditions of the gastrointestinal tract can be derived either from an intrinsic resistance or to an adaptive response (Segal and Ron 1998). The latter may include various modifications, such as morphological changes (Lange and Hengge-Aronis 1991, Bron et al. 2004) or induction of proteins (Volker et al. 1992), appearing after exposure to moderate stresses and the development of cross-resistance to various stresses.

Saarela et al. (2004) reported that sublethal treatments by acid and temperature shocks of stationary phase probiotic lactobacilli improved the viability and enhanced their further survival during lethal treatments. Likewise, the acid tolerance of *Bifidobacterium lactis* (at pH 3.5 in synthetic gastric fluids) increased significantly after decreasing the growth medium pH from 6.0 to 5.2 and under conditions of starvation (Park et al., 1995). Adaptive responses to bile were observed in *Enterococcus faecalis* (Flahaut et al. 1996), *Lactobacillus acidophilus* (Kim et al. 2001), *Propionibacterium freudenreichii* (Leverrier et al. 2003), and *Lactococcus lactis* ssp. *lactis* but not in the three strains of *Lactococcus lactis* ssp. *cremoris* tested (Kim et al. 1999). In *E. faecalis*, bile adaptation was shown to increase the tolerance not only to bile salts but also to heat shock. Inversely, heat adaptation protected it from a lethal bile challenge. In contrast, bile adaptation of *L. acidophilus* increased its resistance to heat stress but not vice versa (Kim et al. 2001). The stress response can at least be specific to bacterial species (Duwat et al. 2000; Rince et al. 2000) if not to every strain. It is thus difficult to extrapolate a general behavior common to all microorganisms.

To study the effects of sublethal pretreatment of probiotics on their viability in the gastrointestinal tract, we used a single reactor gastrointestinal tract model simulator (GITS) (Sumeri et al. 2008), which simulates the environment during the passage of food through the stomach and upper intestinal tract. Ritter et al. (2009) compared the survival of *Lactobacillus gasseri* K7 in a similar simulator with in vivo measurements and obtained results that confirmed the reliability of this model.

In our previous studies on the survival of probiotic bacteria in vitro using GITS, we found that survival was very low in some cases and that survival might depend significantly on the physiological state of the culture. In the current study, we tested the possible technological implications of stress pretreatment of *L. acidophilus* La-5, *Lactobacillus rhamnosus* GG, and *Lactobacillus fermentum* ME-3 using GIT simulations with the LN_2 fast frozen pH-auxostat (Adamberg et al. 2003)-derived stressed cells of these cultures.

Materials and methods

Microorganisms and media

Three strains of probiotic lactobacilli were used in this study: *L. acidophilus* La-5 (Chr. Hansen, Denmark), *L. rhamnosus* GG (ATCC 53103) isolated from Gefilus daily dose drink (Valio Ltd.), and *L. fermentum* ME-3 (DSM 14241) isolated from Hellus kefir (Tere AS, Estonia). De Mann–Rogosa–Sharp (MRS) agar medium (LAB M, UK) was used for isolation and storage of the bacteria.

pH-auxostat cultivation

Bacterial cultures for freezing and GITS experiments were prepared under controlled conditions using pH-auxostat technology. The cultivation system consisted of an Applikon 1 l fermenter; controlled by an ADI 1030 biocontroller ("Applikon," The Netherlands) and cultivation control program "BioXpert" ("Applikon"). The system was equipped with pH, pO_2, and temperature sensors. Two variable speed pumps (feeding and culture take-out) were controlled by control algorithms similar to those described by Adamberg et al. (2003). MRS medium (LAB M, UK) adjusted to pH 7.0 with 2 N NaOH was used as feed. The anaerobic cultivation was performed at 37 °C and pH=6.0 with a culture volume of 900 ml. After passing through at least five culture volumes, the culture was defined to be in steady state and samples of 200 ml were gathered from the fermenter (Fig. 1) for further stress treatment and GITS modeling.

Stress treatments

In order to prevent decrease of pH during incubation, stress treatment was carried out at controlled acidity conditions in pH-stat. The pH-auxostat culture (200 ml) was transferred into another reactor containing 200 ml of fresh MRS medium, adjusted to a pH of 6.0 for bile and temperature treatments, and 4.5 or 3.5 for acid treatments. For bile treatment, a 20% solution of bile salts (Sigma Aldrich) was added to achieve final bile salt concentrations of 0.1%, 0.04%, or 0.02%. The cell suspensions were incubated at 37 °C for 2 h in all experiments except those that made use of a 43 °C temperature stress. After incubation, stress treated cells were harvested by centrifugation at 4 °C, 5,800 rpm for 15 min and resuspended in the same volume (400 ml) of fresh MRS medium at pH 6.3±0.1 as a "model food," which was further frozen in liquid nitrogen as beads.

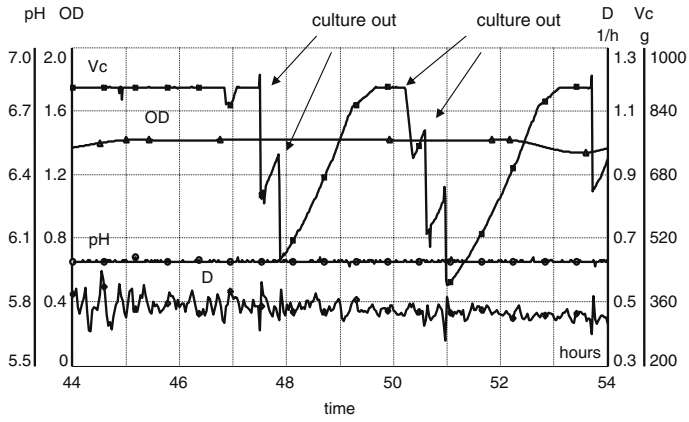

Fig. 1 pH-auxostat auxostat culture of *L. acidophilus* La-5. *Vc* current weight of culture in fermenter, *D* current dilution rate (per hour), *pH* the pH value in fermenter, *OD* optical density in fermentor. The *arrows* show *time points* when about 200 ml of culture was transferred into other vessels for subsequent stress pretreatments of GITS testing

As a control, 100 ml of nontreated cells from the auxostat culture was mixed with 100 ml fresh MRS medium and immediately subjected to the GITS experiment.

Preparation of frozen beads

The bacterial cell suspensions prepared as described above were slowly dripped into liquid nitrogen using a peristaltic pump. The beads (1–2 mm size) were harvested from liquid nitrogen (LN_2) and stored at −40 °C. For each GITS experiment, 100 g of frozen MRS beads were thawed at room temperature and mixed with 100 ml of MRS media.

