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APPLICABILITY OF FRUCTOPHILIC LACTIC ACID BACTERIA IN FOOD INDUSTRY

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The aim of this study was to discover fructophilic lactic acid bacteria in Finnish samples and to see their potential for use as probiotics in the food industry. Fructophilic lactic acid bacteria use fructose as the energy source but in some circumstances they can also use other carbohydrates. This study was conducted at the Functional Foods Forum, University of Turku.

The bacteria used in the food industry should survive stresses during food processing and storage. Usually the process includes heating and during storage, so called post-acidification may occur because lactic acid bacteria produce lactic acid. Also, if bacteria are used as probiotics, they should survive to the intestines. Four methods were used to simulate stressful situations which bacteria may encounter in the food industry and after that in the humans gastric and intestinal conditions. These methods were heat shock at 60 and 70 °C, acid tolerance at pH 3.3 and simulated gastric juice and bile juice tolerance.

Based on the results there are only a few strains which survived the tests be considered for follow-up tests in different kind conditions. The heat shock test indicated that some fructophilic lactic acid bacteria have quite good heat tolerance, but the acid tolerance study indicated that all of the fructophilic lactic acid bacteria prefer neutral pH. Based on the result, fructophilic lactic acid bacteria might be suitable for food use.

KEYWORDS:

fructophilic lactic acid bacteria, probiotic, stress tolerance

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FRUKTOFIILISTEN MAITOHAPPOBAKTEERIEN MAHDOLLINEN KÄYTTÖ ELINTARVIKETEOLLISUUDESSA

Opinnäytetyön tavoitteena oli tutkia, onko fruktofiilisiä maitohappobakteereja mahdollista käyttää elintarviketeollisuudessa, jossa niitä mahdollisesti käytettäisiin probiootteina. Fruktofiiliset maitohappobakteerit käyttävät energianaan fruktoosia tai tietyissä olosuhteissa muitakin monosakkarideja. Sokerien fermentointiin fruktofiiliset maitohappobakteerit käyttävät kahta eri fermentointipolkua, homo- sekä heterofermentatiivista. Opinnäytetyö tehtiin Turun yliopiston Funktionaalisten elintarvikkeiden kehittämiskeskuksella.

Tutkimuksessa käytettiin neljää menetelmää, joilla mallinnettiin elintarviketeollisuuden prosessien ja varastoinnin ja ihmiskehon asettamia stressiolosuhteita bakteereille. Bakteerien tulisi selvitä stressiolosuhteista monilukuisina ja lisääntymis- ja elinkykyisinä, jos niitä halutaan käyttää elintarviketeollisuudessa probiootteina. Menetelmät olivat lämpöshokki 60 ja 70 °C:ssa, haponkestävyys pH 3,3:ssa ja stimuloitu maha- ja sappinestekestävyys. Tutkimuksessa käytettiin kuuttatoista fruktofiilistä maitohappobakteerikantaa ja kahta paljon tutkittua probioottikantaa: *Lactobacilus rhamnosus* GG:tä ja *Bifidobacterium animalis* subsp. *lactis* Bb12:ta.

Suurin osa fruktofiilisistä maitohappobakteereista kuoli kaikissa tutkimuksissa. Kaikista tutkimuksista selvisivät aina samat kannat parhaiten. Näitä kantoja voidaan harkita jatkotutkimuksien tekemiseen, koska niillä voisi olla todella hyvät mahdollisuudet selvitä esimerkiksi korkeammassa pH:ssa. Tulosten pohjalta on mietitty, millaisissa elintarvikkeissa fruktofiilisiä maitohappobakteereita voitaisiin käyttää.

.

ASIASANAT:

fruktofiilinen, maitohappobakteeri, probiootti, stressin sieto

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LIST OF ABBREVIATIONS (OR) SYMBOLS

a_w	Water activity value; indicates how much free water the product contains. The more free water, the easier it is for bacteria, yeasts and molds to grow in it. The water activity scale is from 0 to 1, where 0 is no water at all and 1 is pure water. (Stolaki *et al.*, 2012)
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
Bb12	Probiotic, *Bifidobacterium animalis* subsp. *lactis* strain Bb12.
CFU	Colony forming unit
FYP	Cultivation broth containing fructose as the carbohydrate.
GYP	Cultivation broth where is glucose as a carbohydrate.
LAB	Lactic acid bacteria
LG	Decimal logarithm. Value increase by factor ten.
LGG	Probiotic, *Lactobacillus rhamnosus* strain GG.
NADH	Nicotinamide adenine dinucleotide.
PBS	Phosphate buffer saline
pH	pH value rates alkalinity and acidity. The pH value is from 1 to 14. Value seven is neutral, which means in a solution that alkalinity and acidity are in balance. Values which are smaller than seven are acidity and values above are alkaline One step up or down increases or decreases by a factor of ten. (Tekniikan kemia, 2008)
ROS	Reactive oxygen species.

1 INTRODUCTION

From ancient times people have been using soured milk products as part of their nutrition. Early on they noticed the health benefits of fermented foods (Stolaki *et al.*, 2012). People did not know that it was the antimicrobials (e.g. lactic acid, acetic acid, hydrogen peroxide and bacteriocins) of lactic acid bacteria, which were inhibiting the growth of pathogenic and spoilage microorganisms (Silva *et al.*, 2001). In the early 20th century lactic acid bacteria (LAB) were identified and their first taxonomical classification (cellular morphology, mode of glucose fermentation, optimal temperature for growth and sugar utilization patterns) was provided by Orla-Jensen in 1919 and it is still part of the identification of LAB (Gueimonde *et al.*, 2012).

Recent studies have identified new lactic acid bacteria strains from fructose rich niches (e.g. from fruit peels, flowers, plants and the gut of bees). These LAB are so called fructophilic lactic acid bacteria because they prefer fructose over glucose. Fructophilic lactic acid bacteria can also use glucose as a source of energy but they need an external electron acceptor for that purpose. They can be divided into two groups: ”obligately” and facultatively fructophilic LAB. (Endo *et al.,* 2009; Koch & Schmid.Hempel, 2011)

Nowadays there are many different probiotic foods and feeds, and in all products probiotic bacteria have to cope with different stress situations during the process and storage. These stresses are related to different temperatures during process and storage (high and low), water activity (a_W), acidity, oxygen, and presence of other microorganisms or harmful chemicals. Of course, before the bacteria strains can be used, they have to be capable of being stored and remaining viable after storage. Usually either freezing or drying is used for stocking. It is also important to know how the process and storage may affect the strains; not only should they survive each stress situation, but they should still be viable and able to multiply. (Stolaki *et al.*, 2012)

2 LACTIC ACID BACTERIA

Lactic acid bacteria (LAB) can be found in fermented food, plants, fruits and berries. Usually LAB live in nutrition rich niches, and so LAB can be found in many foods, but they are also part of the normal human gut flora. LAB are a group of bacteria which share several common characteristics, e.g. in metabolism and physiology. There is no one way to describe lactic acid bacteria. Of course there are some general descriptions for LAB, which are good for many genera. Such descriptions are accurate in the standard or normal situation. A “typical” lactic acid bacterium is a Gram-positive, non-respiring, non-sporing coccus or rod. They are catalase-negative, acid-tolerant, and fastidious, and their major end product from fermentation of carbohydrates is lactic acid. Typical LAB are aerotolerant anaerobic. (Axelsson, 1998)

The LAB can be divided into two groups by the difference in the way they metabolize glucose. The first convention is the use of glycolysis (Embed-Meyerhof pathway), which produces 2 moles of lactic acid from 1 mole of glucose. This type of fermentation is called homofermentative. The other way is heterofermentative, where the LAB convert glucose to lactic acid, carbon dioxide and ethanol or acetic acid. Of course these pathways need standard conditions, a non-limited concentration of sugar and another growth factors (e.g. amino acids, vitamins and nucleic acid precursors) and limited oxygen availability (Axelsson, 1998). The fermentation pathway of LAB can be tested with a gas (CO_2) production test, which can be performed e.g. by using the Durham tube (Endo *et al.*, 2009).

2.1 Fructophilic lactic acid bacteria

Fructophilic lactic acid bacteria inhabit fructoce-rich niches, e.g. berries and fruits, but fructophilic LAB can also be found in the guts of bumblebees (Koch & Schmid-Hempel, 2011), in the gut lumen of ants (i.e. *Camponotus japonicus)* (He *et al*., 2011) and in the crop and midgut of some fruit flies (i.e. Australian tropical fruit fly) (Thaochan *et al*.,2010).

Fructophilic LAB, e.g. *Fructobacillus fructosus*, grow well on D-fructose (He *et al*., 2011) and are most likely heterofermentative when the end product from D-fructose is lactic acid, acetic acid and carbon dioxide (Endo *et al*., 2009). Fructophilic lactic acid bacteria can be divided into at least two different groups. The first group contains e.g. the *Lactobacillus kunkeei* and *Fructobacillus* species, which grow on D-fructose and also on D-glucose if there is pyruvate or oxygen available as external electron acceptor. Strains in the first group are classified as "obligatory" fructophilic lactic acid bacteria. The second group contain *Lactobacillus florum.* (Endo *et al.*, 2010) These bacteria grow well on D-fructose and on D-glucose if electron acceptors are present. Still, without the electron acceptors, these bacteria are able to grow, but at a delayed rate. Strains in the second group are classified as facultative fructophilic lactic acid bacteria. Even though all LAB are identified as heterofermentative, there are differences in the end products. "Obligatory" fructophilic lactic acid bacteria mainly produce lactic acid, acetic acid and a small amount of ethanol from D-glucose. Facultative fructophilic LAB produce lactic acid, acetic acid and ethanol from D-glucose, but there is a difference in the ratio which is recorded for heterofermentative lactic acid bacteria. (Endo *et al.*, 2009)

2.2 Fermentation pathways

Lactic acid bacteria use different kind of carbohydrates to produce cellular energy e.g. the *Lactococcus* genus uses lactose (Todar, 2008a) and the *Fructobacillus* genus uses fructose (Endo *et al.*, 2009).

