IS: Survival of lactic acid bacteria (LAB) in a human model digestive system. Faye et al.

The survival of LAB (as washed cells or in fermented milk) was investigated both under conditions similar to human digestion using human gastric and duodenal juices, and with traditional methods using acidic conditions and bile salts. The *Lactobacillus* strains showed the highest survival rate. However, the *Enterococcus hirae* and some of the *Lactococcus* strains benefited significantly from the fermented milk. The human model digestive system comprises an in vitro testing regime suitable for evaluation of the survival of candidate probiotic bacteria in human digestion.

Running head: SURVIVAL OF LAB IN HUMAN GASTRIC AND DUODEMUM JUICE

Survival of lactic acid bacteria from fermented milks in an in vitro digestion model exploiting sequential incubation in human gastric and duodenum juice.

T. Faye, A. Tamburello, G.E. Vegarud and S. Skeie[1]

Department of Chemistry, Biotechnology and Food Sciences,

Norwegian University of Life Sciences,

P.O. Box 5003, NO-1432 Ås, Norway

Department of Chemistry, Biotechnology and Food Sciences

The Norwegian University of Life Sciences

P.O. Box 5003, N-1432 Ås, Norway

Phone: +47 64965844 Fax: +47 64965901

[1]Corresponding author: siv.skeie@umb.no

ABSTRACT

In the present study the survival of nine lactic acid bacteria; five *Lactococcus* strains, three *Lactobacillus* strains and one strain of *Enterococcus hirae*, was investigated in vitro under conditions similar to human digestion using human gastric and duodenal juices. The tolerance of the bacteria was also tested with traditional methods using acidic conditions and bile salts. The strains were subjected to a model digestive system comprising sequential incubation in human gastric and duodenal juices, in a two-step digestion assay at 37°C simulating the human upper gastrointestinal tract with human gastric juices at pH 2.5 and human duodenal juices at pH 7. The bacterial strains were tested either as washed cells from culture media or in fermented milk. The initial in vitro testing in acid and bile salts showed that *Lactobacillus* strains and the *Enterococcus hirae* strain displayed a significantly higher acid tolerance than the lactococci. The lactobacilli and the *Enterococcus* numbers increased, while the lactococci decreased at least 1 log during the bile salt treatment. The *Lactobacillus* strains showed the highest survival rate in the model digestive system when washed bacterial cultures were used with a minor log reduction while the lactococci numbers were reduced by at least log 4. However, when using fermented milks in the model digestion system it was demonstrated that the *Enterococcus* strain and two strains of *Lactococcus lactis* ssp. *cremoris* benefited significantly from the presence of the fermented milk as food matrix, with log numbers > log 7 and 5 respectively after digestion of the fermented milk. The analyses reported comprise a comprehensive in vitro testing regime suitable for evaluation of the survival of candidate probiotic bacteria in human digestion as an initial prescreen to clinical trials.

Key words: Lactic acid bacteria, survival, human digestive model system, gastric juice, duodenum juice, fermented milk.

INTRODUCTION

In recent decades there has been growing interest in functional foods containing bacteria with beneficial effects. Products containing “functional bacteria” or definite probiotics are popular among the consumers and include capsules, tablets, juices, yogurts, fermented milks and other dairy products. Among food products with probiotics added, fermented milks and yogurts are especially acceptable. An explanation of this popularity can be that these products initially contain relatively large amount of bacteria, among them several strains with well known functional properties which have been regarded as healthy products for a long time. However, the criteria of being considered as a probiotic bacteria are several and strict (Borchers et al., 2009; de Vrese and Schrezenmeir, 2008). These criteria include that the bacteria should be of human origin and at least one clinical phase study must have been conducted. This implies that several bacterial strains with a long history in food fermentations and with conferred probiotic properties like good survival through the digestive tract, production of exopolysaccharides, antagonism against pathogenic bacteria, or the capability to adhere to the epithelium in the gut, cannot be considered as declared probiotic bacteria. Nevertheless, food related bacteria with stated positive effects for the consumer are generally accepted as functional ingredients in food products (Pfeiler and Klaenhammer, 2007; Zhu et al., 2009).

Scandinavian ropy milks are traditional fermented milk products from northern Scandinavia (Fondén et al., 2006; Furuset, 2005; 2008). The main characteristic of these products is the slimy texture of the milks caused by growth of exopolysaccharide-producing *Lactococcus lactis* ssp. *cremoris* in the fermenting product. According to the tradition in Norway, this ropy milk was produced by adding leaves of the herb butterwort (*Pinguicula vulgaris*) into the milk before incubation (Furuset, 2005; 2008). Nowadays, a commercial product called “Tjukkmjølk” is produced based on old starter cultures that are supposed to

originate from this herb. Similar products are available in Sweden (Långfil), Finland (Viili) and Iceland (Skyr) (Fondén et al., 2006).

Traditionally, the Norwegian ropy milk has a public image of having especially good health related properties. Examples are health sites and debate forums on the internet reporting that people are consuming ropy milks without any adverse effects despite suffering of milk allergy or intolerance. However, although ropy milk may have a great potential as a functional dairy product worldwide, only a few scientific studies have investigated ropy milk products or examined the functional potential of the exopolysaccharide-producing lactococci (Kitazawa et al., 1991; Kitazawa et al., 1993; Nakajima et al., 1992; Nilsson and Nilsson, 1958).