Gastrointestinal tract simulator

The gastrointestinal tract simulator set up and control algorithm was the same as that used by Sumeri et al. (2008). Briefly, the stomach step of this simulator is characterized by a pH profile changing from empty stomach pH 2.0 up to a level depending on the food buffering capacity followed by a decrease in the pH back to 3.0 using 1 N HCl. In the duodenum control step, the contents of the reactor is neutralized by 1 M $NaHCO_3$ to pH 6.0 and 4% bile salt solution is added to obtain a final concentration of 0.4%, which is retrained for 30 min. Finally, the bioreactor content is diluted (D=0.4 h^{-1}) with dilution medium (lactose, 5 g l^{-1}; tryptone, 2.5 g l^{-1}; yeast extract, 1.25 g l^{-1}; Tween 80, 0.5 g l^{-1}; K_2HPO_4, 1 g l^{-1}; pH was set to 5.0 by 5 M HCl) to simulate the absorption of bile acids and food components in the jejunum and ileum.

Viability assessment

Viability of bacteria was assessed by colony forming ability using the plate count method on MRS agar (LAB M, UK). The viable bacterial counts were determined from the fresh pH-auxostat culture, culture before and after stress treatment, before and after freezing, and at the several points during the GIT simulation: food before injection, at the end of stomach phase of GITS, after the duodenum step, i.e., incubation with 0.4% bile solution 30 min (see "Results" section).

Results

To investigate the effect of different stress pretreatments on the survival of probiotic lactobacilli under gastrointestinal conditions, we first obtained steady-state pH-auxostat (pH= 6.0) cultures of *L. acidophilus* La-5 (Fig. 1), *L. rhamnosus* GG, and *L. fermentum* ME-3. Two auxostat cultivations were carried out for *L. acidophilus* La-5 and *L. rhamnosus* GG and three for *L. fermentum* ME-3. After achieving steady state, the cell density and specific growth rate remained constant in all experiments, while the viable cell count and specific growth rate for the same strains in parallel cultivations varied slightly. The biomass concentrations (log colony forming units per milliliter) and specific growth rates (per hour) of these cultures were as follows: 7.5±0.3 and 0.63±0.18 for *L. acidophilus* La-5; 8.1±0.24 and 0.9±0.14 *L. rhamnosus* GG; and 8.36±0.25 and 1.2± 0.14 for *L. fermentum* ME-3.

The stress treatments of steady-state pH-auxostat cultures were performed at constant pH (in pH-stat). During stress treatments, significant bacterial growth was observed with the exceptions of pH 4.5 and pH 3.5 induced stress for *L. rhamnosus* GG and pH 3.5 or 0.1% bile stress for *L. fermentum* ME-3.

Survival of probiotics during LN_2 freezing and inside GITS was affected by the previous stress pretreatments (*T*, low pH, or presence of bile) of the culture. The results of the effect of stress treatment and subsequent freezing on the viability of pH-auxostat-derived cultures are summarized in

Table 1. Average survival after LN_2 freezing of stress untreated auxostat cells of three strains studied was 91.5±3.4%. LN_2 freezing of stressed cultures of *L. acidophilus* decreased the survival of bacteria significantly; the average survival after freezing prestressed cells was 30.8±8.5%. Average survival of stress-treated *L. rhamnosus* GG and stress-treated *L. fermentum* ME-3 was 73.3±19.2% and 87.3±7.5%, respectively. Gradual change in viable counts of LN_2 frozen cells of all studied strains was observed depending on amount of bile or acid applied during stress treatment, i.e., the treatment with 0.02% bile or at pH 4.5 caused less decrease in viability than 0.04% and 0.1% bile or pH 3.5, respectively.

A series of gastrointestinal tract simulator experiments were performed comparatively with stressed and non-stressed LN_2 frozen cells of *L. acidophilus* La-5, *L. rhamnosus* GG, and *L. fermentum* ME-3. Since the focus of this study is on the effect of sublethal stress treatment to improve acid and bile resistance of probiotic bacteria in gastrointestinal tract, the final bile dilution phase of GITS was, in most experiments, omitted. Results of survival of nontreated and treated *L. acidophilus* La-5, *L. rhamnosus* GG, and *L. fermentum* ME-3 during GIT transit are shown on Fig. 2.

L. acidophilus La-5 was found to be the most resistant strain to simulated gastrointestinal conditions. Viability of La-5 cells derived directly from auxostat culture decreased by 0.36±0.09 log units after acid treatment and by 1.5±0.4 log units after bile addition. The frozen nonstressed *L. acididophilus* La-5 cells showed a similar decrease in viability: 0.49±0.2 and 1.25±0.35 log units, respectively. *L. acidophilus* La-5 showed slightly better acid tolerance after treatment at pH 3.5 compared to nontreated cells in GITS simulator (decrease of viable counts was 0.18±0.13 log units). In the stomach phase, average loss of colony forming ability of cells treated with bile, temperature 43 °C or pH 4.5 was 0.32±0.04 log units, was not found to differ significantly from the survival rate of auxostat cells and that of frozen nonstressed cells. *L. acidophilus* La-5 cells pretreated with 0.02% bile and frozen in LN_2 beads improved the tolerance to bile in GITS compared to cells without freezing and stress pretreatment (loss of colony forming units was 0.63±0.40 log units compared to that of 1.25±0.35 log units without pretreatment). Pretreatment with 0.1% bile or pH 4.5 or pH 3.5, (decrease of viable counts 1.05±0.25, 0.97±0.15, and 1.05±0.36 log units, respectively) only slightly improved if at all the survival after the bile phase in GITS.

The viability of *L. rhamnosus* GG auxostat cells decreased after the stomach phase in GITS to 0.39±0.38 and after bile addition 3.96±1.48 log units. The frozen nonstressed *L. rhamnosus* GG cells showed a similar decrease in viability of 0.14±0.33 and 3.61±2.11 log units, respectively.

In general, stress treatment of *L. rhamnosus* GG bacteria did not improve their acid tolerance in GITS model. Only bacteria incubated at pH 3.5 showed better resistance to stomach acid (decrease of viable cells was 0.09±0.31 log units). Treatment with 0.02%, 0.04%, or 0.1% bile solution rather impaired tolerance of *L. rhamnosus* GG to acid in GIT simulator (decrease of colony forming cells of 0.55±0.31, 0.61±0.46, 0.64±0.78 log units). Survival of bacteria incubated at pH 4.5 was comparable to frozen nonstressed cells, i.e., loss of colony forming units of 0.19±0.04 log and temperature stressed *L. rhamnosus* GG showed similar results with cells from auxostat culture (i.e., decrease of viable bacteria of 0.34±0.17 log units). Also the bile pretreatment of *L. rhamnosus* GG did not increase its tolerance to simulated digestive stresses. Survival of 0.02% bile-treated cells in GITS was comparable to that of the nontreated frozen cells (loss of colony forming cells of 3.73±0.78). Treatment with 0.1% and 0.04% bile solution decreased the colony forming unit number by 4.02±0.94 and 5.25±0.55 log units, respectively. Viability of pH 3.5 and temperature-treated GG bacteria decreased by 4.22±1.33 and 4.73±1.39 log units, respectively. Only pH 4.5 treatments somewhat improved survival in GITS compared to untreated cells, i.e., loss of colony forming units of 2.41±1.36 log versus 3.61±2.11 log.