2.2.1 Homofermentation pathway

In the homofermentation pathway one glucose molecule is converted into one molecule of lactic acid. The conversion follows the Embden-Meyerhof-Parna's (EMP) glycolytic pathway whereby glucose, the sixcarbon molecule, is first phosphorylated and after that isomerized. Aldolase cleaves fructose-1,6-diphosphate into glyceraldehyde-3-phosphate and dihydroxyacetonephosphate, which are converted to pyruvate. During the conversion two molecules of ATP are produced by substrate-level phosphorylation at two sites. (Todar, 2008a)

Also, two molecules of NAD^+ are reduced to NADH (Axelsson, 1998). To reduce pyruvate to lactic acid bacteria uses NADH molecules by reoxiditation. The balance of a redox is thus obtained and the lactic acid is the only end product. (Adams & Moss, 2000)

2.2.2 Heterofermentation pathway

Heterofermentation bacteria produce roughly equimolar amounts of lactate, carbon dioxide and ethanol or acetate from glucose. They do not have any aldolase enzyme and they transform glucose into a pentose by oxidation and decarboxylation. Phosphoketolase enzyme cleaves the pentose into glyceraldehyde-3-phosphate and acetyl phosphate. The triose phosphate follows the same pathway as in homofermentation and gives also two molecules of ATP. What happens to the acetyl phosphate depends on which electron acceptor is available. At the same time two molecules of NAD^+ are regenerated from NADH. If there is oxygen present NAD^+ can be regenerated by peroxidase and NADH oxidase and acetyl phosphate is left available for conversion to acetate. The use of oxygen as an electron acceptor increases the overall ATP yield from one to two molecules of ATP. When this happens, a higher cell mass is yielded. The same effect can be achieved with other electron acceptors. (Adams & Moss, 2000)

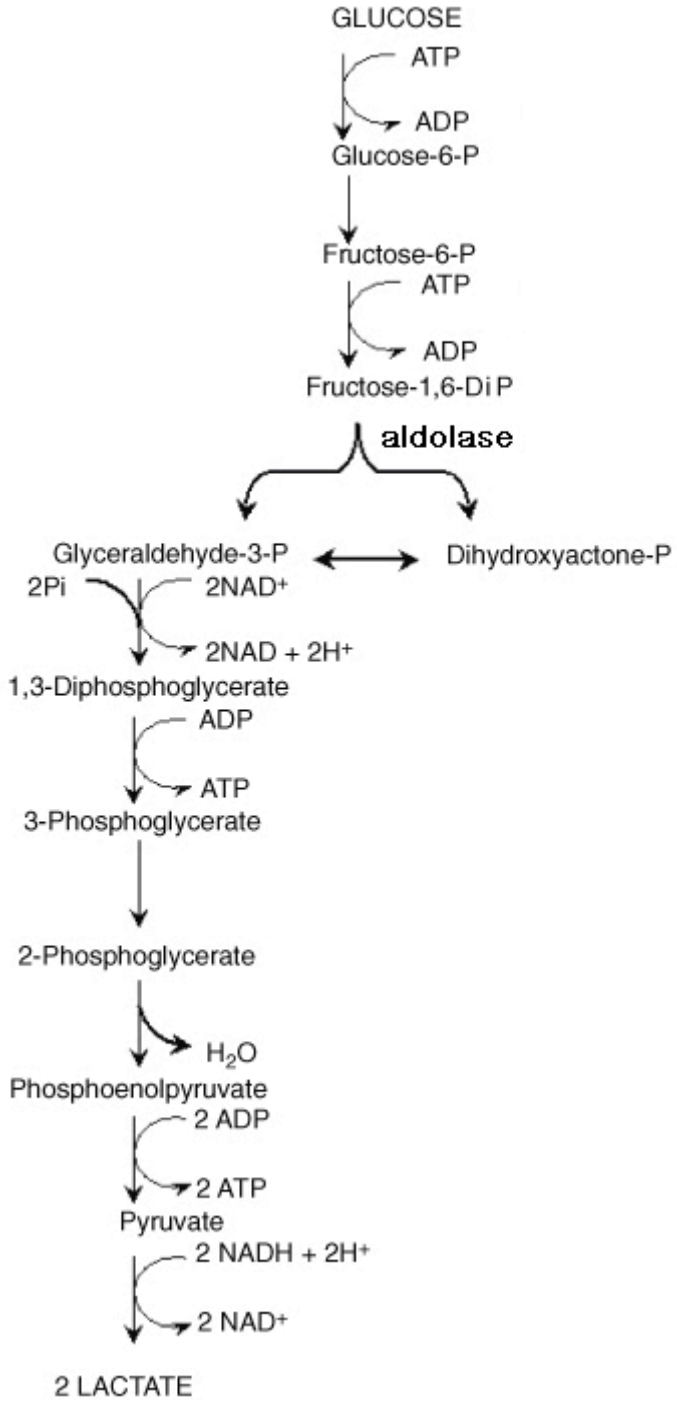

Figure 1 Homofermentation pathway in lactic acid bacteria (Todar, 2008a)

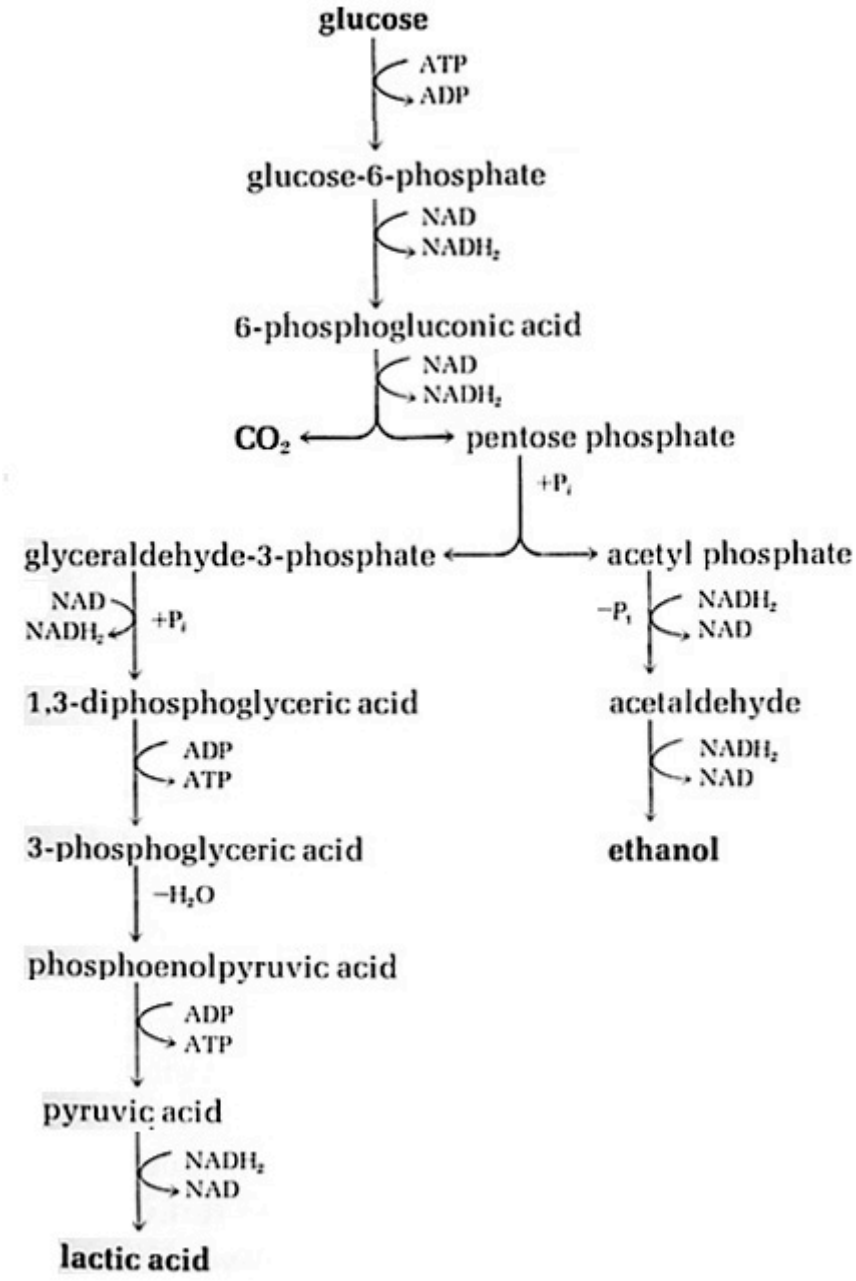

Figure 1 Heterofermentation pathway in lactic acid bacteria (Todar, 2008b)

3 PROBIOTICS AND PROBIOTIC PRUDUCTS

3.1 Lactobacillus rhamnosus GG

Bacteria in the genus of *Lactobacillus* are Gram-positive and microaerophilic. They can inhabit many different niches such as human mucosal surfaces, dairy environments and soils and plants. *Lactobacillus rhamnosus* can be found in the human oral cavity, where it provides protection against harmful bacteria but can also contribute to dental erosion. The sugar fermentation of *Lactobacillus* genus can be of three different kinds: Bacteria in the first group are obligately homofermentative species, but they can switch from homofermentation to hetefofermentation under some conditions. Lactobacilli in the group two are facultatively heterofermentative, so their major end produce is lactic acid but they also produce ethanol and CO_2 in equimolar amounts if any electron acceptors are available. Group three comprises bacteria, which are obligatorily heterofermentative. They can use the pentose phosphate pathway. (Barrangou, 2012)

Lactobacillus rhamnosus GG (ATCC 53103, LGG®) was isolated from infant feces in 1983 and it has been used in foods from 1990. It is the most studied probiotic bacterium in humans and experimental animals. There are many studies which show that *Lactobacillus rhamnosus GG* has heath promoting effects, such as i.e. decreasing respiratory infections in children and reducing the duration of acute diarrhoea. (Kekkonen *et al.*, 2009)

3.2 Bifidobacteria and *Bifidobacterium animalis* subsp. *lactis* BB12

The history of the *Bifidobacterium* genus started as early as 1899 when Tissier identified bifidobacteria from stool samples of breast-fed infants. It was then named *Bacillus bifidus.* Since the year 1973 bifidobacteria have been a distinct genus (i.e. *Bifidobacterium).* By then it had been possible to identify 11 species.

(Ventura *et al.*, 2012) By today 33 species have been identified (Ventura *et al.*, 2007). One of these species is *Bifidobacterium animalis* subsp. *lactis BB12*, which was used in this thesis as a comparison for fructophilic lactic acid bacteria.

Bifidobacteria are Gram-positive, non-spore-forming, non-motile (Solano-Aguilar, 2008) and obligately anaerobic (Biavat *et al.*) or microaerophylic (Ventura *et al.*, 2012) organisms. They are also sensitive to heat (Sun & Griffiths, 2000). Bacteria of the *Bifidobacterium* genus can be found in humans (i.e. in the vagina or in feces), some animals feces, sewage (i.e. *B. minimum* and *B. subtile*) (Biavat *et al.*), oral cavity, food and the insect intestines (Ventura *et al.*, 2012). For each *Bifidobacterium* species is known the optimum temperature, pH, how they manage in oxygen, which kind of cell wall structure they have, what they need for nutrition, how their antagonist activity is and what is their susceptibility to antibiotics. (Biavat *et al.*)

Bifidobacterium animalis subsp. *lactis* is the most detected bifidobacterium in probiotic foods, even though other bifidobacteria have probiotic properties also. *Bifidobacterium animalis* subsp. *lactis* is more stable in fermented milk products and more resistant to environmental stress than other *Bifidobacterium species.* (Gueimonde *et al.*, 2012)

3.3 Probiotics in food

Health effects are achieved if probiotic bacteria survive during processing of food, in storage conditions and through human digestion to the gut (Gueimonde *et al.*, 2012). At the gut they should adapt and be viable (Stolaki *et al.*, 2012). Probiotic strains need to be tested in stress conditions such as changes in temperature, as well as with regard to their tolerance of oxygen, acid, bile, NaCl and chemicals, and bacteria are able to survive if the a_w (active water) value is high or whether they need a low a_w value before strains can be used in food. LAB can grow in high water activity and maintain viable conditions with low a_w. It is also important to know if it is possible to genetically modify the strain and how the different strains of specific genus are different from each other,

because there can be a significant difference in the tolerance of stresses. Probiotic strains are often supplied as a frozen culture when they for food applications, but the strains can be also supplied as a dried culture. That means the strain should survive under frozen or dry conditions and be also viable in the product. (Gueimonde *et al*., 2012)

3.3.1 Culture manufacture

Food technology uses many bacteria strains which are carefully tested to be sure they can cope in stressful environments. Stresses cause loss of viability. Good viability guarantees adequate biomass and survival of strain during production and storage. It is relatively easy to test whether the strain is sensitive to stressing environments, such as heat, acid, gastric juice, bile salt, oxygen, freezing, drying, NaCl. Sometimes, if a strain is resistant to one stress, it is resistant to some other stresses, too. Stresses may change the strain's functional and physiological properties, which can be changes in carbohydrate fermentation or difficulties in adhering to the human intestinal mucus. Gene modification is one possibility for enhancing the stress tolerance of a strain, but this is not applicable to bacteria used for food. (Gueimonde *et al.*, 2012)

3.3.2 Freezing and drying

Freezing and drying are used to store strains for food applications, so it is important for the probiotic strain to maintain its viability under these stresses. Nowadays drying is more common than freezing.