One of the main criteria for the assessment of probiotic bacteria is that they survive the hostile environments in the digestive tract in order to perform their actions in the gut. During the gastrointestinal passage, the probiotic bacteria must tolerate the presence of pepsin and the low pH of the stomach. Although the pH in the stomach will increase to a higher level (pH 4-6) depending on the buffering capacity after food intake, it generally stabilizes after some time to 2.5 to 3.5 (Holzapfel et al., 1998). Thereafter, the bacteria must survive the effects of bile salts and the protease-rich conditions of the duodenum (Ekmekcioglu, 2002).

Normally, potentially probiotic bacteria are exposed to standard in vitro testing to examine if they can survive in the digestive tract (Borchers et al., 2009). These tests include exposure of the bacteria to acidic conditions normally between pH 2.0 and pH 3.5, mimicking the environment in the stomach, and to bile salts. However, these tests give only an indication of the bacterial tolerability of the digestive conditions. During digestion the bacteria encounter multiple severe stress factors that ultimately might affect their survival and subsequent performance in the gut. In order to determine the actual capability of a bacterium to survive passage through the digestive tract, it is necessary to apply model systems mimicking the

human digestive system accompanied by human clinical trials. In a model system it is possible to copy the subsequent exposure to all the conditions bacteria must tolerate in real digestion. Furthermore, the metabolic state and fitness of ingested bacteria in the digestive tract will most probably be influenced by the method used for delivery. Pure bacterial cultures will presumably be more exposed to the severe conditions during digestion than bacteria embedded in food. Although the moment the bacteria are exposed to the human digestive tract environment, a dramatic metabolic adjustment will be necessary for them to survive regardless of how they are administered. However, bacterial cultures might be in a favorable metabolic condition after growth in appropriate media at optimal conditions regarding temperature and oxygen supply, compared to bacterial cells stressed by factors in the foods like for instance limited carbon supply, low pH, unfavorable red-ox potentials and incubation at non-optimal temperatures.

In this paper we compare the potential of selected lactic acid bacteria to survive gastrointestinal conditions through classical in vitro experiments (testing their tolerance to acidic environments and to bile salts) with a digestive model system utilizing gastric and duodenal juices of human origin, mimicking an in vivo gastrointestinal digestion. By using this digestive model, the bacteria were sequentially exposed to all the conditions present in the upper part of the human digestive tract. Therefore, this model seems to represent a realistic evaluator of the functional or probiotic potential of the bacteria tested. The suitability of fermented milk as a protective delivery matrix for the bacterial strains through the digestive model system was also investigated.

MATERIALS AND METHODS

Bacterial Strains

A total of nine strains, five lactococcal, three lactobacilli and one enterococcal strain, were examined and propagated as indicated in Table 1. All strains, except for the commercial probiotic strain *Lactobacillus rhamnosus* GG, are of food origin (Table 1). The four strains of *Lactococcus lactis* ssp. *cremoris* originate from Norwegian ropy milks, and produce the exopolysaccharides responsible for the characteristic texture of these products. The strains *Lactococcus lactis* ssp. *cremoris* Bf-2 and Bf-3 originate from ropy milks made from sterile skimmed milk inoculated with *Pinguicula vulgaris*. All strains were identified to species level using 16S rDNA gene sequencing (Østlie et al., 2004) and sequences were edited using BioEdit software and analyzed using BLAST sequence search tool.

Tolerance to Temperature, Low pH and Bile Salt

The bacterial strains were tested for their ability to survive and grow at 37^{o}C, and to survive acidic conditions and the presence of bile salt. Overnight cultures of the bacteria were inoculated in the appropriate medium (Lactobacilli: MRS broth (Difco, Becton Dickinson and Company, Sparks, USA), Lactococci: M17 broth (Merck, Darmstadt, Germany)) and in the same medium acidified to pH 2.0 or pH 3.0 with 0.1M HCl, or with 0.3% bile salt (≈ 6-7 mM) (Sigma Aldrich, St. Louis, USA) added. The bacterial cultures were incubated at 37^{o}C for three hours before plating on their respective optimal media. All experiments were made in three replicates.

Tests with Human Gastric and Duodenal Juices Using an In Vitro Model System

Human gastric juice (HGJ) (19.2 U/ml) and duodenal juices (HDJ) (12.9 U/ml) were collected from 20 individual healthy volunteers and pooled in two separate batches, one for HGJ and one for HDJ to avoid variability. The aspiration procedures are described by Holm et al. (1988), in brief, a three-lumen silicone tube (Maxter Catheters, Marseilles, France) enabled

simultaneous instillation of a stimulation solution in the duodenum and aspiration of gastric (HGJ) and duodenal (HDJ) juice. Correct placement of the tube was monitored by radiology. Continuous instillation, 100 ml/h of the isotonic stimulatory solution (17.5 g/l sucrose, 450 mg/l NaCl, 800 mg/l L-phenylalanine and 575 mg/l L.valine in H_2O) and a continuous aspiration of gastric and duodenal fluids were performed simultaneously. The stimulation solution was instilled close to the papilla of Vater while the duodenal juice was aspirated some 10 cm distally. Juices from the stomach were aspirated from the canalis ventriculi. The juices were collected in 50 ml tubes on ice, inspected and pH was measured periodically to avoid samples of mixed duodenal and gastric juices. The samples were centrifuges (4500 g for 10 min) to remove mucous and cell debris before aliquots were frozen at -20 and then stored at -80 ◦C. The enzyme activities were calculated by pepsin activity assay for HGJ using haemoglobin as substrate according to Sánchez-Chiang et al. (1987) and by the total proteolytic activity assay for HDJ with casein as a substrate according to Krogdahl and Holm (1979). The enzyme activity (1 U) is defined as the amount (ml) of HGJ or HDJ giving a difference in absorbance of 1.0 at 280nm in 10 min at 37 ◦C.