Experiments with *L. fermentum* ME-3 also showed more negative than positive influence of stress pretreatments and freezing on survival in GITS irrespective of the imposed stress condition. The untreated pH-auxostat culture showed the highest survival, i.e., decrease of colony forming cells in GITS after acid addition was 0.19±0.16 log units and after bile addition 2.69±1.73. The number of frozen

Table 1 Survival % of *Lb acidophilus* La-5, *Lb. rhamnosus* GG and *Lb. fermentum* ME-3 during stress treatment and LN_2 freezing

Stress treatment	*L. acidophilus* (%)	*L. rhamnosus* (%)	*L. fermentum* (%)
LN_2 frozen pH-auxostat cells	94.2±15.0	87.7±29.9	92.5±21.8
2 h 0.02% bile-stressed LN_2 frozen cells	43.3±32.8	91.1±9.3	89.8±17.3
2 h 0.04% bile-stressed LN_2 frozen cells	34.2±20.7	66.7±11.3	84.6±20.3
2 h 0.1% bile-stressed LN_2 frozen cells	21.7±8.5	38.9±14.1	77.8±12.0
2 h pH 4.5-stressed LN_2 frozen cells	27.3±6.5	81.8±23.6	97.7±6.3
2 h pH 3.5-stressed LN_2 frozen cells	22.2±12.9	73.3±23.1	80.8±15.4
2 h T=43 °C-stressed LN_2 frozen cells	36.1±18.6	88.1±11.8	92.8±26.9

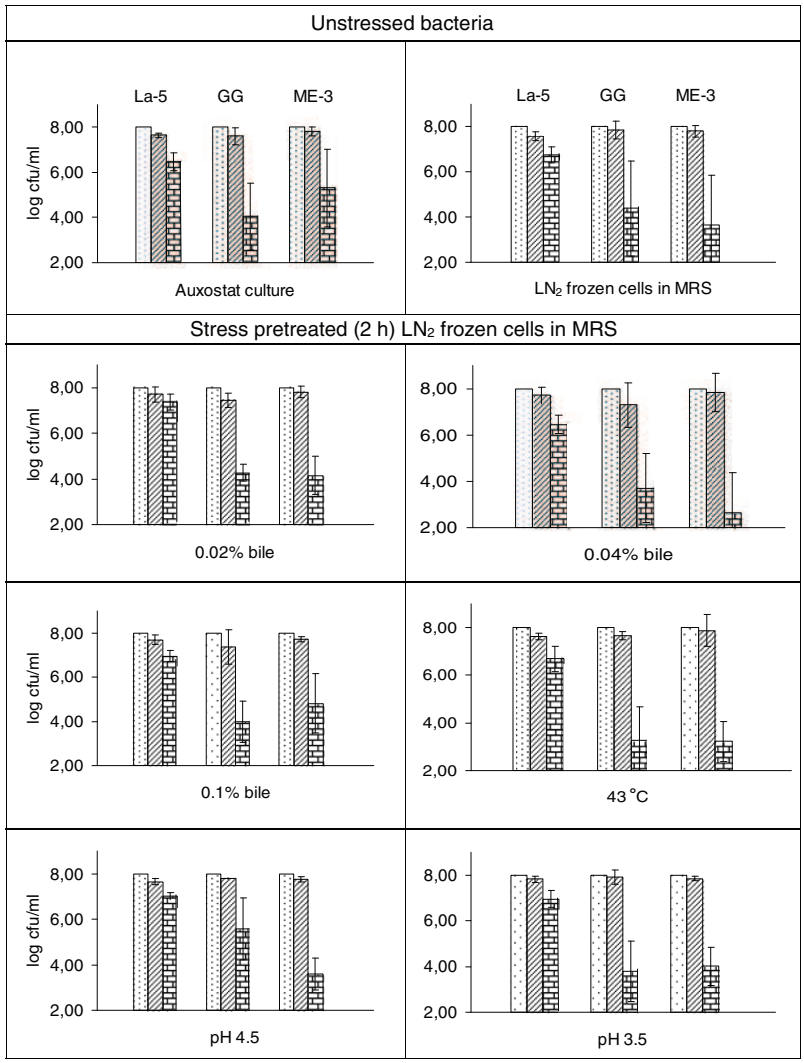

Fig. 2 Survival (normalized data) of stress pretreated pH-auxostat cells of *L. acidophilus* La-5, *L. rhamnosus* GG, and *L. fermentum* ME-3 in gastrointestinal tractsimulator (GITS): *Dots* indicate initial count. *Stripes* indicate counts after acid addition. *Bricks* indicate counts after bile addition

bacteria without pretreatment decreased by 0.19±0.25 log units after stomach step of GITS and by 4.08±1.87 log units after the duodenum step. Sublethal treatment of *L. fermentum* ME-3 did not alter survival of cells in the stomach phase of GITS (average loss of colony forming units was 0.19±0.19 log). None of these stresses improved the resistance of ME-3 to bile in GITS. Treatment with 0.1% bile had a less negative effect than other sublethal treatments, which resulted in a decrease of colony forming units by 3.19±1.34 log units in the GITS duodenum phase. Incubation at pH 4.5, 3.5, and with 0.02% bile gave similar survival results to those of frozen nonstressed bacteria (decrease of colony forming cells was 4.41±0.71, 3.99±0.85, and 3.85±0.83 log units, respectively). Pretreatment with 0.04% bile solution as well as that of T=43 °C had a clear negative effect on survival (decrease 5.36±0.83 and 4.77±2.68 log units).

Discussion

Survival of microorganisms during processing and in the gastrointestinal tract depends on the physiological state of the culture, thus the production and maintenance of the bacteria in the same physiological state is crucial for comparative survival testing.

The probiotic strains for our experiments *L. acidophilus* LA-5, *L. rhamnosus* GG, and *L. fermentum* ME-3 were produced in continuous steady-state culture pH-auxostat at pH 6.0 (Fig. 1). Although auxostat fermentation may be more difficult to operate under industrial conditions, this technology is worth the investigation and could be used to produce cells with different physiologies and to apply stresses under well-controlled conditions (Lacroix and Yildirim 2007).

The specific growth rates and steady-state biomass concentrations of the same strain varied slightly between different cultivations. These variations might be explained by using different slots of yeast extract influencing the buffering capacity and substrate profiles of the media. Nevertheless, pH-auxostat cultures obtained can be considered to be in a nonstressed state since this method sustains the exponential growth at the maximum growth rate of the culture.