The most important issue with freezing is to select a suitable cryoprotectant, which is usually incorporated into the culture medium (Gueimonde *et al.*, 2012). The cryoprotectant is described as "*any additive which can be provided to cells before freezing and yields a higher post-thaw survival than can be obtained in its 'absence'*" (Fulle, 2004).

The drying process comprises water removal. The bacterial suspension can be dried by freeze-drying or spray-drying. Spray-drying is the most commonly used drying method. (Gueimonde *et al.*, 2012) Spray-drying starts with the

atomization of the bacterial suspension. The purpose of atomization is to create optimum conditions for the water to evaporate. The next step is bringing atomized liquid into contact with hot gas in the chamber. (Patel *et al.*, 2009) This is the part which is harmful for bacteria, because the temperature has to be very high for water evaporation and osmotic stress can be relatively harsh. Nonfat milk or trehalose are usually used as protectants to minimize damage in spray-drying. (Gueimonde *et al.*, 2012) Spray-drying is good for producing large amounts of viable bacteria for dairy manufacturing, as bacteria powders can be transported easily and at a low cost and the strains stay viable for prolonged periods. (Silva *et al.*, 2001)

Another drying method is freeze-drying, which is used for the preservation and storage of bacteria and biological samples. Freeze-drying also has some problems. It causes denaturation of some sensitive proteins and it leads to a decrease in bacterial viability. Protectants e.g. glycerol, skimmed milk, trehalose and sucrose are used to protect the bacteria from such damage. (Leslie *et al.*, 1995)

3.3.3 Product manufacture

It is common that in functional foods the number of probiotic bacteria is low because there are some parameters in food production which have a negative impact on the viability of the bacteria. Water activity (a_w) and oxygen concentration have a significant impact on bacterial viability. Chemical and microbiological compositions also cause troubles in surviving. (Gueimonde *et al.*, 2012)

$$a_w = \frac{p}{p_0}$$

Water activity a_w is defined as “the ratio of the vapor pressure of water in a material (p) to the vapor pressure of pure water (p_o) at the same temperature” (Decagon Devices, 2011). a_w can get values between 0 and 1 (Gueimonde *et al.*, 2012). Water activity can be described as “free”, “bound” or “available water”. These three stages are more like energy states than real “boundness”.

The a_w value shows how much free water there is in the sample. Bound water is bound with weak chemical bonds such as hydrogen bonds and ion-dipole bonds. Available water is bound less tightly but microorganisms are still not able to use it, i.e. it is not available. Temperature affects the a_w value due to a chance in the solubility of solutes in the water, water binding, state of the matrix, or dissociation of water. (Decagon Devices, 2011) In spray-drying a_w value is directly proportional to the temperature: the lower outlet temperature is, the higher is the a_w value, because more moisture is left in the product. If the a_w value is higher than 0.8, the product is “moist” (Chaplin, 2012) and it is a good growth matrix for moulds, when most of the bacteria need an a_w of above 0.90 (Decagon Devices, 2011). If the value is below 0.7 it is low and the product is “dry” and it is not a good growth matrix for bacteria or moulds or yeast (Chaplin, 2012), but with probiotics a low a_w value is good for stability; dried functional products ($a_w < 0.25$) can have shelf-life of months (Gueimonde *et al.*, 2012).

Oxygen has a highly negative impact on the viability of lactic acid bacteria and bifidobacteria, which are catalase-negative (Axelsson, 1998). The damage which is due to oxygen is usually caused by so-called reactive oxygen species (ROS) (Gueimonde *et al.*, 2012). ROS are a problem of any aerobic organism because they are caused by the use of oxygen. ROS are very harmful for cells, DNA or any molecule, because they are highly reactive and therefore they can mutate DNA or break molecules. For example hydroxyl radical, superoxide and hydrogen peroxide are reactive oxygen species. (Nordberg & Arnér, 2001)

Chemicals and microbiological components are able to influence the probiotic strain both positively and negatively. Therefore, the effects of using of chemicals or microorganisms should always be considered. For example, low pH and food additives (i.e. colorants and flavourings) may have a negative effect on the growth of the probiotic strain. (Gueimonde *et al.*, 2012) Some chemicals have a positive impact on the stability of the strain, such as prebiotics, which are non-digestible carbohydrates (i.e. inulin, lactulose and some oligosaccharides). They stimulate the proliferation and/or activity of the probiotic strains in intestinal tracts. (Mattila-Sandholm *et al.*, 2002)

3.3.4 Storage

As regards storage conditions the keys to the survival of strains are temperature and oxygen content. A temperature under refrigerator temperature might be the best option for the strains to survive in foods. Low oxygen content can be dealt with by some methods such as oxygen-scavenging agents, active packaging and modified atmosphere. Also a phenomenon called post acidification is known to cause harm to the probiotic strains in dairy products. Usually it is caused by starter and adjunct strains (i.e. *Lactobacillus delbrueckii* subsp. *bulgarius*), which are added to enhance the fermentation process. (Gueimonde *et al.*, 2012)

4 MEDIA

4.1 FYP Broth and agar

1 % FYP broth contains 10 g/L of fructose and 30 % FYP broth contains 300 g/L of fructose, 10 g/L of yeast extract, 5 g/L of polypeptone, 2 g/L of sodium acetate trihydrate, 10 ml/L of Tween 80, 0.02 g/L of $MgSO_4 \bullet 7H_2O$, 0.02 g/L of $MnSO_4 \bullet 4H_2O$, 2 g/L of $FeSO_4 \bullet 7H_2O$, 2 g/L of NaCl, 0.01 g/L of cycloheximide and 0.01 g/L of sodium azide. In agar plates is also 12 g/L of agar and 5 g/L of calcium carbonate.

In both broth and agar the hexose used is fructose because the strains which were used in this study are fructophilic. They prefer fructose over glucose as a carbon source. They grow poorly on glucose. (Endo *et al.*, 2009)

Yeast extract and peptone are both used for better growth because they contain vital compounds for the living cells. Yeast extract provides the bacteria with vitamins and amino acids and peptone provides the bacteria with amino acids. (Todar, 2008a; Neogen Corporation, 2011)

Tween 80, or Polysorbate 80, is a polyethylene sorbitol ester. It contains 20 ethylene oxide units, 1 sorbitol and 1 oleic acid, which is a primary fatty acid:

usually 70 % of the fatty acids of Tween 80 are oleic acid (RLG, 2006). Tween80 is required for the growth of certain LAB.

$MgSO_4 \bullet 7H_2O$, $MnSO_4 \bullet 4H_2O$ and $FeSO_4 \bullet 7H_2O$ are used because of the minerals which they contain. From $MgSO_4 \bullet 7H_2O$ bacteria get sulphur and magnesium ions, from $MnSO_4 \bullet 4H_2O$ they get manganese ions and $FeSO_4 \bullet 7H_2O$ is a source of iron ions. (Todar, 2008c)

Cycloheximide (CHX) is an antibiotic produced by *Streptomyces griseus*. It inhibits the protein synthesis of eukaryotic cells, which leads to cell growth arrest and cell death. CHX is used to inhibit the growth of yeast and fungi. Most of the bacteria are tolerant to cycloheximide. (Sigma Aldrich, 2006)

Sodium azide, NaN_3, is used to inhibit the growth of aerobic bacteria. (Lichestein & Soule, 1943) In the isolation it has the desired effect, because lactic acid bacteria are facultatively anaerobic so sodium azide does not affect them (Axelsson, 1998).

Agar is used as a firming agent in culturing media. Chemically, agar is a polymer, which is composed of subunits of the sugar galactose. It is an extract from some red-purple marine algae, which are usually harvested in eastern Asia and California. Agar is better in media than gelatin, because bacteria do not degrade agar. (Science Buddies, 2012)

Calcium carbonate does not dissolve in water but it can be dissolved by acid (Advanced Aquarists, 2002). Lactic acid bacteria produce lactic acid when they metabolize carbohydrates as a source of energy. Lactic acid is strong enough to dissolve calcium carbonate and that can be seen as a clear area in the agar. Thus, in the isolation process the clear areas indicate lactic acid bacteria. (Endo *et al.*, 2009)

4.2 GYP Broth

GYP broth is similar to FYP broth, but in the GYP broth is used glucose as a hexose. With this bacteria can be separated which can use both fructose and glucose as an energy source.

4.3 Phosphate buffer saline (PBS)

Phosphate buffer saline contains 8.5 g/L of NaCl, 1.21 g/L of K_2HPO_4 and 0.3 g/L of KH_2PO_4. It is commonly used to dilute substances, because it is isotonic, or its osmolarity and ion concentration are similar to those of the human body, and non-toxic to cells. It does not dry cells as water would. (Protocols Online, 2010)

5 METHODS

5.1 Isolation

First in this thesis was attempted to isolation of fructophilic lactic acid bacteria from Finnish foods and honeys but it did not succeed. After that attempts were made with foreign fruits, but nothing was found. The isolation method (Endo *et al.*, 2009) was the same method which was used to isolate the strains used in this thesis.

Peels or crushed fruits were added to five to 10 ml of 1% FYP broth. The tubes were incubated stabile at 30 °C for 24 hours. Honeys were taken with a 1 µl loop and added to 2 ml of 1% FYP broth. From all suspensions, 20 µl to 2 ml of 1% and 30% FYP were pipetted at 30 °C. 1 µl catalase enzyme of bovine were also pipetted to the broths to protect the cells from H_2O_2 exposure. The incubation took place in aerobic conditions on an orbital shaker whose speed was 300 rpm. Tubes were kept in the shaker as long as the growth could be seen with the bare eye. 30 % FYP broth was used in the isolation as a selective isolation tool, because it is known that a high concentration of sugar inhibits the

growth of some bacteria, but some fructophilic lactic acid bacteria (i.e. *Fructobacillus fructosus and F. pseudoficulneus*) can grow in the presence of the high D-fructose concentration. The orbital shaker was also used because of the selectivity: aerobic conditions inhibit the growth of some LAB, but do not affect the fructophilic lactic acid bacteria, even though they are catalase-negative. (Endo *et al.*, 2009)

The bacterial suspensions were inoculated with a 1 μl loop to 1% FYP agar plates. The plates were incubated for 24 hours at 30 °C. Colonies were selected and inoculated to 1 ml of 1% GYP and 1% FYP broths and incubated at 30 °C for 24 h. The selection of colonies was based on morphological differences such as shape and size of colonies or size of the clear zone around the colonies. The clear zone was formed from the hydrolysis of the $CaCO_3$ by lactic acid. The isolation would have been successful, if bacteria had grown only in the FYP broth and poorly in the GYP broth. (Endo *et al.*, 2009)

5.2 Heat shock

The objective of the heat shock study was to discover if the fructophilic lactic acid bacteria are able to survive in high temperatures. The heat shock study was performed first with all sixteen strains at 60 °C and after that at 70 °C with strains which survived from the first study. A twenty microliter glycerol bacteria suspension was inoculated into two millilitres of 1 % FYP broth and incubated at 30 °C for 24 hours. After the cultivation 50 μl of bacteria suspension was inoculated to 5 ml of 1% FYP broth and incubated at 30 °C for 24 hours. Bacteria were harvested by centrifugation at 1800 g for 5 minutes, washed with PBS buffer and centrifuged again. Supernatants were discarded and the bacteria were re-suspended in 1 ml of PBS. 500 μl of the new bacterial suspension was pipetted to 500 μl of PBS buffer. The bacterial suspensions were held in both temperatures for 10 and 20 minutes and after both durations serial dilutions were made from 10^0 to 10^{-4} (for strain NRIC1058T dilutions were up to 10^{-5} and 10^{-6}) and the dilutions were plated onto FYP agar. The dilutions were performed as in Figure 3 Dilution pathway. The plates were incubated

under aerobic conditions at 30 °C for 72 hours. After incubation colonies were counted and colony forming units per millilitre and decimal logarithm values were calculated. The results from heat shock at 60 °C are shown in Figures 4 – 8 and the results the from heat shock at 70 °C are shown in Figures 9 – 13.