Model Digestion of Bacterial Cultures. An overnight culture of each bacterial strain, 1% inocula in 40 ml growth medium, were used in all the experiments. After centrifugation for 30 min at 2400*g* (Centrifuge 2010, Kubota, Fujioka, Japan), the bacterial cell pellet was diluted with 40 ml Ringer's solution. Ten ml of cell suspension was transferred to each of two plastic tubes equipped with sterilized magnetic stirrers and incubated in a water bath circulator (Julabo MB 7A (JULABO Labortechnik GmbH, Seelbach, Germany) at 37^{o}C. One of the tubes was equipped with a pH meter (PHM 210, MeterLab Radiometer Analytical, Lyon, France) in order to check the pH during the digestion. The pH in the first step of digestion was adjusted to 2.5 with 1M HCl. After 5 minutes, 328 µl of HGJ (19.2 U/ml) was

added. After 60 minutes of digestion at pH 2.5, the pH in the sample was adjusted to 7.0 with 1M NaOH and then 1440 µl HDJ (12.9 U/ml) was added followed by incubation for another 60 minutes. Samples for determination of viable cell counts (**cfu**) were taken after 0, 5, 65 and 125 minutes. The lactococci were enumerated on M17 agar plates, and the *Lactococcus lactis* ssp. *lactis* strains were incubated aerobically for 48 hours at 30°C. The *Lactococcus lactis* ssp. *cremoris* strains were however incubated anaerobically for 72 hours at 22°C. The lactobacilli were enumerated on MRS agar plates and incubated anaerobically for 48 hours at 30°C. Anaerobic conditions were created using Gaspak™ holding jar and Oxoid Atmosphere Generation System AnaeroGen™ (Oxoid Ltd, Basingstoke, Hampshire, UK).

Digestion of Fermented Milk as a Carrier Matrix for the Bacteria. In order to evaluate the viability of the bacteria in a carrier matrix, fermented milk was used. Milk was fermented with a single strain of each bacterium. In order to improve the growth of lactobacilli, 1% sterilized (121°C, 15 min) D+glucose (Merck, Darmstadt, Germany) was added to the milk with these strains. With the aim of removing any interference due to the MRS/M17 medium, the cultures were back slopped twice. In the first step, 10 ml sterilized skimmed milk (95°C, 10 minutes), prepared from distilled water and 10% of skimmed milk powder (Tine BA, Norway) was inoculated with 1% (100µl) of an overnight broth culture and incubated. All the *Lactococcus lactis* ssp. *cremoris* strains were incubated for 20 hours at 22°C, while the rest of the strains were incubated for 15 hours at 30°C. Subsequently, an aliquot equal to 1% (400 µl) was inoculated in 40 ml sterilized (95°C, 10 minutes) full fat milk (Tine BA, Norway) with 3.2% protein and 3.9% fat and incubated under the same conditions as the previous step. After incubation, the fresh fermented milk was refrigerated for 24 hours at 4°C.

198 The fermented milk samples were then submitted to the in vitro digestive model
199 system with HGJ and HDJ, following the protocol described previously (Model digestion of
200 bacterial cultures). The amount of human juices added was calculated on the basis of enzyme
201 activity/g protein (20U HGJ/g protein and 62.4 U HDJ/g protein). Fermented milk (10mL)
202 was transferred to 50 ml sterile plastic tubes equipped with sterilized magnetic stirrers and
203 incubated in the water bath circulator at 37°C. The pH was adjusted to 2.5 with 1M HCl, and
204 after 5 minutes 300 µl of HGJ (19.2U/ml) was added. After 60 minutes incubation, the pH
205 was adjusted to 7.0 with 1 M NaOH and 1316 µl of HDJ (12.9 U/ml) was added followed by
206 60 minutes of incubation. Samples of 0.5 ml were again drawn after 0, 5, 65 and 125 minutes,
207 and the viable cell count was evaluated by plating on M17/MRS agar and incubated in the
208 conditions shown in Table 1.
209
210 ***Statistical Analyses***
211 Analysis of variance (ANOVA) was performed using the SAS Enterprise guide 4.0
212 (SAS Institute, Cary, NC, USA). The treatment factors bacterial strain and media and their
213 interaction formed the statistical model for the tests of the effects in adjusted media and in the
214 digestion model, respectively. Tukey's simultaneous test was used to find differences between
215 means (Snedecor and Cochran, 1989). For the statistical calculations the following computed
216 values were used:
217 $\Delta \log_{3h}$ =(log cfu/ml at time 0 - log cfu/ml after 3 h incubation),
218 $\Delta \log_{digestion}$ =(log cfu/ml at time 0 - log cfu/ml after 125 min digestion (both gastric juice and
219 duodenum juice)),
220 $\Delta \log_{gastric}$ =(log cfu/ml at time 0 - log cfu/ml after 60 min in gastric juice)
221 $\Delta \log_{duodenum}$ =(log cfu/ml after 60 min in gastric juice - log cfu/ml after 60 min in duodenum
222 juice).