However, the growth at optimal environmental conditions might not always give the maximum resistance to stress conditions (for example in GI tract), possibly because of an absence or low concentrations of stress-induced proteins (e.g., against bile and low pH stress). It is generally recognized that cell growth conditions in nature are often suboptimal compared to controlled conditions provided in the laboratory. Adaptation to different stresses often requires protein synthesis, indicating that stress conditions induce expression of newly synthesized genes (Rallu et al. 1996). The viability/resistance of the cells could be improved by various stress treatments.

Fast freezing in liquid nitrogen fixes the structural properties of cells to resist the stress and was considered as reliable method for preparation of comparable starting samples to study the effects of stress pretreatment on survival of lactobacilli in GIT model. It enables also to perform sufficient number of GITS experiments with the cells derived from the same culture.

The LN_2 frozen MRS beads of *L. acidophilus*, *L. rhamnosus*, and *L. fermentum* were used as model products. The viability of pH-auxostat grown cells in GITS did not decrease after LN_2 freezing and subsequent storage at −40 °C. This was confirmed by similar survival in GITS model of frozen and unfrozen and non-stress pretreated cells (see Fig. 2). So we consider the conclusions drawn from experiments with LN_2 beads to be valid also for the corresponding unfrozen cultures or products.

Bile and acid stresses applied to auxostat cultures of *L. rhamnosus* and *L. fermentum* before LN_2 freezing did not show a statistically relevant positive effect on survival in the GITS. The previous study by Saarela et al. (2004) also demonstrated that sublethal pretreatment at pH 4.0 had no effect on the survival of *L. rhamnosus* E800 cells during the lethal bile treatment.

The resistance to bile stress of *L. acidophilus* beads was a few log units better than that of *L. rhamnosus* and *L. fermentum*, and we observed some improvement in bile tolerance of stress pretreated cells in GITS. Kim et al. (2001) reported that treatment of LA1-1 (CRC Culture Collection) with sublethal bile concentration of 0.05% improved the tolerance against lethal concentration 0.5% of bile 100 times. However, our study could not statistically confirm the effect of such pretreatment.

This study verifies that tolerance to bile stress is a critical factor for survival of probiotic bacteria in the gastrointestinal tract and that bacterial species respond diversely to the bile in GITS: the death rate can be several magnitudes. This observation is important not only for probiotic food design but also with respect to the pathogenic potential of microorganisms in food. The GIT survival of probiotic strains might not only depend on their number and physiological state but also food matrix and habits of food consumption which affect bile excretion. This importance of bile excretion to bacterial survival demonstrates the need for further studies on the effect of food matrix on the bile tolerance of probiotics as well as physiological and nutritional studies in human.

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ARTICLE IV

Sumeri, I., Adamberg, S., Sarand, I., Paalme, T. Survival of cheese bacteria in gastrointestinal tract simulator (manuscript submitted to *International Dairy Journal*)

Survival of cheese bacteria in gastrointestinal tract simulator

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Keywords: gastro-intestinal simulator (GITS), cheese, lactic acid bacteria, *Lactobacillus*, survival, bile salts, gastric acid, digestive enzymes

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Abstract

The microbial consortium of 3-month old semi-hard cheeses from two dairies was studied before and after transit through gastrointestinal tract simulator. In total 8 species (22 different genotypes) were identified from one cheese (survival 4.9 ± 3.6 %) and 9 species (13 genotypes) from the second cheese (2.9 ± 2.3 %). GITS survival between different species varied significantly. The most abundant nonstarter species *Lactobacillus casei/paracasei* showed the highest survival (up to 24%) while survival of the most resistant starter species *Lactococcus lactis* subsp. *lactis* remained below 3%. However, GITS survival of *Lc. lactis* greatly exceeded that of *L. casei/paracasei* when applied as stationary phase MRS cultures. Addition of digestive enzymes pepsin and pancreatin into GITS decreased remarkably the survival and variability of LAB from both cheeses. The GIT simulator is an efficient tool for isolating acid, bile and digestive enzyme tolerant potentially probiotic strains from complex food ecosystems.

1. Introduction

Probiotic lactic acid bacteria (LAB) have naturally been delivered to the human gastrointestinal tract (GIT) via milk, cheese and fermented milk systems (Klaenhammer, Azcarate-Peril, Altermann & Barrangou, 2007) and possibly with other foods like fermented vegetables or meat for centuries. To increase the probability of reaching sufficient amount of viable bacteria to human colon, consumption of probiotics with food is recommended by Pacheco, del Toro, Martínez & Durán-Parámo (2010). Food may buffer the environment by protecting bacteria during transit through stomach (Kailasapathy & Chin, 2000; Vinderola, Costa, Regenhardt & Reinheimer, 2002; Ranadheera, Baines & Adams, 2010). However, depending on the amount of ingested food, its buffering capacity and fat content, the holding time in the acidic environment of stomach and bile secretion in small intestine may vary in great extent. Concentration and type of proteins, fats and carbohydrates in the product are some of the factors that influence microbial growth and survival in food and under GIT environment (Gänzle, Hertel, van der Vossen & Hammes, 1999; Dommels et al., 2009). Functionality of the same probiotic strain could vary in the presence of different food ingredients (eg prebiotics) as well as at different physiological stages of the bacterium (Sumeri, Arike, Adamberg & Paalme, 2008).

Cheese has been reported as a good vehicle to deliver viable probiotic bacteria in numbers providing therapeutic benefit (Boylston, Vinderola, Ghoddusi & Reinheimer, 2004, Settanni & Moschetti, 2010). Nevertheless, development of probiotic cheese requires stringent selection of strains to maintain their viability in the product during processing, ripening and shelf life as well as during transit through upper GIT after ingestion (Boylston et al., 2004) at the same time not affecting organoleptical properties (flavor, texture and appearance) of the cheese (Mc Brearty, Ross, Fitzgerald, Collins, Wallace & Stanton, 2001). Contrary to relatively short shelf life of yogurt and fermented milk, the time from production to consumption of hard cheeses can range from several months up to years. Compared to liquid fermented products, cheese has lower acidity and water activity. Its high buffering capacity, protein matrix and high fat content could also protect bacteria against environmental changes occurring in GI tract (Kailasapathy & Chin, 2000; Vinderola et al., 2002, Phillips, Kailasaphaty & Tran, 2006). Potentially probiotic strains including representatives of species *Lactobacillus (L.) plantarum*, *L. rhamnosus* and *L. casei* have been selected from microflora of various cheese types (Kask et al., 2003; Succi et al., 2005; Ugarte, Guglielmotti, Giraffa, Reinheimer & Hynes, 2006, Georgieva et al., 2008). Tolerance of these non-starter

lactobacilli to low pH, bile salts, pepsin and pancreatin at biological concentrations has been shown to be strain-dependent (Haller et al., 2001, Georgieva et al., 2008) and even comparable to that of well-studied probiotic strain *L. rhamnosus* GG (Succi et al., 2005). Several studies have proven that probiotic lactobacilli of human and cheese origin added to various cheeses, survive well the ripening period (Gardiner, Ross, Collins & Stanton, 1998; Vinderola & Reinheimer, 2003; Songisepp et al., 2004; Vinderola, Prosello, Molinari, Ghiberto & Reinheimer, 2009: Phillips et al., 2006; Järvenpää et al., 2007, Sharp, Mc Mahon & Broadbent, 2008; Bergamini et al., 2010) as well as GIT barriers *in vitro* and *in vivo* (Gardiner et al., 1999; Saxelin et al., 2010; Lahtinen et al., 2011;).