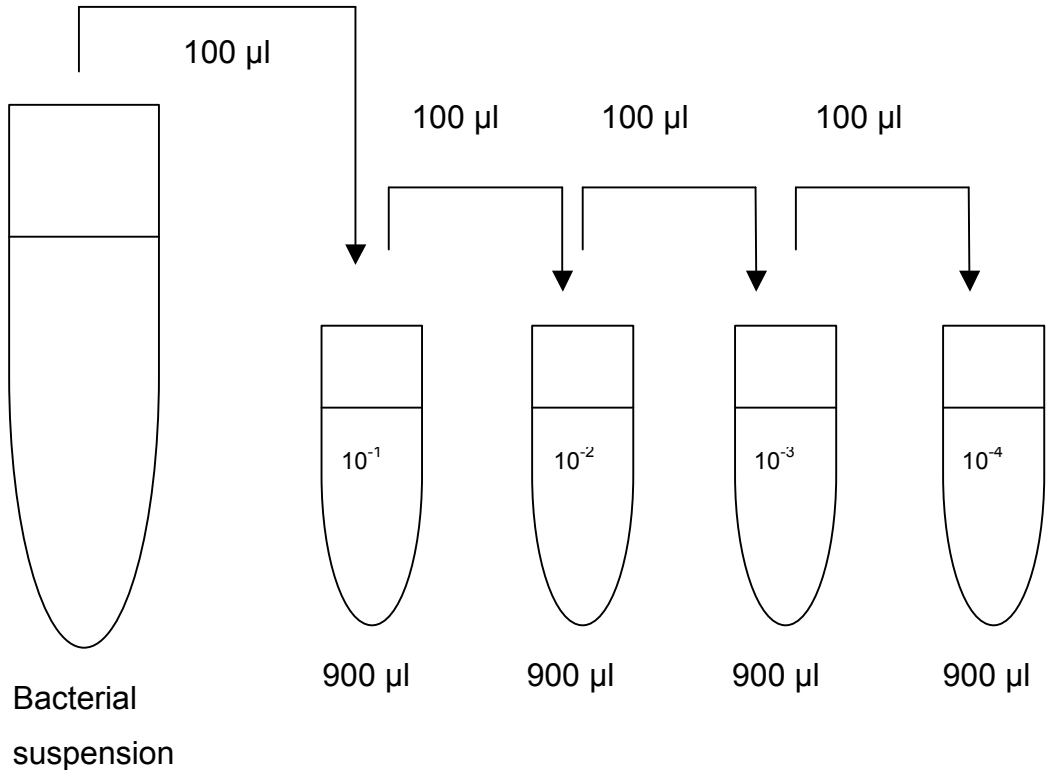

Figure 2 Dilution pathway.

Strains F9-1, F189-1, F214-1 and NRIC1058T were worked twice, for comparison to the first results. These results are shown in Figures 8 and 12.

5.3 Acid tolerance

The acid tolerance study is important, because it adduce whether fructophilic lactic acid bacteria are able to survive in the presence of acid. Start cultivations were made similar to those in the heat shock study. After the harvesting of bacteria they were washed with PBS and centrifuged again. Supernatants were

discarded and bacteria pellets were suspended to 5 ml of FYP whose pH was adjusted to 3.3 with lactic acid. Bacteria were inoculated to 1% FYP agar plates on day zero, one, three and seven. Day three may also have been day two or four. Dilutions were made from the bacterial suspensions on day zero to 10^{-6} and from dilutions 10^{-5} and 10^{-6} were inoculated to the agar plates, on day one dilutions were made from zero to 10^{-4} and on days two, three or four and seven dilutions were made as necessary. Dilutions were made as shown in the figure 3 Dilution pathway.

Bacteria were incubated under aerobic conditions at 30 °C for 72 hours. After the incubation colonies were counted and the decimal logarithm value was calculated. The figures from 10 to 13 show the results of the acid tolerance study.

Lactobacillus rhamnosus GG (LGG) and *Bifidobacterium animalis* subsp *lactis* BB12 strains were used as references because they are known to survive in the presence of acid. The acid tolerance studies were made in the same way as other strains to LGG and BB12. The figures 10 – 13 also show how LGG and BB12 survived at pH 3.3. LGG and BB12 strains were used also in simulated gastric juice and bile juice studies.

5.4 Simulated gastric juice tolerance

The simulated gastric juice tolerance study was performed in a solution similar to human gastric juice. The simulated gastric juice was consisted of 125 mM NaCl, 7 mM KCl, 45 mM $NaHCO_3$ and 3 g/L of pepsin and its pH was adjusted to 2.49 with HCl. (Arboleya, 2010)

In this study strains were grown over night on the orbital shaker (300 rpm) at 30 °C. The next day 50 µl was inoculated to 5 ml of FYP and the solution was grown over night as above. Cells were harvested by centrifugation at 3000 g for 5 min. They were washed with PBS and harvested again by centrifugation. Bacteria were suspended into 500 µl of PBS. 100 µl of bacterial suspension were added to 900 µl of simulated gastric juice at 37 °C for 90 minutes.

Inoculations to FYP plates were made before and after incubation. Before incubation the plates were made from dilutions from 10^{-5} to 10^{-7} depending on how the bacteria were growing and after incubation the plates were made from dilutions 10^{0} – 10^{-4}. The dilutions were performed as shown in Figure 3 Dilution pathway. The agar plates were incubated at aerobic conditions for 72 hours at 30 °C, cells were counted and decimal logarithm results were calculated.

The results of the simulated gastric juice study are shown in Figures 19 – 23.

5.5 Bile juice tolerance

In the bile juice study a solution was prepared, which contained 45 mM of NaCL, 1 g/L of pancreatin and 3 g/L of pepsin (Arboleya, 2010). pH of the solution was adjusted to 8.01 by NaOH.

Strains were cultivated and harvested as in the gastric juice study. The bacteria pellet was suspended into 500 µl of PBS and100 µl of that suspension was pipetted to 900 µl of bile juice. The bile juice bacteria suspensions were incubated for 180 minutes at 37 °C. Inoculations were made before incubation to FYP agar as gastric juice study. After incubation dilutions were made from 10^{0} to 10^{-7} depending on how the bacteria were growing. Dilutions were made as shown in Figure 3 Dilution pathway. The agar plates were incubated under aerobic conditions for 72 hours at 30 °C.

After incubation colonies were counted and decimal logarithm results were calculated. The results from bile juice study are shown in Figures 24 – 28.

6 RESULTS AND DISCUSSION

This chapter shows all the results from the studies. The results of each study are shown in decimal logarithm value from colony forming unit per milliliter and from each study the percentage of survivals has been calculated.

6.1 Results of isolation

Fifty samples were used in the isolation study. First the samples from Finland were used, but since the isolation did not succeed samples from other countries were used. 19 samples out of 50 did not show any bacterial activity. Maybe there were no bacteria or the bacteria did not grow well in fructose. Some lactic acid bacteria could be isolated from 19 samples and incubated to the FYP broth and the GYP broth, but none of these strains were fructophilic.

6.2 Results of heat shock in 60 °C

Eight strains survived the heat shock study in 60 °C, Figures 4 – 9. The strains were *Lactobacillus kunkeei* F20-1,, , *Lactobacillus florum* F9-1 and F17, , *Fructobacillus fructosus* NRIC1058T, *Fructobacillus pseudoficulneus* F189-1, , *Fructobacillus tropaeoli* F214-1, , *Fructobacillus durionis* NRIC0663T, and, *Leuconostoc mesenteroide* subsp. *mesenteroide* NRIC1541T. The heat shock study bring out that the fructophillic lactic acid bacteria strains differ in heat tolerance. It can be said that most fructophillic lactic acid bacteria do not survive heat processing well. Figure 5 shows that *Fructobacillus fructosus* strain NRIC1058T survived the heat shock well, as many as 98 % had survived after 10 minutes and 97 % after 20 minutes, which means an only 0.2–0.3 logarithm unit decrease in the growth. Also *Lactobacillus florum* strain F9-1 is potential heat shock survivor. Its surviving percentages were 89 % after 10 minutes and 77 % after 20 minutes and finally the decrease in the growth was 2 logarithm units. In the literature it is said that the final product should have 10^6-10^7 CFU/ml of bacteria (Gueimonde *et al.*, 2012) and both strains survived that well from the heat shock.

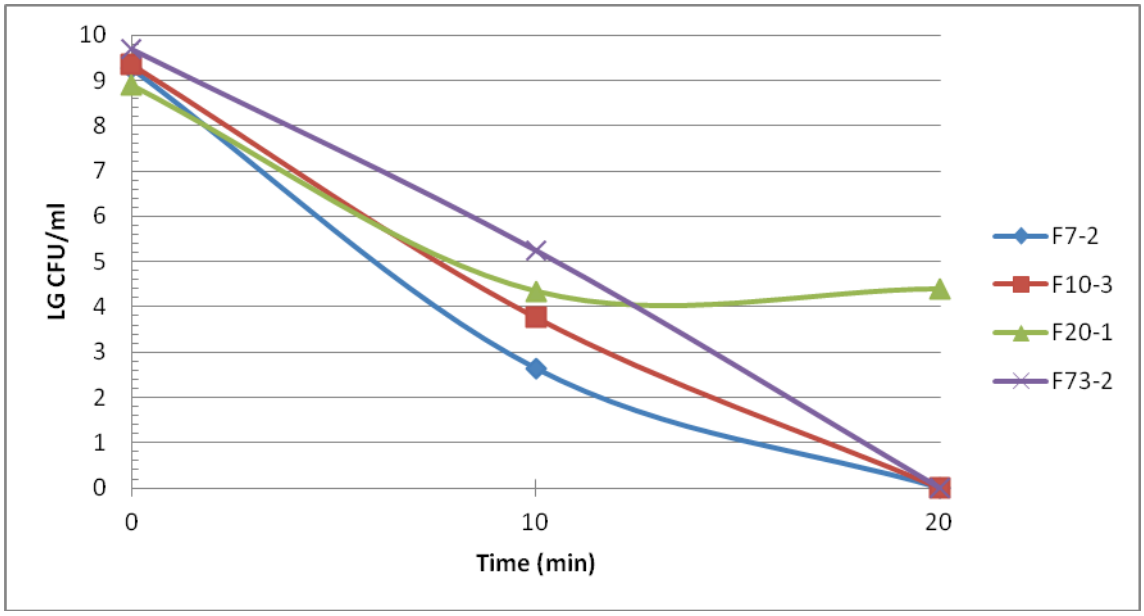

Figure 3 Heat shock results for four *Lactobacillus kunkeei* strains in 60 °C as a function of time.