RESULTS

Growth and Survival of Single Bacterial Cultures in Broth and Adjusted Medium Containing 0.3 % Bile Salt and with pH 2 and 3

Incubation for 3 h at 37°C in normal and adjusted M17/MRS broths showed differences ($P<0.001$) in $\Delta \log_{3h}$ between both the strains and the media (Table 2). In normal media the *Lactobacillus* strains and *Lactococcus lactis* ssp. *lactis* ML8 and *Enterococcus hirae* INF E1 increased their cell numbers during 3 hours at 37°C, while the *Lactococcus lactis* ssp. *cremoris* strains had reduced viability ($P<0.05$).

None of the *Lactobacillus* strains or *Enterococcus hirae* INF E1 were significantly affected by the bile salts, while the *Lactococcus* strains except *Lactococcus lactis* ssp. *cremoris* Af-1 and Bf-2 showed reduced numbers ($P<0.05$) when grown in bile salts. At reduced pH most of the tested strains had considerably reduced viability. However, after incubation at pH 3 for 3 hours at 37°C, the *Lactobacillus* strains (GG, INF448 and INF456) survived and maintained an approximately constant viable cell count, while the viable cell counts of *Lactococcus* ssp. and *Enterococcus hirae* decreased ($P<0.05$) and were reduced by about 2-3 logs. In particular, incubation at pH 3 had a strong reducing effect on both the *Lactococcus lactis* ssp. *cremoris* strains, Bf-2 and Bf-3, and the enumerated numbers were less than 10^2 cfu/ml. After incubation at pH 2 for 3 hours at 37°C, none of the strains were able to maintain a good survival rate. The enumerated numbers of all the *Lactococcus* strains and *Enterococcus hirae* were less than 10^2 cfu/ml after 3 hours of incubation. The $\Delta \log_{3h}$ was less than two for *Lactococcus lactis* ssp. *cremoris* strains Bf-2 and Bf-3. However, the numbers after inoculation at pH 2 were very low and less than log 4. The *Lactobacillus* strains showed better tolerance ($P<0.05$) to the acidic environment compared to the lactococci and *Enterococcus* strain investigated, although their numbers were reduced from log 7 to about

log 4 cfu/ml.

Digestion of Single Bacterial Cultures and Fermented Milks with Human Gastric and Duodenal Juices in a Digestive Model System

The production of the fermented milk and the following digestion were principally performed in order to study how fermented milk act as a carrier matrix that could influence the viability of a strain during digestive conditions. During the model digestion using HGJ/pH 2.5 for 60 minutes and HDJ/pH 7 for 60 minutes, differences ($P<0.001$) in viability were shown between the groups of lactococci and lactobacilli throughout the digestion process (Figure 1 and Figure 2). In general, the *Lactococcus lactis* ssp. *cremoris* strains Af-1 and Bf-2 and *Lactococcus lactis* ssp. *lactis* ML8 had a poorer ($P<0.01$) survival rate measured as viable cell count after digestion compared to the other strains investigated. The *Lactobacillus* ssp. retained a high cell number throughout the digestion and decreased by only one log regardless of the matrix. After two hours of digestion (125 minutes), *Lactococcus lactis* ssp. *cremoris* Ar-1 and Bf-2 and *Enterococcus hirae* benefitted ($P<0.01$) from the presence of a fermented milk carrier matrix. On the other hand, *Lactococcus lactis* ssp. *cremoris* Af-1, *Lactococcus lactis* ssp. *lactis* ML8 and *Lactobacillus paracasei* ssp. *paracasei* INF448 survived better as pure cultures than in fermented milk.

In gastric juice (**HGJ**), the viability of the washed cells of *Lactococcus* ssp. and *Enterococcus hirae* decreased ($P<0.001$) during the first hour simulating the gastric tract. However, during the following hour at pH 7 with human duodenal juice (**HDJ**) (duodenum tract simulation), they were able to resume growth and the cell numbers increased approximately 1-2 logs (Figure 1).

DISCUSSION

Five strains of *Lactococcus lactis* sp., three strains of lactobacilli and one strain of *Enterococcus* were tested for their tolerance to acid, commercial bile salt and to gastric conditions through the exposure to gastric and duodenal juices in a digestive model system. One of the main purposes of this study was to compare the traditional in vitro tests with a model system simulating human digestion. Furthermore, all the selected lactococci and lactobacilli strains have interesting functional properties related to different food products. We therefore wanted to evaluate whether these strains could contribute with beneficial health functions, or even have the potential as probiotics for human consumption. *Lactobacillus rhamnosus* GG, one of the most studied probiotic bacterial strains on the market, was chosen as a probiotic control strain. All other strains than *Lb. rhamnosus* GG were food isolates and are thus not considered true probiotics. The one strain of *Enterococcus* was chosen because these are often found as non starter lactic acid bacteria in dairy products. They often dominate the intestine microbial flora and they might be potentially pathogenic (Klein, 2003). The analyses reported comprise a comprehensive in vitro testing regime for evaluation of the survival of candidate probiotic bacteria during human digestion.