A single vessel gastrointestinal tract simulator (GITS) was developed in our laboratory to imitate physiological barriers of stomach and small intestine (Sumeri et al., 2008). The model was successfully used for evaluation of viability of probiotic bacteria and functionality of probiotic food (Sumeri et al., 2008, 2010). The aim of the current study was to compare survival of different species and strains of complex population of lactic acid bacteria in semi-hard cheese using GITS in combination with molecular identification methods.

2. Materials and methods

2.1. Cheese samples

Cheeses subjected to study were commercial 90-day-old open texture cheeses produced in two cheese factories of Valio Ltd. (Finland) situating in Estonia and Finland. All cheeses were produced from pasteurized cow's milk with Chy-Max rennet and mesophilic DL starter cultures (Chr. Hansen A/S, Denmark) composed of undefined proportions of *Lactococcus (Lc.) lactis* subsp. *lactis, Lc. lactis* subsp. *cremoris, Lc. lactis* subsp. *lactis* biovar *diacetylactis* and *Leuconostoc (Ln.) mesenteroides* subsp. *cremoris.* The cheeses were brine salted and had (an average) NaCl content of 1.1–1.4 %. The average fat content in FIN cheese was 31.7% and in EST cheese 26.3% (w/w) and dry matter 60.7 and 58.2, respectively.
Cheese samples from two different batches from both dairies, totally 2 x 12 samples, were analyzed in the current study. The cheeses were kept at -20° C until experimental use. 20 grams of sample was aseptically taken from the cheese interior and homogenized with 200 ml sterile distilled water. This cheese homogenate was used as a "model food" in GITS experiments.

2.2. Gastrointestinal tract simulation experiments

The gastrointestinal tract simulator (GITS) and control algorithm is described in detail in Sumeri et al. (2008). Briefly, the stomach step of this simulator is characterized by a pH profile changing from empty stomach pH 2.0 up to a level depending on the food buffering capacity followed by a decrease in the pH back to 3.0 with 1 N HCl. In the duodenum step, the contents of the reactor is neutralized by 1 M $NaHCO_3$ to pH 6.0 and 4% bile salt solution is added to maximum concentration of 0.4% (w/v) that is kept for 30 minutes. The bioreactor content is then diluted ($D=0.4\ h^{-1}$) with dilution medium (g l^{-1}: lactose, 5; tryptone, 2.5; yeast extract, 1.25; Tween 80, 0.5; K_2HPO_4, 1; pH was set to 5.0 by 5 M HCl) to simulate the absorption of bile acids in the jejunum and ileum. The model was complemented with digestive enzymes in some experiments: pepsin (4 g/l) was added in conjunction with hydrochloric acid in stomach phase and pancreatin (9 g/l) along with bile salts in duodenum phase.

2.3. Enumeration and isolation of lactic acid bacteria

Viable bacterial counts were determined from cheese homogenate, the content of simulation vessel in the beginning and end of GITS experiment, by plate count method on MRS agar (LAB M, UK). Plates were incubated anaerobically (AnaeroGen™, Gas Pack System, Oxoid, Inc., Basingstoke, UK) at 30°C for 72 hours.

About 10 – 15 colonies per sample were randomly picked from MRS agar plates and purified by 2 successive subcultures on the same medium. Culture from a single colony in MRS broth (LAB M, UK) was subjected to DNA extraction and stored at -80°C in MRS supplemented with 25% (v/v) of glycerol.

Distribution of LAB species and genotypes in the cheese before and after GITS was studied by molecular typing and species identification of the selected colonies from plates. The identification data were extrapolated onto the whole LAB population (total numbers by plate counts) to get numbers of each species and genotype before and after GITS experiment.

2.4. DNA extraction and PCR analysis

For DNA collection 30 µl of cell cultures were applied to FTA™ Cards (Whatman,

Maidstone, UK). Bacterial DNA was extracted from FTA™ Cards according to the manufacturers procedure. For strain typing PCR amplification was performed with (GTG)-5 (5′ -GTGGTGGTGGTGGTG - 3′) oligonucleotide primer (Versalovic et al., 1994) using Taq DNA polymerase (Fermentas UAB, Vilnius, Lithuania). All PCR-reactions were carried out in an Eppendorf Mastercycler thermal cycler (Eppendorf, Hamburg, Germany).

The cycling program comprised: initial denaturation of DNA for 6 min at 95°C; 30 cycles each consisting of 1 min at 94°C, 1 min at 40°C and 8 min s at 65°C; and final extension for 16 min at 65°C.

The PCR products were separated by electrophoresis on 2.0 % agarose gel containing 0.05 μg ml^{-1} ethidium bromide, for 1 h in 1×TAE buffer and 100 V (constant voltage) at room temperature and a 1 kb Gene Ruler ladder (Fermentas) was used as molecular weight marker. Electrophoresis was performed using Bio-Rad Power Basic power supply (Biorad, USA). The buffer 50×TAE prepared from 242 g TRIS BASE, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA pH 8.0 and demineralized water to 1000 ml. The rep-PCR profiles were visualized under ultraviolet light, followed by digital image capturing using a camera ImageQuant 400 + IQuant Capture 400 (Amersham Biosciences, USA).

2.5. 16S rDNA sequence analysis

The 16S rDNA region was amplified with universal primers 27- f – YM1 and 16R-1522 1U (20 pmol both) using Taq DNA polymerase (Fermentas UAB, Vilnius, Lithuania). The cycling program consisted as follows: initial denaturation of DNA for 1 min at 95°C; 34 cycles each consisting of 20 s at 94°C, 1 min at 65°C and 1.5 min at 72°C; and final extension for 5 min at 72°C. The PCR products were purified with MSB Spin PCRapace 50 kit (Invitek GmbH, Germany). The isolated PCR products were sequenced using 27- f – YM1 primer and the obtained partial 16S rRNA gene sequences were aligned with those in GenBank using Blast program.