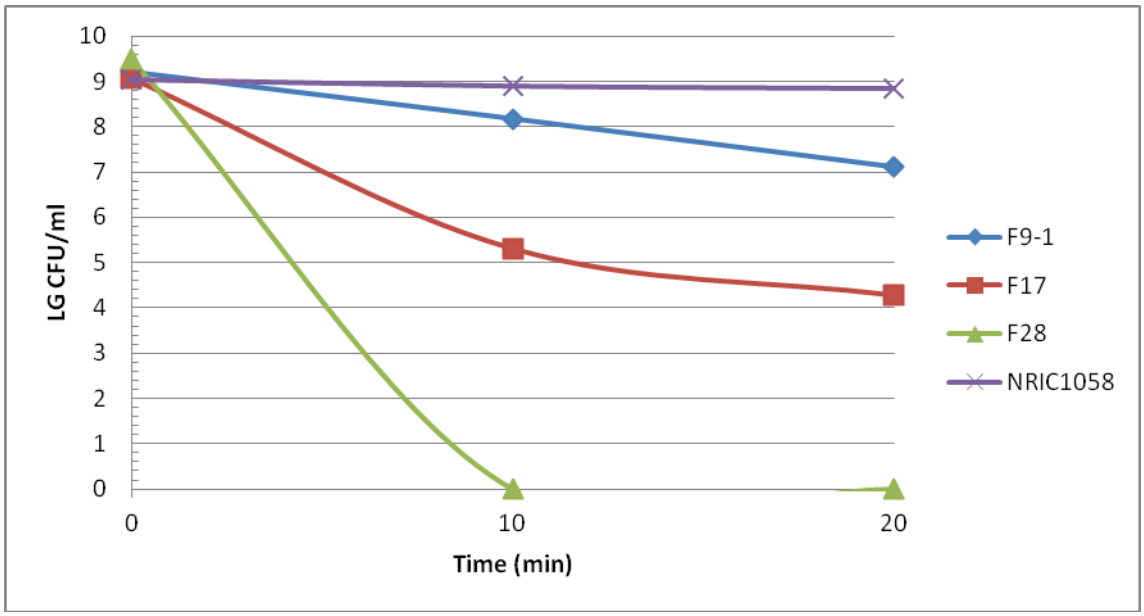

Figure 4 Heat shock results for *Lactobacillus florum* and *Fructobacllus fructosus* in 60 °C as a function of time.

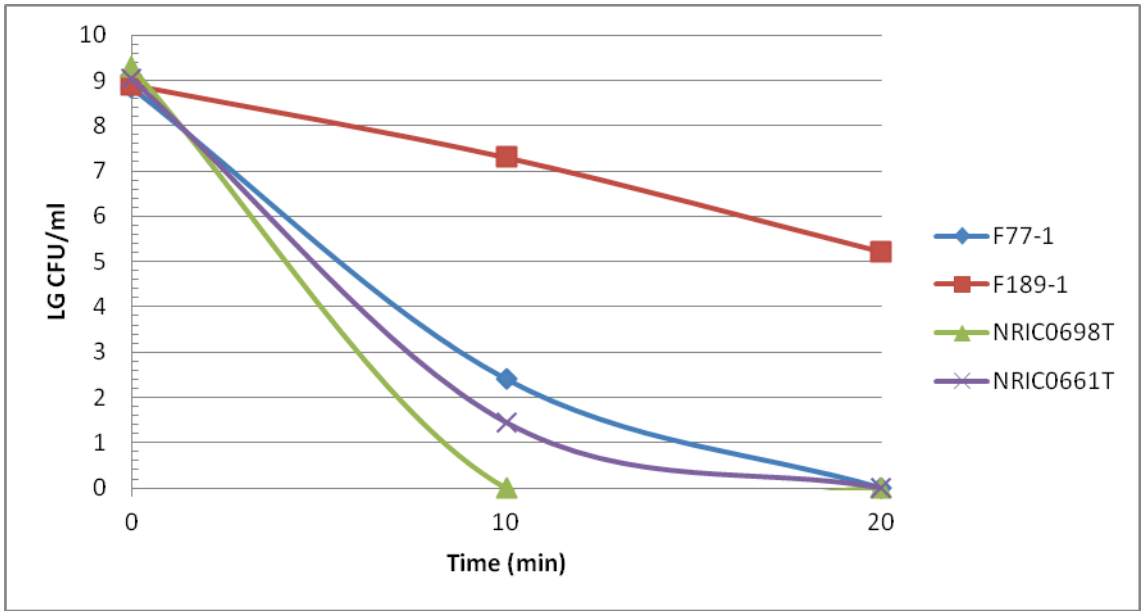

Figure 5 Heat shock results for *Fructobacillus pseudoficulneus* and *F. ficulneus* in 60 °C as a function of time.

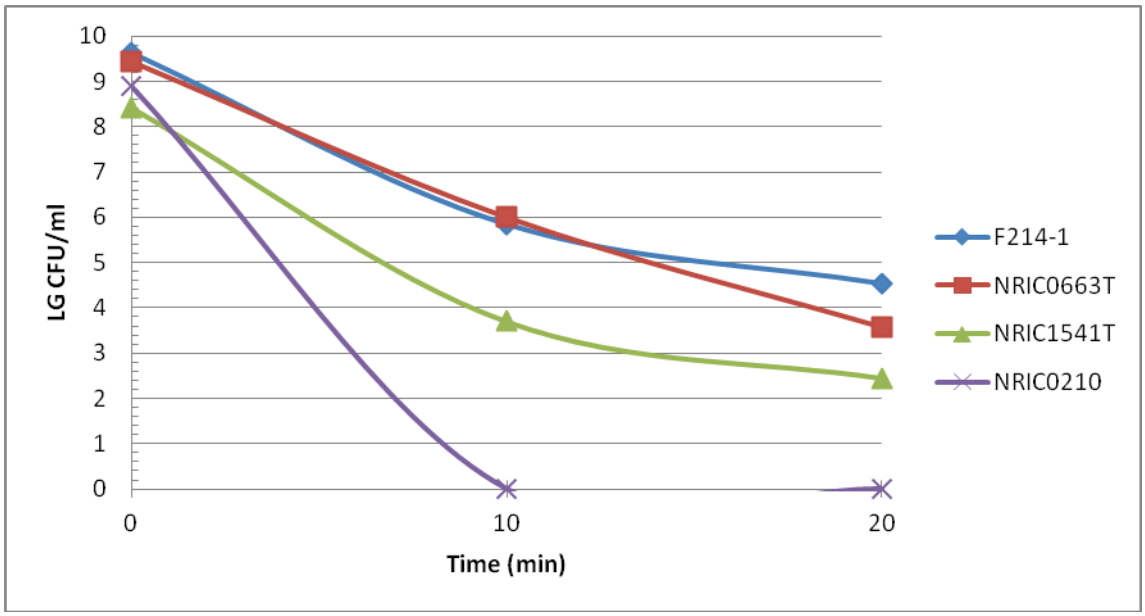

Figure 6 Heat shock results for *Fructobacillus tropaeoli, F. durionis, Leuconostoc mesenteroide* subsp. *mesenteroides* and *L. fallax* in 60 °C as a function of time.

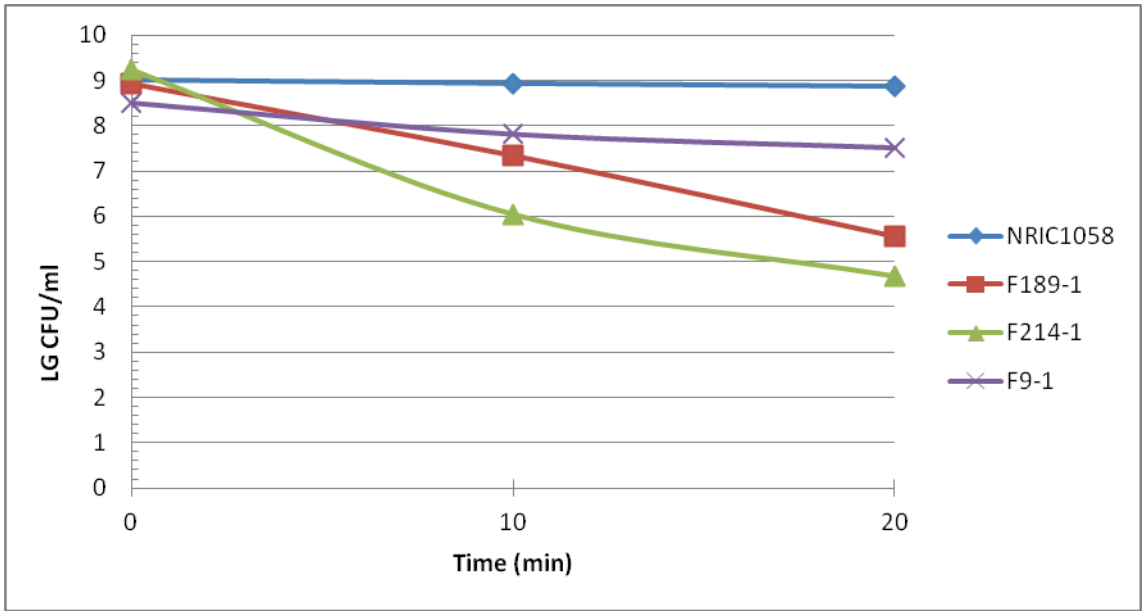

Figure 7 Heat shock results at the second time for *F. fructosus*, *F. pseudoficulneus*. *F. tropaeoli* and *L. florum* in 60 °C as a function of time.

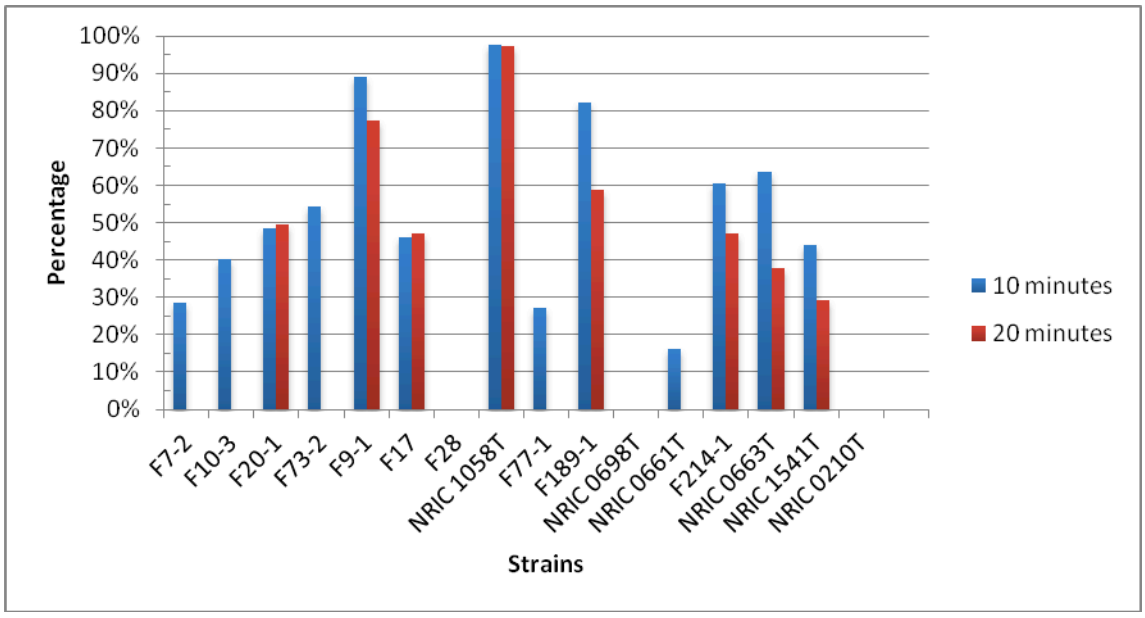

Figure 8 Results of heat shock study in 60 °C as a percentage of survived bacteria.