Evaluation of the probiotic properties of a strain requires extensive in vitro and in vivo investigation. It has been observed that many in vitro models can predict, with good approximation, the ability of a strain to survive in the human gastrointestinal tract and thereby confer a health benefit for the host. Such tests include investigations of the resistance to gastric acidity, bile salts and pancreatic enzymes, the adherence to human epithelial cells using the cell lines, the immunomodulating effects, the antibiotic resistance profile and the hemolytic properties, the antimicrobial activity against pathogens as well as competition with them for the sites of adhesion (Tannock, 2005).

The present study investigated the ability of nine strains, five lactococci, three lactobacilli and one enterococcal strain, to survive conditions that mimic the digestive

environment through a digestive model using human gastrointestinal juices. Human gastrointestinal enzymes differ from purified commercial non-human enzymes in the way that human gastrointestinal enzymes are complex and contain a mixture of proteases, amylases and lipases that exist in different isoforms in combination with inhibitors, bile salts, bilirubin, cell mucus and other minor components that may all influence the survival of bacteria (Ulleberg et al., 2011). Survival of digestive conditions is one of the fundamental properties of bacteria that are supposed to have effects beneficial to the consumers, and it is usually one of the first characteristics evaluated.

The acidity tolerance test showed that the *Lactobacillus* strains had a significant better acid tolerance than the lactococci under defined conditions. The two lactobacilli investigated, *Lactobacillus paracasei* INF448 and *Lactobacillus paracasei* INF456 showed similar acid tolerance (confirmed by the Tukey's test) as the well-known probiotic strain *Lactobacillus rhamnosus* GG (Alander et al., 1999), used as a control. They maintained a high survival rate (~10^7 cfu/ml) after 3 hours at pH 3, but their numbers were reduced to about 10^4 cfu/ml when tested at pH 2. This loss of viability observed in vitro between pH 3 and pH 2 is reported in several studies (Jacobsen et al., 1999; Schillinger et al., 2005). Among lactococci, it was possible to distinguish between the two subspecies. The *Lactococcus lactis* ssp. *cremoris* strains already decreased significantly in M17 broth (pH 7.2) at 37°C for 3 hours, demonstrating their inability to survive for this length of time at the human body temperature. Thus, as expected, at pH 3 and pH 2 their numbers of cells decreased to numbers less than 10^3 cfu/ml, lower than any levels generally accepted for probiotic bacteria (Kimoto-Nira et al., 2007). On the other hand, in the same tests at pH 7.2 and 37°C *Lactococcus lactis* ssp. *lactis* ML8 and *Enterococcus hirae* INF E1 were able to grow, and at low pH they showed a survival rate slightly better than the strains of *Lactococcus lactis* ssp. *cremoris.* This seems to confirm earlier reports that state the subspecies *Lactococcus lactis* ssp. *lactis* as generally

more robust and less affected by environmental changes than *Lactococcus lactis* ssp. *cremoris* (Kim et al., 2001).

Few of the strains were affected by the presence of bile salts, and only *Lactococcus lactis* ssp. *cremoris Bf-3* seemed to be severely affected, confirming the results of similar work (Jacobsen et al., 1999; Lee et al., 2007), although other studies have concluded that bile salts had severe influence on bacterial survival (Coeuret et al., 2004). Surprisingly, five strains showed a weak increase in numbers of viable cells during the 3 hours of incubation in 0.3 % bile salt, a result not reported from other studies. Furthermore, as in the acid tolerance tests, the lactobacilli generally had a better performance than the lactococci, with the exception of the strains *Lactococcus lactis* ssp. *cremoris* Bf-2 and *Enterococcus hirae* INF E1, which also showed a high survival rate.

The survival of the lactobacilli was much better in the human model digestion system with gastric and duodenum juice as compared to the standard acidity and bile tolerance tests while the cocci, with some exeptions, performed similarly in both systems. The model digestion experiments gave the opportunity to simulate with more precision the gastrointestinal events occurring in the upper gastrointestinal tract, distinguishing the two phases: stomach and duodenum. The digestion presented two sequential phases in the same trial, the first part in acid conditions, pH 2.5, with human gastric juices, and the second step at neutral pH 7 with human duodenal juices (including natural pancreatic enzymes, inhibitors and bile salts). The digestion of the pure bacterial strains demonstrated significant differences between lactobacilli and lactococci with the latter being less tolerant than the lactobacilli strains. The digestion of the lactococcal stains and *Enterococcus hirae* INF E1 gave some surprising results. As expected, none of them survived in significant numbers after exposure to the conditions in the stomach, pH 2.5 with human gastric juice. In fact, after the gastric phase, their viable cell numbers were below10^2 cfu/ml. However, after the subsequent

duodenal phase, the cell numbers increased by approximately 1-2 logs. This could mean that lactococci and enterococci after a period of acid stress are able to resurrect their viability if they are exposed to more suitable conditions like those in the small intestine. This aspect is indeed very interesting since it is in the intestine that functional or probiotic bacteria confer their health benefit to the host. It also implies that bacteria not regarded as probiotic but yet with potential health-related useful properties, like the exopolysaccharide-producing lactococci, still may have the possibility of a positive impact on the consumer.