2.6. Calculation of GITS survival

Viable bacterial counts in the end of experiments (from plate counts) were normalized taking into account the dilution in duodenum phase of GITS to determine survival rate of total LAB and individual species and genotypes during GITS passage. Survival of bacteria - SUR - was calculated as described by Sumeri et al. (2008) according to the formula:

$$SUR\ \% = e^{D\tau} * (CFU_t/CFU_F) * 100 \quad (1)$$

where CFU_t is the number of colony-forming bacteria in the end of GITS and CFU_F is the number colony-forming bacteria in the food used for simulation . The $e^{D\tau} = 10$ is the dilution rate correction factor, where τ is the time of dilution phase (5.75 h) and $D = 0.4\ h^{-1}$ is the dilution rate during this phase.

3. Results and discussion

3.1. LAB species in semi-hard cheeses and their survival in GITS

The changes of microbiota of 90 days old semi-hard open texture cheeses from two dairies were studied in GITS. The average numbers of colony forming lactic acid bacteria in cheeses from Finnish factory was $8.5 \pm 3.4 \times 10^6$ CFU/g and that of cheeses from Estonian factory $2.4 \pm 1.1 \times 10^7$ CFU/g. Proportions of different species within total LAB before and after GITS are presented in Table 1. According to 16S rDNA sequence data, totally 8 different species containing 22 different genotypes were detected from FIN cheese (among 315 isolates analyzed) and 9 species containing 13 genotypes were detected from EST cheese (among 284 isolates analyzed). The species *Lactobacillus casei* and *Lactobacillus paracasei* are presented as a combined *L. casei/paracasei* species since we could not differentiate them with the current methods. In general, the species of non-starter lactobacilli in the studied cheeses were similar to those described by other authors for semi-hard cheeses (Lindberg, Christiansson, Rukke, Eklund & Molin, 1996; Fitzsimons, Cogan, Condon & Beresford, 1999; Williams, Choi & Banks, 2002).

The starter lactococci (*Lc. lactis* subsp. *lactis* and biovar. *diacetylactis, Lc. lactis* subsp. *cremoris*) comprised 30.7 % of determined LAB population in cheeses from Finnish factory and 63.6 % in cheeses from Estonian factory (Tab. 1) while *Lc. lactis* biovar *diacetylactis* was found as dominating starter species in cheeses from Finnish factory only. After GITS the percentage of lactococci decreased down to 18.1 and 34.7 % corresponding to GITS survival of 2.7-3.1 and 1.6 % for cheeses from Finnish and Estonian dairies, respectively. *Lc. cremoris* did not survive GIT conditions in detectable amounts.

Table 1. Percentage of species from whole LAB consortium in cheese before and after GITS

	Cheeses from Finnish factory		Cheeses from Estonian factory	
Species	Before	After GITS	Before	After GITS
Starter	**30.7**	**18.1**	**63.6**	**34.7**
Lc. lactis	10.8	6.0	63	34.7
Lc. diacetylactis	19.3	12.1	ND	ND
Lc. cremoris	0.6	ND	0.6	ND
Non-starter	**69.2**	**81.9**	**35.6**	**65.3**
Ln. mesenteroides	6.6	0.7	12	20.7
L. casei/paracasei	39.1	76.5	20	34.7
Ln. pseudomes.	18.7	1.3	0.6	2.5
L. curvatus	ND	ND	2.4	5.8
L. diolivorans	ND	ND	0.6	ND
L. rhamnosus	1.8	0.7	ND	0.8
L. plantarum	3.0	2.7	ND	ND
St.thermophilus	ND	ND	ND	0.8
Total LAB per cheese, cfu g^{-1} [a]	$8.5 \pm 3.4 \times 10^6$	$4.2 \pm 0.3 \times 10^4$	$2.4 \pm 1.1 \times 10^7$	$7.0 \pm 1.1 \times 10^4$
Number of analyzed colonies	166	149	163	121

[a]corrected by dilution factor

In the Finnish cheese *L. casei/paracasei* dominated both in the beginning and end of GITS transit comprising 39.1 and 76.5 % of the population respectively (Tab. 1) with 9.7% survival clearly exceeding the average for total LAB - 4.9%. Survival of *Ln. mesenteroides, L. plantarum* and *L. rhamnosus* is hard to determine in this study due to very low numbers detected.

In cheeses from Estonian dairy the most dominant NSLAB was *L. casei/paracasei* followed by *Ln. mesenteroides* representing 20% and 12 % of population, respectively. The species *L. curvatus* comprised 2.4%, *Lc. cremoris, Ln. pseudomesenteroides* and *L. diolivorans* altogether 2.6 % from total colony forming units. After GIT simulation the proportion of *L.casei/paracasei* increased up to 34.7% (survival 4.9%). In different from Finnish cheese, *Ln. mesenteroides* showed as good survival (4.9%) constituting 21% of total counts after GIT simulation. The percentage of *L. curvatus* and *Ln. pseudomesenteroides* after GITS increased slightly remaining however subdominant. Few colonies of *L. rhamnosus* and *Streptococcus thermophilus* were also detected after GITS transit suggesting their survival. Neither *Lc. cremoris* nor *L. diolivorans* survived GIT conditions in detectable amounts.

The average survival of total LAB was somewhat higher for Finnish cheeses compared to that for Estonian cheeses (4.9± 3.6 vs 2.9± 2.3 %). That can be explained with higher content of *L. casei/paracasei* having better survival (in population of Finnish cheese) compared to *Lc. lactis* with relatively lower survival that dominated in Estonian cheese.

3.2. Distribution and survival of different genotypes of Lb. casei/paracasei

The most abundant and most resistant NSLAB species during GIT transit was *Lb. casei/paracasei*. Average extrapolated numbers of *L. casei/paracasei* per gram of cheese before and after GITS were $3.3 \times 10^6 - 3.2 \times 10^4$ cfu/g and $4.8 \times 10^6 - 2.4 \times 10^4$ cfu/g, for Finnish and Estonian factories, respectively.

According to rep-PCR analysis 13 different genotypes of *L. casei/paracasei* were detected from Finnish cheese and 6 from Estonian cheese, whereas none of the genotypes were found in both cheeses (Figure 1).

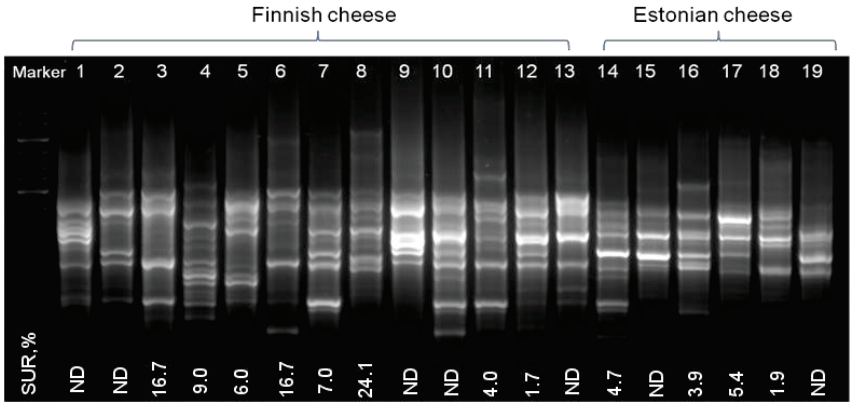

Figure 1. rep-PCR patterns of different genotypes of *L. casei/paracasei* obtained with GTG5 primer and their survival (%) in GITS. Marker - Gene Ruler™ 100 bp DNA Ladder ; Lines 1-13 isolates from Finnish cheese; Lines 14-19, isolates from Estonian cheese.