6.3 Results of heat shock in 70 °C

The heat shock study in 70 °C were performed with eight strains, which survived the first heat shock study. Only four of eight survived in 70 °C for 20 minutes. Again in this study can be seen difference between the same species strains because both *Lactobacillus florum* strains did not survive at 70 °C. The results also show some strains survived differently at 60 °C and 70 °C: The strain NRIC 1058T did not survive as well as strain F9-1 at 70 °C. In turn strain F9-1 did not survive as well at 60 °C.

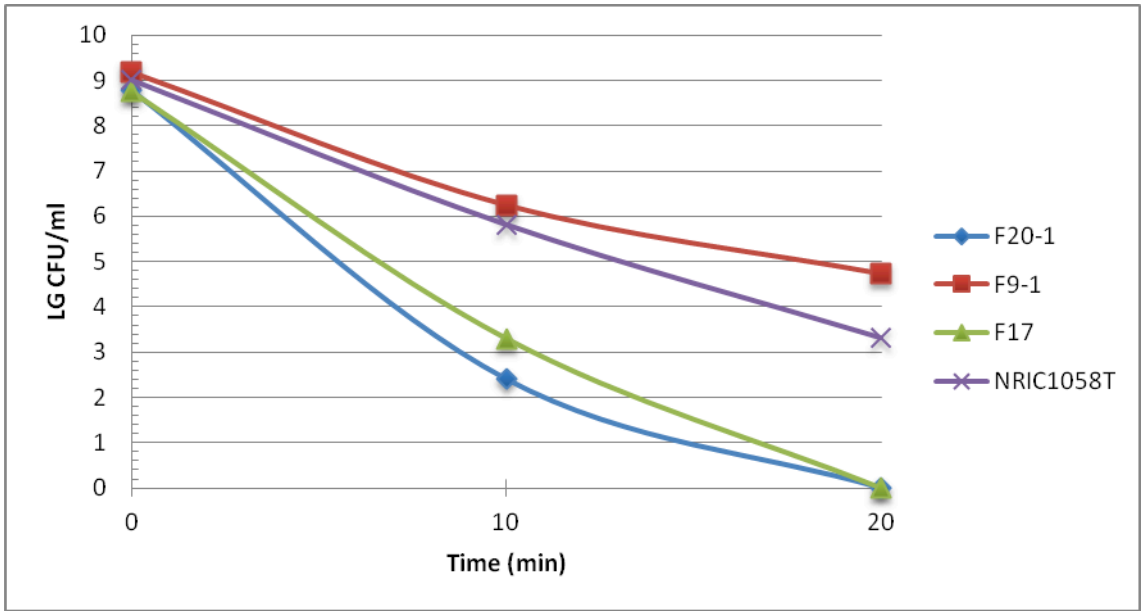

Figure 9 Heat shock results for *Lactobacillus kunkeei, L. florum* and *Fructobacillus fructosus* in 70 °C as a function of time.

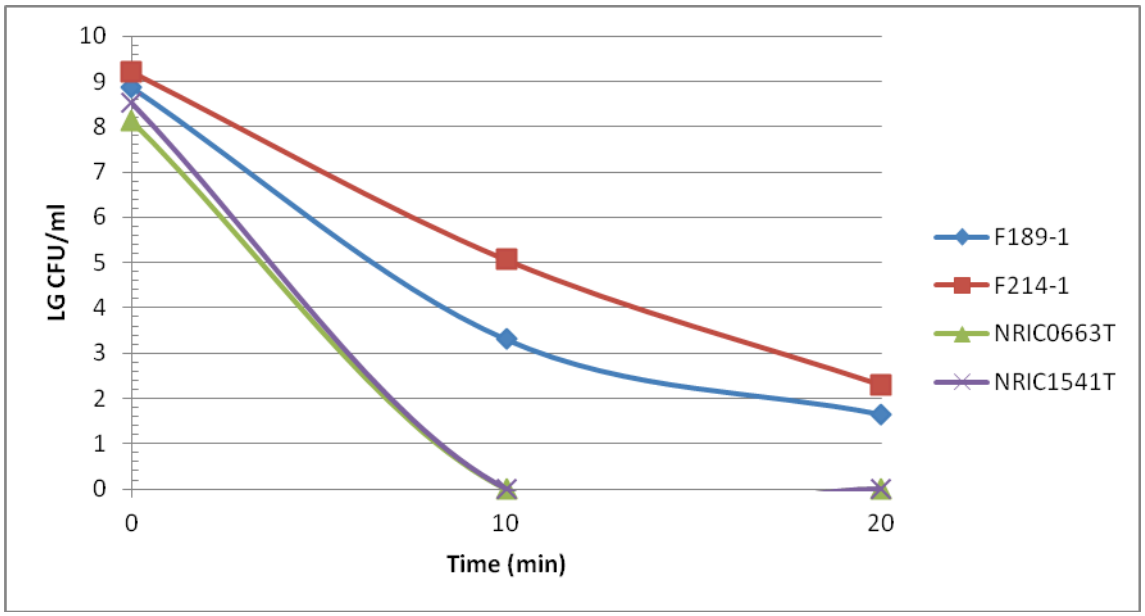

Figure 10 Heat shock results for *Fructobacillus tropaeoli, F. durionis* and *Leuconostoc mesenteroides* subsp. *mesenteroides* in 70 °C as a function of time.

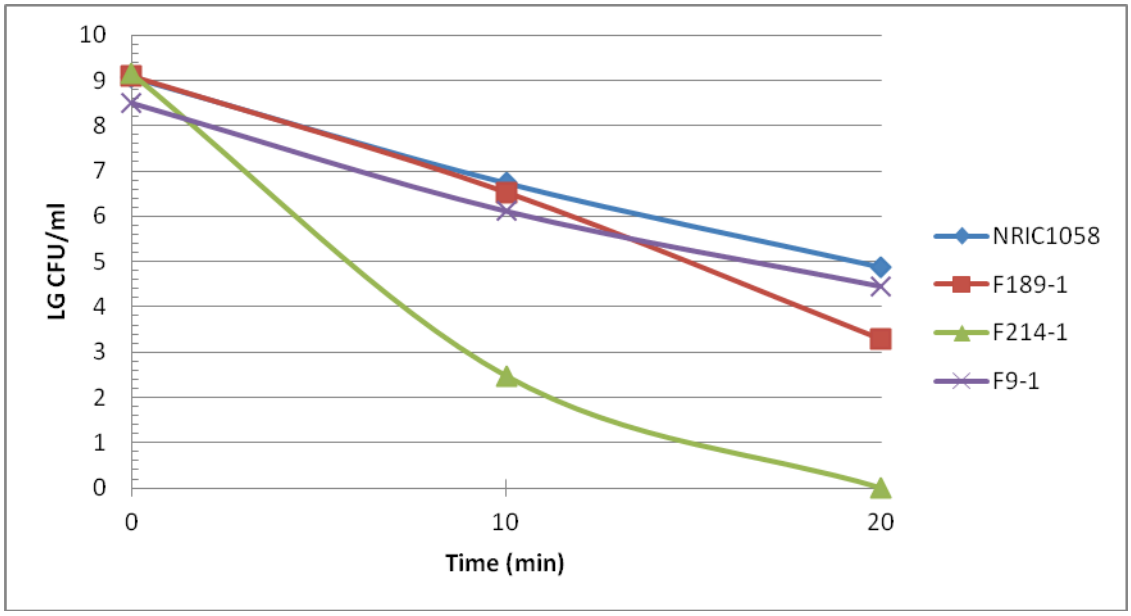

Figure 11 Heat shock results at the second time in 70 °C for *Fructobacillus tropaeoli, F. durionis* and *Leuconostoc mesenteroides* subsp. *mesenteroides* as a function of time.

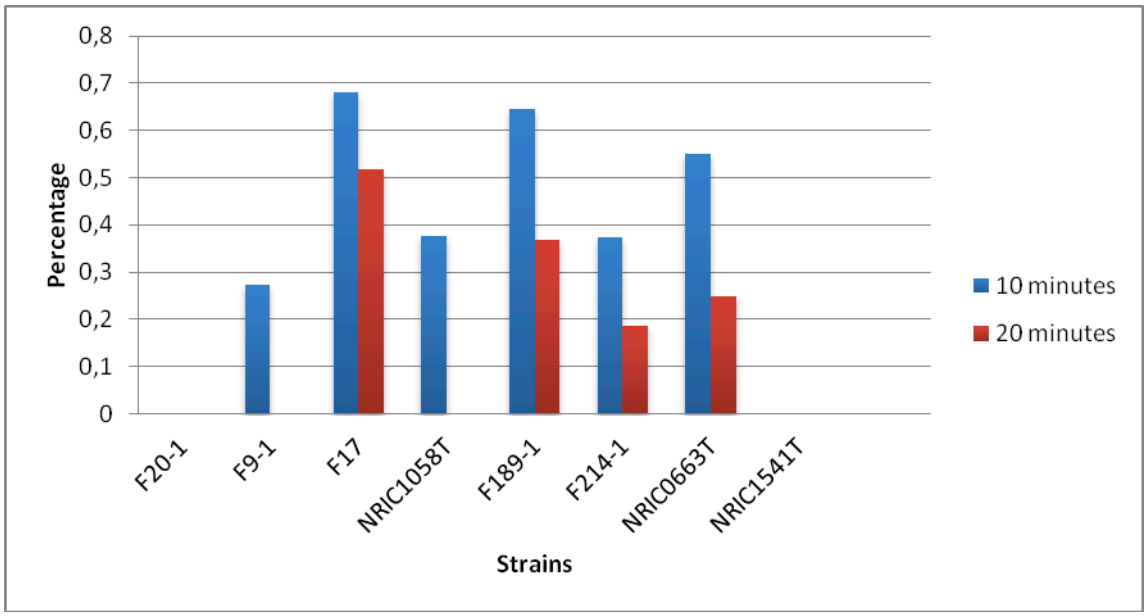

Figure 12 Results of heat shock study in 70 °C as a percentage of survived bacteria.

6.4 Results of acid tolerance in pH 3.3

Only one strain survived the acid tolerance study. It was *Fructobacillus tropaeoli*, F214-1. Still it did not survive so well that there would be living bacteria at least 10^6 CFU/ml after seven days. That is too little for shelf life, which should be a few weeks. It is not surprising that bacteria from *Lactobacillus* genus did not survive at pH 3.3 because they are not able to grow under pH 3.7 (Mortazavian *et al.*, 2012). All the results of the acid tolerance study can be found in Figures 14 – 17 and the results as a percentage of survived bacteria are in Figure 18. Appendixes two and three show results in CFU/ml and as a percentage of survived bacteria from the tables 6 and 10.