The digestions of the fermented milks were performed with a protocol similar to the digestion of pure bacterial cultures in order to compare them and to evaluate how fermented milk as a carrier matrix could influence the viability of the strains. The initial hypothesis was that the fermented milks should give protection to the bacteria through the digestive tract, but the tests revealed strain dependent results. At the end of the fermented milk digestion, the *Lactococcus* strains Af-1 and ML-8 and *Lactobacillus paracasei* INF448 showed lower numbers of viable cells compared to digestion of pure bacterial cells. In contrast, the *Lactococcus* strains Ar-1, Bf-2 and *Enterococcus hirae* INF E1, *Lactobacillus paracasei* INF456 and the commercial probiotic strain *Lactobacillus rhamnosus* GG showed higher numbers of viable counts compared to the results from the digestion of the pure cultures. In particular, the carrier matrix improved the viability of the *Lactococcus* strains Ar-1, Bf-2 and *Enterococcus hirae* INF E1 during the incubation in gastric conditions. These results seem to confirm that foods, such as fermented milks, could be a protective matrix enhancing survival of bacteria (Schillinger et al., 2005). In addition, as reported by Kim et al. (Kim et al., 1999) and others (De Angelis and Gobbetti, 2004), this improvement could be an effect of the adaptive responses to acid stress. The bacteria were exposed to sublethal acid conditions (the acid pH of the fermented milk) prior to digestion and this could contribute to the observed protection against the subsequent exposure to normally lethal acidic levels (pH of the

stomach).

Until now species in the *Lactobacillus* and *Bifidobacterium* genera commonly isolated from the intestine have received most attention in the search for bacteria with positive health related properties and as candidates for new probiotic strains. This study confirms that lactobacilli have potential as functional and even as probiotic strains since they showed better tolerance overall to the conditions mimicking the environment in the digestive tract. The *Lactobacillus paracasei* INF448 and *Lactobacillus paracasei* INF456 strains tested, showed very good acid and bile tolerance and high numbers of viable cells after digestion both as pure bacterial cultures and in a food matrix such as fermented milk. These strains were originally isolated from ripened cheese and have been used successfully as adjunct cultures in experiments developing cheese. Several aspects of their metabolism have been investigated (Skeie et al., 2008). Therefore, it would be interesting to evaluate functional properties of these two strains further for future exploitation of their potential as functional bacteria in food, or even as probiotics.

Enterococci are versatile bacteria that are part of the commensal human microbiota and are generally regarded as highly adapted to surviving the human digestive system. In addition enterococci occur frequently in fermented meat and milk products, including cheese. Certain strains of enterococci are included in probiotic formulas and, as such, are regarded as beneficial to human health. We investigated an *Enterococcus hirae* strain isolated from milk for its performance in an in vitro digestion model. Our results showed that this strain benefitted from the protective matrix of fermented milk through good survival during and after the digestion. These results are comparable to what has been reported for the closely related species *Enterococcus faecium* (Klein, 2003).

One of the aims of the current study was to investigate the capacity of *Lactococcus* spp. as functional bacteria in foods and whether they are able to enter the intestine, although

they are formally not considered normal inhabitants of the intestine. Until now only a few investigations have been conducted, showing that some lactococcal strains were able to survive at low pH and in the presence of bile salts (Kimoto-Nira et al., 2007; Lee et al., 2007). Among the lactococci tested in the present study, none have clearly shown a strong tolerance both to low pH and bile salts, although the strains *Lactococcus lactis* ssp. *cremoris* Ar-1 and Bf-2 have displayed a good survival rate in presence of the fermented milk as carrier matrix. This result is especially interesting since these specific strains are exopolysaccharide-producing bacteria in ropy milk, a traditional Nordic product reported to have beneficial impact on the consumers' health (Kitazawa et al., 1991; Kitazawa et al., 1993; Nakajima et al., 1992; Nilsson and Nilsson, 1958).

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505 **Table 1.** Bacterial strains and conditions for growth used in this study

Bacterial species	Strain[1]	Origin	Media	Growth temperature°C
Lactococcus lactis* ssp. *cremoris	Ar-1	Milk	M17	22
Lactococcus lactis* ssp. *cremoris	Ar-1	Milk	M17	22
Lactococcus lactis* ssp. *cremoris	Bf-2	Plant[2]	M17	22
Lactococcus lactis* ssp. *cremoris	Bf-3	Plant[2]	M17	22
Lactococcus lactis* ssp. *lactis	ML-8[3]	Milk	M17	30
Enterococcus hirae	INF E1	Milk	M17	30
Lactobacillus rhamnosus	GG	Commercial strain	MRS	30
Lactobacillus paracasei	INF-448	Cheese	MRS	30
Lactobacillus paracasei	INF-456	Cheese	MRS	30

506 [1] All strains belong to the collection of the Department of chemistry, biotechnology and food science, Norwegian University of Life Sciences.
507 [2] Isolated from sterile milk inoculated with *Pinguicula vulgaris*.
508 [3] Given as a gift to the Norwegian University of Life Sciences from University of Cork, Ireland.

509 **Table 2.** Survival of the strains in normal medium (MRS/M17) or medium with added 0.3 % bile salt or pH adjusted to pH 2 and pH 3 given as
510 change in cfu[1] of the strains during 3h incubation at 37°C: $\Delta \log_{3h}$ =log cfu/ml at time 0 - log cfu/ml at 3 h. Significant differences (*P<0.05)
511 between the media for each strain (row factor) are shown with different superscript letters while differences between each strain in each specific
512 media (column factor) are shown by different subscript letters.