In the Finnish cheese, five genotypes comprised over 85% of the whole *L. casei/paracasei* species and a single genotype (no 8) became dominating after GITS (Figure 1 and 2A). This genotype had the highest GITS survival - 24% comprising 7.8 % of total LAB in the beginning and 38.3 % in the end of GIT simulation. Three genotypes (1, 2 and 13) were detected in low numbers only in the beginning of GITS and one (no. 9) appeared only in the end of GITS. Two genotypes (2 and 6) appeared in low numbers only in the end of GITS (not shown in Figure 2).

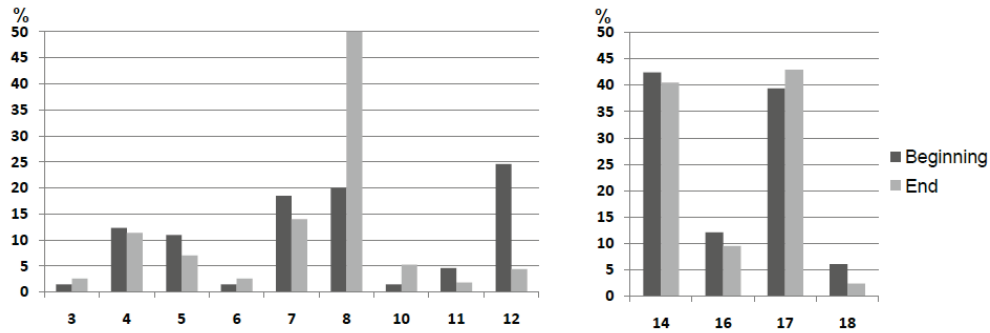

Figure 2. Proportion of dominating genotypes (% of the whole population of *L. casei/paracasei*) before and after GITS, summarized from all experiments. A. Cheese from Finnish factory, Genotypes 3 – 12, and B. Cheese from Estonian factory, Genotypes 14, 16, 17 and 18.

In the Estonian cheese two dominating genotypes (14 and 17, Fig 2B) comprised more than 80% of the whole *L. casei/paracasei* without considerable change of the proportion during GITS (survival about 5%).

Survival of different genotypes of *L.casei/L.paracasei* in GITS experiments varied significantly, while isolates from Finnish cheese showed generally better resistance against GIT stresses (Figure 1). The effect of the cheese matrix and possibly also physiological state of bacteria was demonstrated in GITS experiments with overnight stationary phase culture of *L. casei/paracasei* genotype no 8 in MRS broth (Figure 3).

Figure 3. Survival of *L. casei/paracasei genotype 8* in GITS in cheese homogenate and as pure culture in MRS.

The calculated survival of this strain in GITS when applied in cheese matrix was 24.0% that is about 1000 fold higher compared to survival in overnight MRS culture (0.06%). This difference can be explained beside protective effect of cheese matrix also by different physiological stage of bacteria. However, it is rather difficult to estimate the physiological state of microbes in cheese which is kept at low temperatures in condition of energy source limitation for a long time. Furthermore, freezing of cheese sample may affect survival of LAB in subsequent GIT transit that needs to be elucidated in the future studies. Better survival in GITS when applied in milk or cheese compared to MRS broth was also observed in our previous studies with probiotic *L. rhamnosus* GG (Sumeri et al, 2008).

These results emphasize the importance of food matrix composition as well as design of the probiotic product on survival of probiotics in gastrointestinal tract.

3.3. The effect of digestive enzymes on survival of LAB during GITS transit

The effect of pepsin and pancreatin on the viability of LAB from cheese was studied and compared with the results obtained without enzymes in GITS. Pepsin (4 g/l) was added in the stomach phase and pancreatin (9 g/l) in small intestine.

Results of these experiments are shown in Figure 4. Addition of digestive enzymes into GIT model clearly decreased the variability of microbiota of both cheeses. The only species from those detected in cheeses (Table 1, Fig. 4) with considerable GITS survival were *Lc. lactis* from starter culture and non-starter *L. casei/ paracasei*.

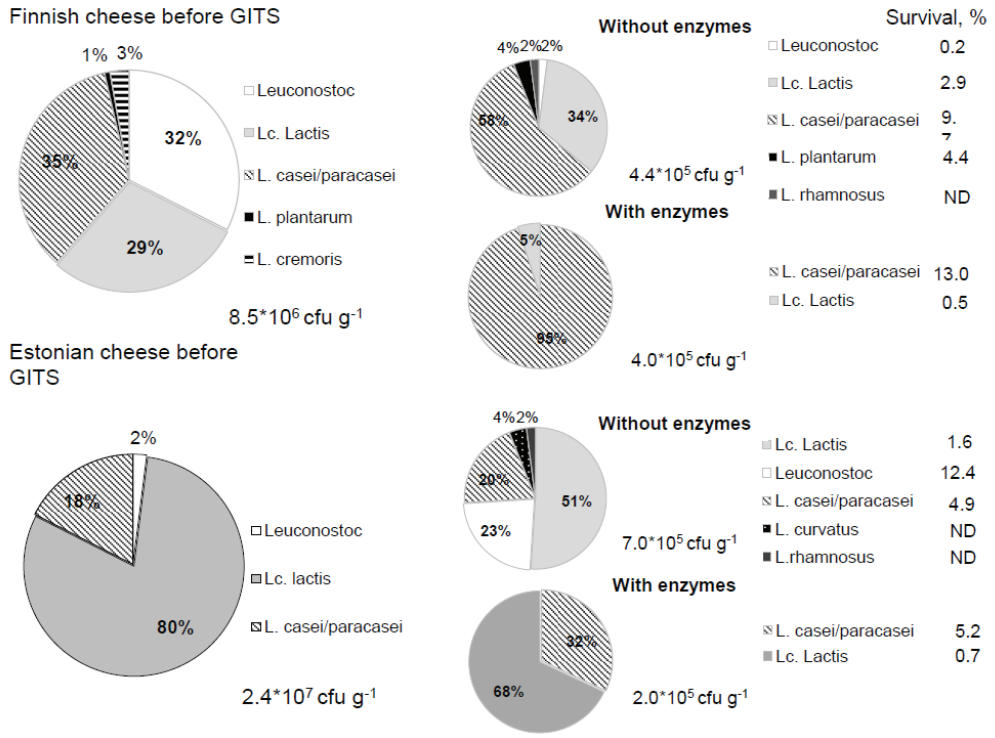

Figure 4. The effect of digestive enzymes pepsin and pancreatin on survival of LAB species in GITS (as mean % of total LAB) summarised from three experiments at both conditions (with and without enzymes). *Leuconostoc – Ln. mesenteroides* subsp. *mesenteroides* plus *Ln. pseudomesenteroides*.