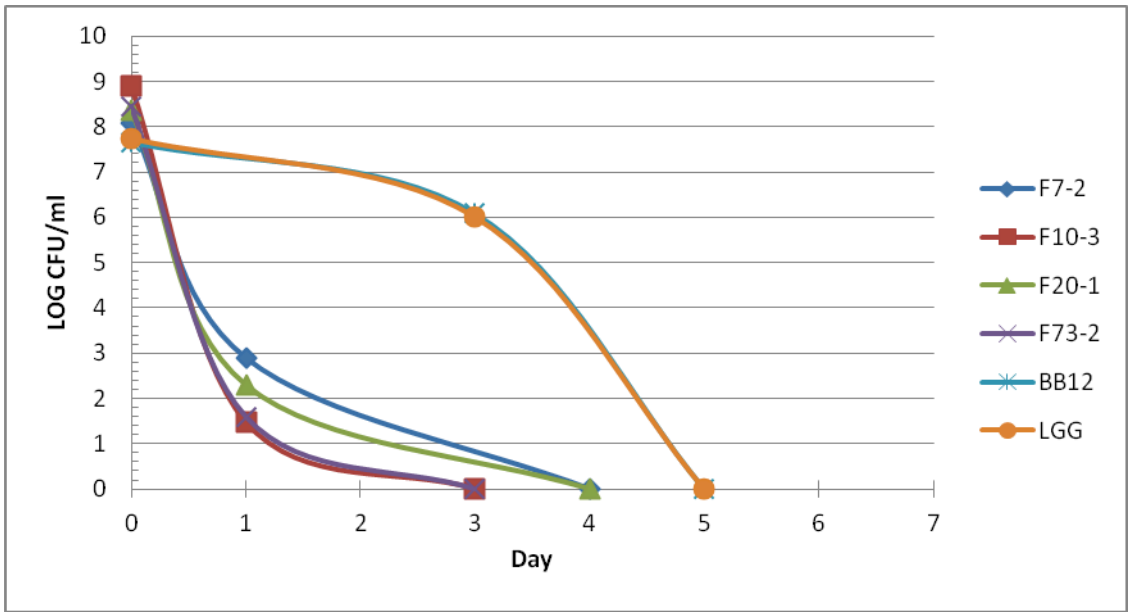

Figure 13 Acid tolerance results, pH 3.3, for *Lactobacillus kunkeei* strains as function of time. BB12 and LGG are known probiotic strains and they are used as

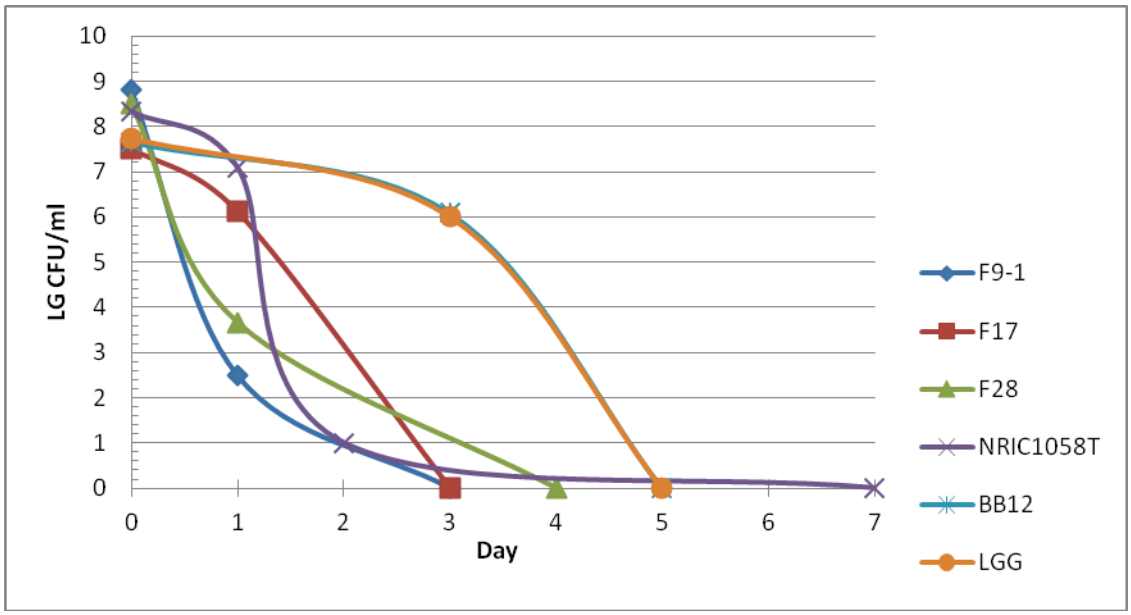

Figure 14 Acid tolerance results, pH 3.3, for *Lactobacillus florum* and *Fructobacillus fructosus* strains as function of time.

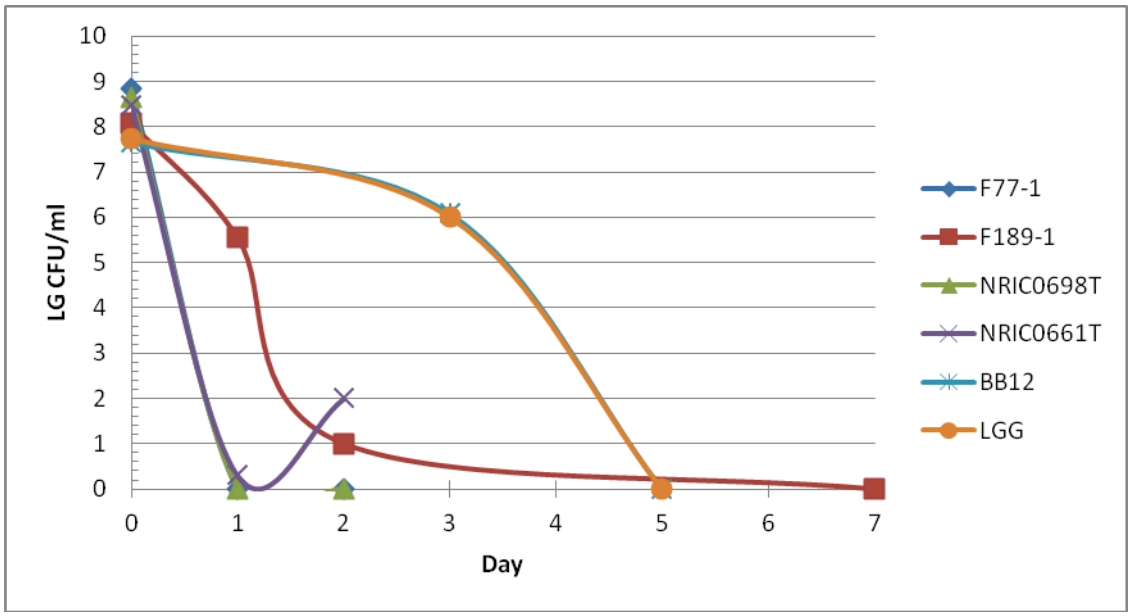

Figure 15 Acid tolerance results, pH 3.3, for *Fructobacillus pseudoficulneus* and *F. ficulneus* strains as function of time.

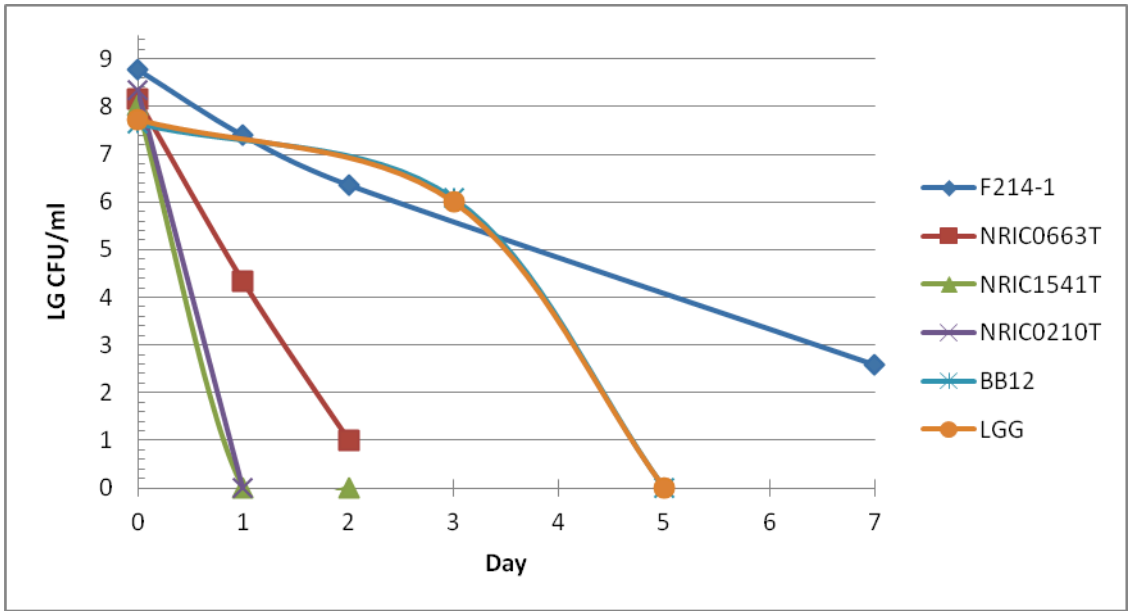

Figure 16 Acid tolerance results, pH 3.3, for *F. tropaeoli, F. durionis, Leuconostoc mesenteroide* subsp. *Mesenteroides* and *L. fallax* strains as function of time.

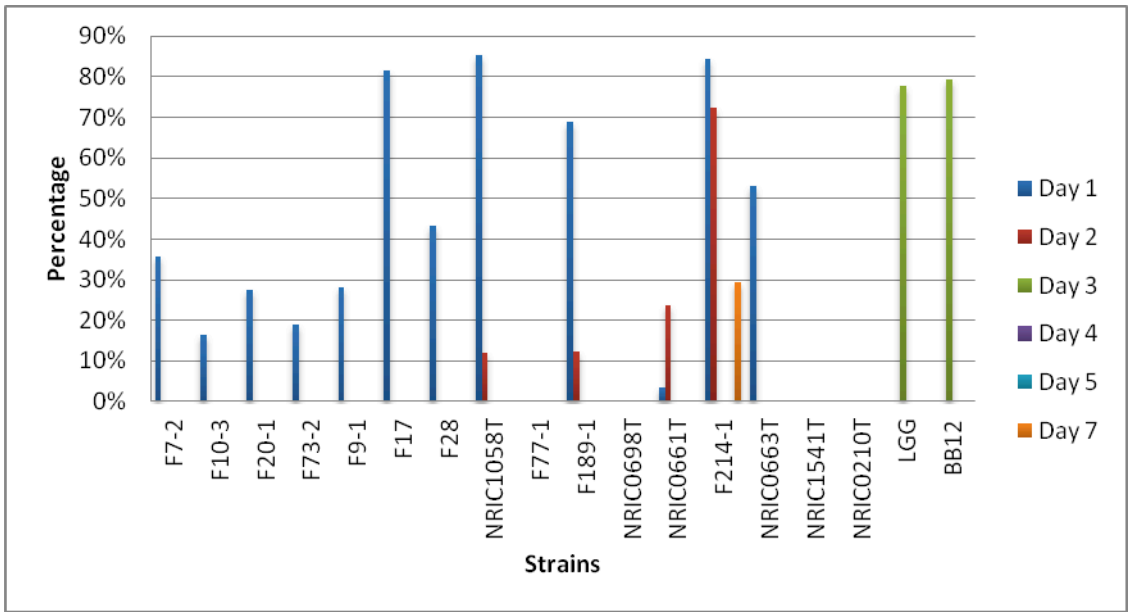

Figure 17 Acid tolerance results as a percentage of survived bacteria at pH 3.3.

6.5 Results of simulated gastric juice study

From the simulated gastric juice study survived seven strains, F10-3, F28, NRIC0661T, NRIC1058T, F189-1, NRIC 1541T and F214-1, and both reference strains. The results of simulated gastric juice study can be found in Figures 19 – 22 and the results as a percentages of survived bacteria can be found in Figure 23. Again the strain F214-1 was the best survivor at this study, it surviving per cent was 66 %.