Bacterial specie	Strain	Normal		Bile		pH 3		pH 2	
		Mean	Std	Mean	Std	Mean	Std	Mean	Std
Lactococcus lactis ssp. *cremoris*	Af-1	1.11^{a}_{c}	0.19	1.1^{a}_{c}	0.06	3.09^{b}_{c}	0.21	4.23^{c}_{c}	0.28
Lactococcus lactis ssp. *cremoris*	Ar-1	1.14^{a}_{c}	0.21	1.58^{b}_{c}	0.22	2.94^{c}_{c}	0.53	4.13^{d}_{c}	0.02
Lactococcus lactis ssp. *cremoris*	Bf-2	-0.41^{a}_{bc}	1.05	-0.68^{a}_{b}	0.04	2.15^{c}_{b}	0.06	1.59^{b}_{a}	0.44
Lactococcus lactis ssp. *cremoris*	Bf-3	0.82^{a}_{c}	0.17	3.03^{c}_{d}	0.22	1.91^{b}_{b}	0.06	1.91^{b}_{a}	0.09
Lactococcus lactis ssp. *lactis*	ML-8	-0.26^{a}_{b}	0.21	0.68^{b}_{c}	0.13	3.8^{c}_{d}	0.55	4.49^{d}_{c}	0.02
Enterococcus hirae	INF E1	-1.54^{a}_{a}	0.12	-1.36^{a}_{a}	0.12	2.38^{b}_{b}	0.09	5.31^{c}_{d}	0.12
Lactobacillus paracasei	INF448	-0.43^{a}_{ab}	0.22	-0.32^{a}_{b}	0.18	-0.16^{a}_{a}	0.23	3.05^{b}_{b}	0.49
Lactobacillus paracasei	INF456	-0.48^{a}_{ab}	0.16	-0.31^{a}_{b}	0.32	-0.04^{a}_{a}	0.33	2.79^{b}_{b}	0.22
Lactobacillus rahmnosus	GG	-0.76^{a}_{a}	0.14	-0.29^{a}_{b}	0.09	0.28^{c}_{a}	0.23	3.18^{d}_{b}	0.19