Proportion of *Lc. lactis* decreased in both cheeses, but remained higher in Estonian cheese. *Lc. cremoris* did not survive neither of the GITS. *L. mesenteroides* in Estonian cheese, which survived well acid and bile treatments, was not detected after GITS complemented with enzymes. Also *L. plantarum*, *L. curvatus* nor *L. rhamnosus* were not detected after GITS with digestive enzymes. The only species which proportion among LAB increased in GITS with enzymes was *L. casei/ paracasei*.

It is important to note that concentration of enzymes used by us correlate well with some references (Pacheco et al., 2010) but are significantly higher than used by others (Kontula et al., 1998; Oomen et al., 2003; Nazzarro, Fratianni, Coppola, Sada & Orlando; 2009 Sanchez et al., 2009). Therefore further studies are needed to elucidate the effect and mechanisms of digestive enzymes on bacterial viability in GIT.

4. Conclusions

The gastrointestinal tract simulator can effectively be used for comparative survival studies of individual probiotic strains in various food matrixes and for selection of acid, bile and digestive enzyme resistant bacteria from complex food ecosystems. Our study showed significant survival differences between LAB species from semi-hard cheese and between strains within one species as well as importance of the matrix in which bacteria are ingested on their survival in conditions of GI-tract. We demonstrated that several strains of lactobacilli that grow in ripening cheese can also survive the physiological stresses of GIT.

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KOKKUVÕTE

Probiootilised bakterid inimese seedetrakti simulaatoris

Tänapäeval on funktsionaalsed, ka probiootilised toidud, kujunenud meie menüü kindlaks osaks. Üheks eelduseks, et bakteritüvi on potentsiaalne probiootikum, on selle bakteri eluvõime seedetrakti tingimustel. Selles vallas on läbi viidud arvukalt uurimistöid, mis peamiselt keskenduvad bakteri resistentsuse kindlakstegemisele happe ja sapi suhtes. Kuna In vivo katsed on raskesti teostatavad, ei kasutata neid üldiselt eeluuringutes. Seetõttu on vajalikud in vitro mudelsüsteemid, mis suudaksid tõepäraselt reaalseid protsesse seedetraktis simuleerida.Tüüpiliselt on sellised simulaatorid mitut anumat ühendatavad süsteemid, mille juhtimine on keeruline. Antud uurimuses on välja töötatud suhteliselt lihtne üheanumaline seedetrakti simulaator, mille abil on võimalik hinnata probiootikumide efektiivsust.

Mudeli hindamiseks testiti selles nelja tuntud probiootilise bakteri eluvõimet. Katsed *L. acidophilus* La-5, *L. Johnsonii* NCC 533, *L. casei* Shirota ja *L. rhamnosus* GG tüvedega teostati,et teha kindlaks, kuidas mõjutab bakterite füsioloogiline seisund nende elujõudu seedetraktis. Tulemused näitasid, et bakterikultuuri füsioloogilise seisundi mõju on tüvespetsiifiline. Statsionaarses või eksponentsiaalses kasvufaasis *L. acidophilus* ja *L. johnsonii* bakterid olid seedetrakti-simulaatoris vastupidavamad kui 2 nädalat 4°C juures säilinud kultuur. Need kaks tüve olid sapi suhtes palju vastupidavamad kui *L. casei* ja *L. rhamnosus*, mille elusrakkude arv langes ligikaudu 6 logaritmilist ühikut sõltumata bakterite kasvufaasist. Toidumaatriksi kaitsvat toimet seedetrakti barjääride suhtes näidati katsetes *L. rhamnosus* bakteritega, mis näitasid suuremat eluvõimet piimatoodetesse viiduna võrreldes bakterisöötmes simulaatorit läbinud bakteritega.

Samuti uuriti bakterite eelneva stressitöötluse võimalikku mõju nende eluvõime suurendamiseks seedetraktis. *L. acidophilus* La-5, *L. rhamnosus* GG ja *L. fermentum* ME-3 kultuurid kasvatati ette pH-auksostaadis, inkubeeriti temperatuuril 43°C, pH väärtusel 3,5 või 4,5 ning 0,02; 0,04 või 0,1 % sapikontsentratsioonil. Seejärel bakterid külmutati vedelas lämmastikus ning testiti seedetrakti simulaatoris. Kuigi kirjanduses on andmeid stressitöötluse positiivsest mõjust bakterite elujõule, ei täheldatud sellist efekti meie poolt läbi viidud karsete tingimustel.

Lisaks uuriti antud seedetrakti simulaatori abil lahtise tekstuuriga juustu mikroobikoosluse dünaamikat. Selle uurimuse eesmärk oli eraldada happe ja sapi suhtes vastupidavad piimhappebakterid lahtise tekstuuriga juustudest, mis olid valmistatud kahes piimatööstuses. Tulemused näitavad, et erinevate liikide ja ka ühe liigi (*L.casei/paracasei*) erinevate tüvede eluvõime on antud simulaatoris väga erinev. Isoleeriti mitmed domineerivad tüved *L. casei/paracasei* rühma kuuluvatest mitte-starter piimhappebakteritest. Neid

tüvesid on plaanis edaspidi iseloomustada ning võimaluse korral lisada juustu starterkultuuridele, et parandada produkti kvaliteeti ning tarbija tervist.

Kokkuvõtteks võib öelda, et antud seedetrakti mudel sobib hästi simuleerima tingimusi, millesse bakterid satuvad seedetrakti läbides.

APPENDIX 1 CURRICULUM VITAE

First Name	Ingrid
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Position	researcher
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Dates and sites of earning the degrees	Tallinn Technical University, 1999
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Current grant funding	Project SF0140090s08 - Food systems biology and physics Project EU29994 - Support for Competence Center of Food and Fermentation Technologies

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Teenistuskäik	2004 - Toidu- ja fermentatsioonitehnoloogia Arenduskeskus, teadur 1997-2004 Keemilise ja Bioloogilise Füüsika Instituut, molekulaargeneetika laboratoorium, vaneminsener 1991-1997 TA Keemia Instituut, Bioorgaanilise Keemia Osakond, insener 1989-1991 Tallinna Tehnikaülikool, Majandusteaduskonna dekanaat, sekretär 1988-1989 Tallinna Mererajooni Rahvakohus, kohtutäitur 1985-1987 TA Eksperimentaalbioloogia Instituut, Viroloogia sektor, laborant
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Teaduskraadi välja andnud asutus, aasta	Tallinna Tehnikaülikool, 1999
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