513 [1]Viable cell count

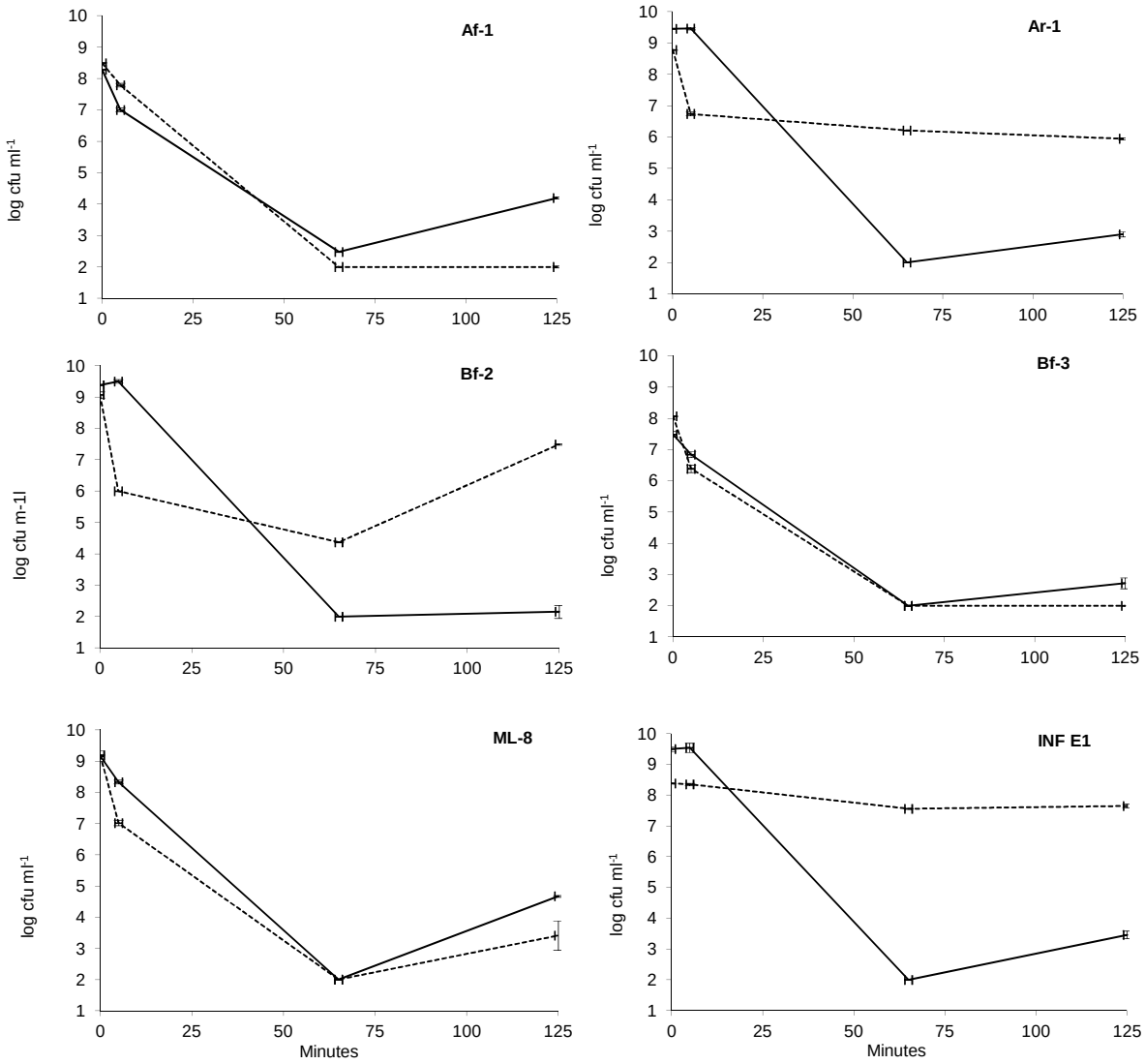
Legends to figures

Figure 1:

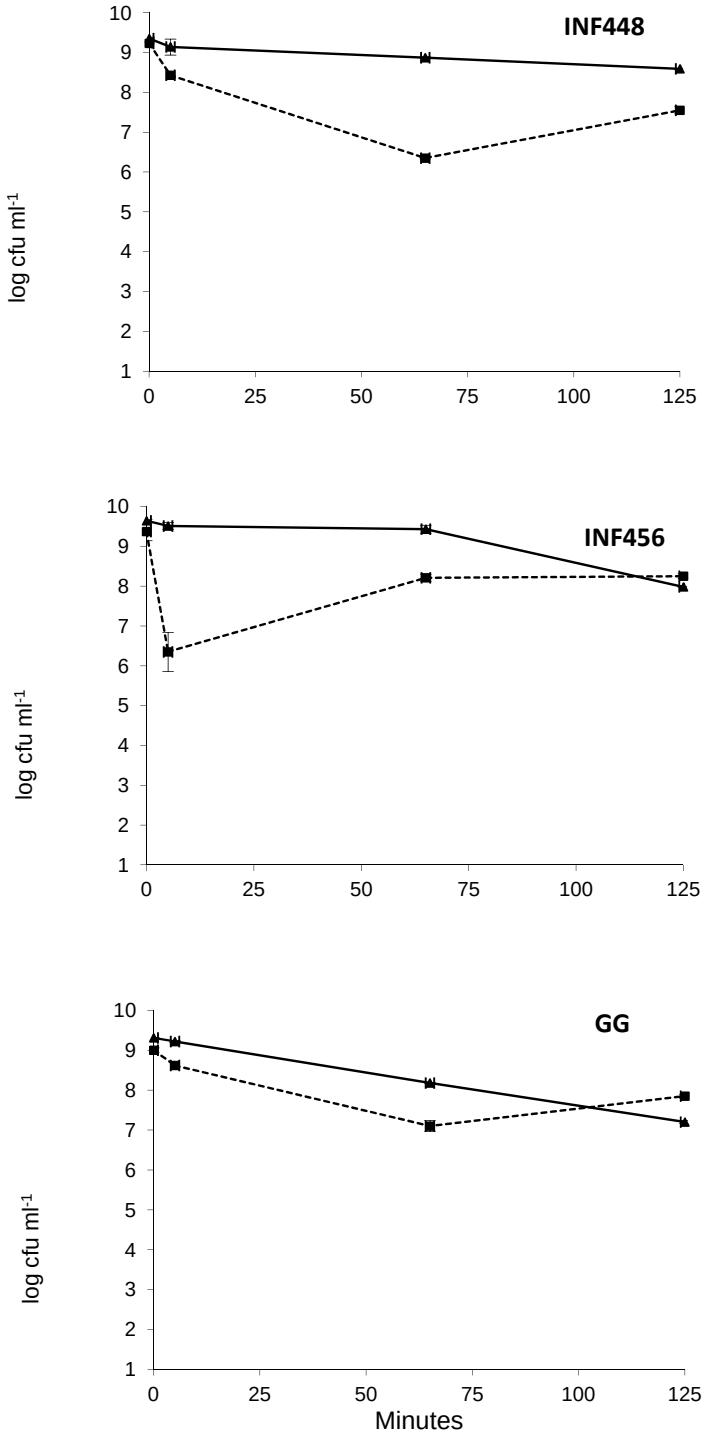
Comparison of the evolution of the *Lactococcus* strains and *Enterococcus hirae* INF E1 during digestion in gastric juice (0 – 65 minutes) and duodenum juice (65-125 minutes) (mean ± SD of n=2 determinations). Solid lines (——) strain as washed cells; dotted lines (------) strain in fermented milk. Log 2 means ≤ 100 colony forming units (**cfu**)/ml, thus log 2 (=100) on the y-axis, might be a lower number than 100 cfu/ml because of the sensitivity of the plating method.

Figure 2:

Comparison of the evolution of the *Lactobacillus* strains during digestion in gastric juice (0 – 65 minutes) and duodenum juice (65-125 minutes) (mean ± SD of n=2 determinations). Solid lines (——) strain as washed cells; dotted lines (------) strain in fermented milk.



Faye et al. Figure 1
JDS-11-4705



Faye et al. Figure 2
JDS-11-4705