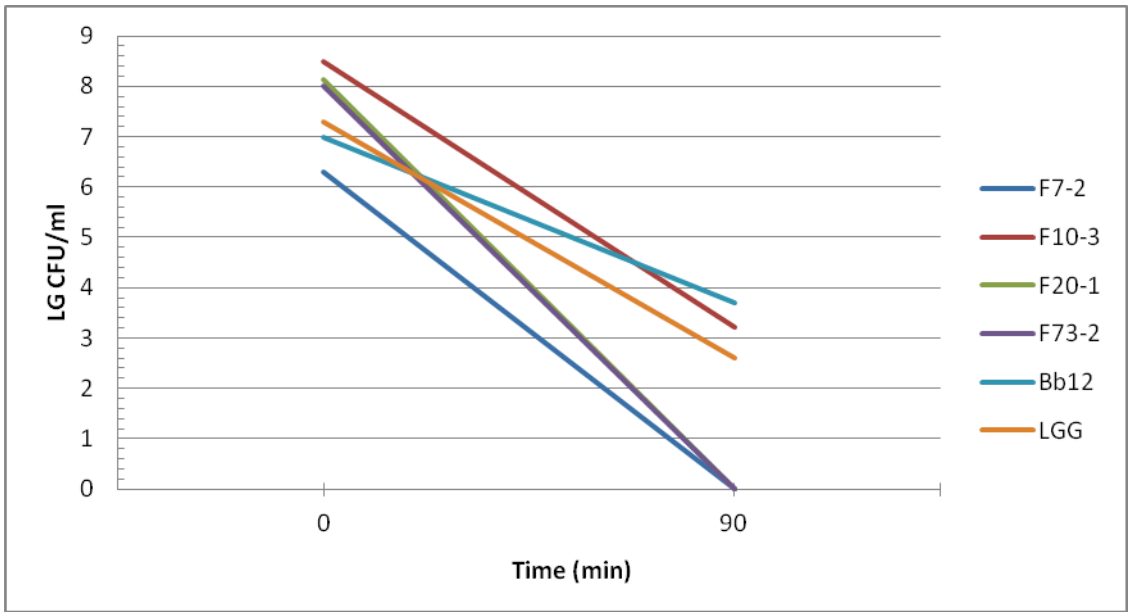

Figure 18 Results of simulated gastric juice study for *Lactobacillus kunkeei* strains as a function of time.

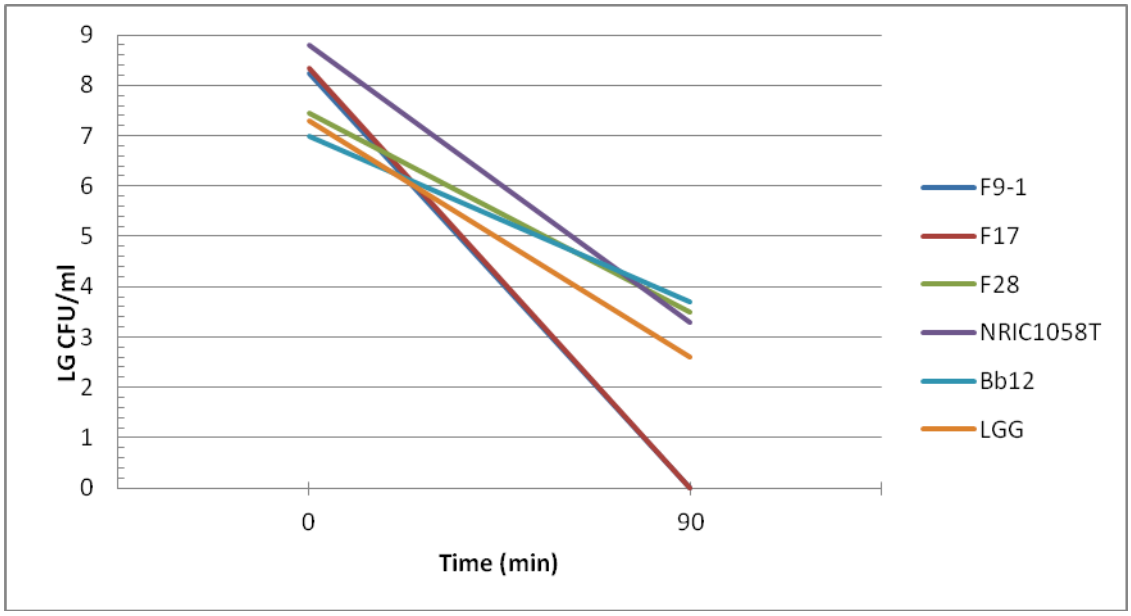

Figure 19 Results of simulated gastric juice study for *Lactobacillus florum* and *Fructobacillus fructosus* strains as a function of time.

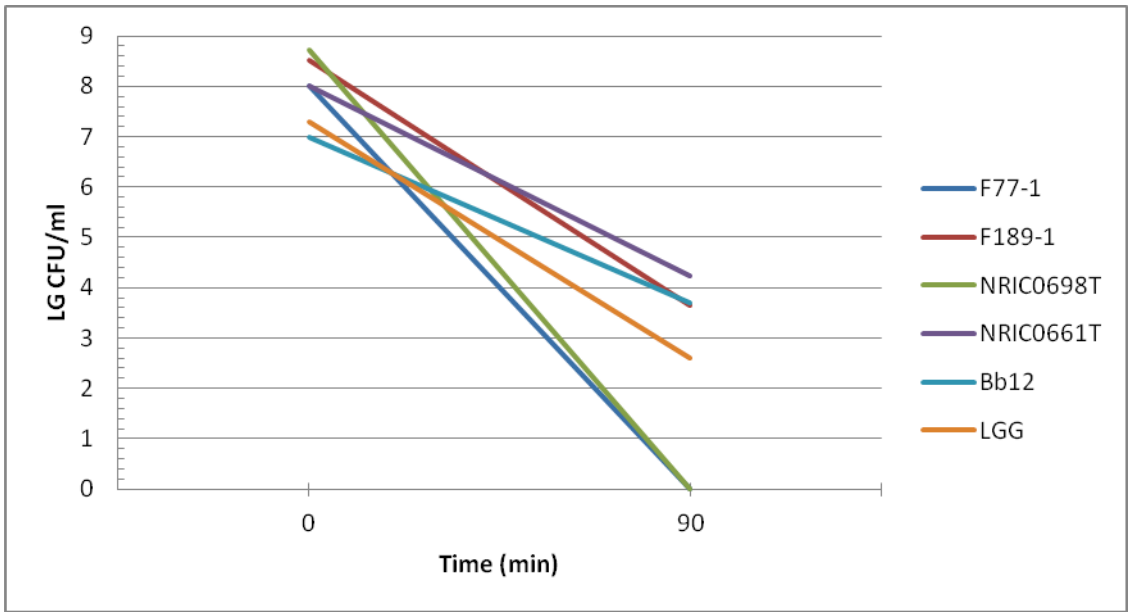

Figure 20 Results of simulated gastric juice study for *Fructobacillus pseudoficulneus* and *F. ficulneus* strains as a function of time.

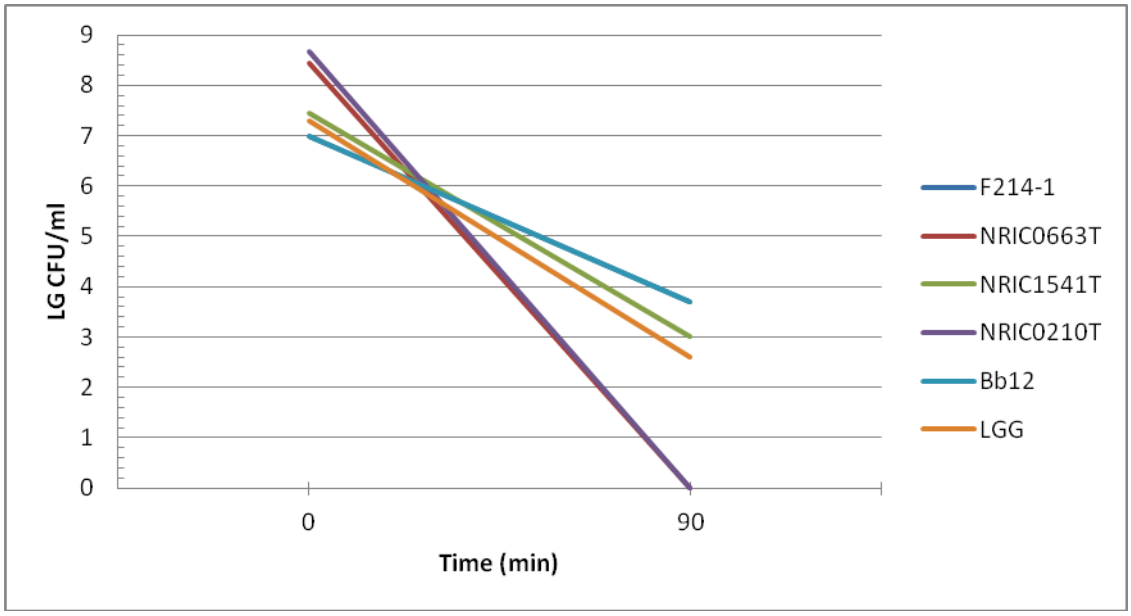

Figure 21 Results of simulated gastric juice study for *F. tropaeoli, F. durionis, Leuconostoc mesenteroide* subsp. *Mesenteroides* and *L. fallax* strains as a function of time.

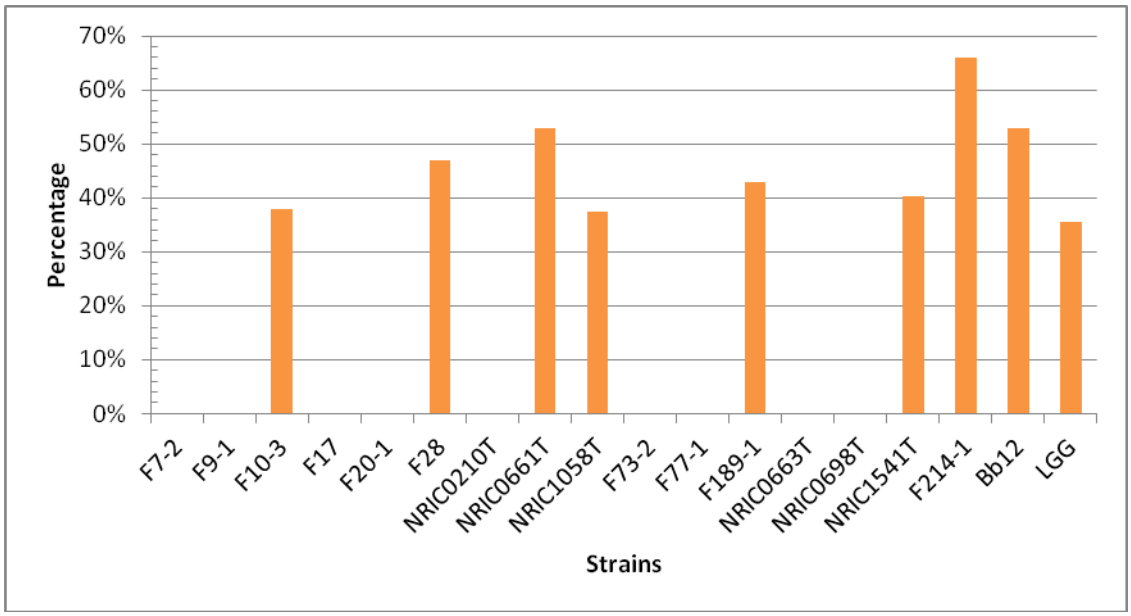

Figure 22 Results of simulated gastric juice study as a percentages of survived bacteria.

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6.6 Results of bile juice study

Most of the strains survived in this study; only three, F28, F77-1 and NRIC0698T, strains did not survive. The results of bile juice study can be found in Figures 24 – 27 and the resutls as a percentages of survived bacteria are in Figure 28. The best survivor was F17, which increased CFU/ml, but it can be caused by bad wash of strain, when there have been fructose and other substances needed to grow. If strain F17 is left out, the survivor is NRIC1058T, whom surviving per cent is 98 %.

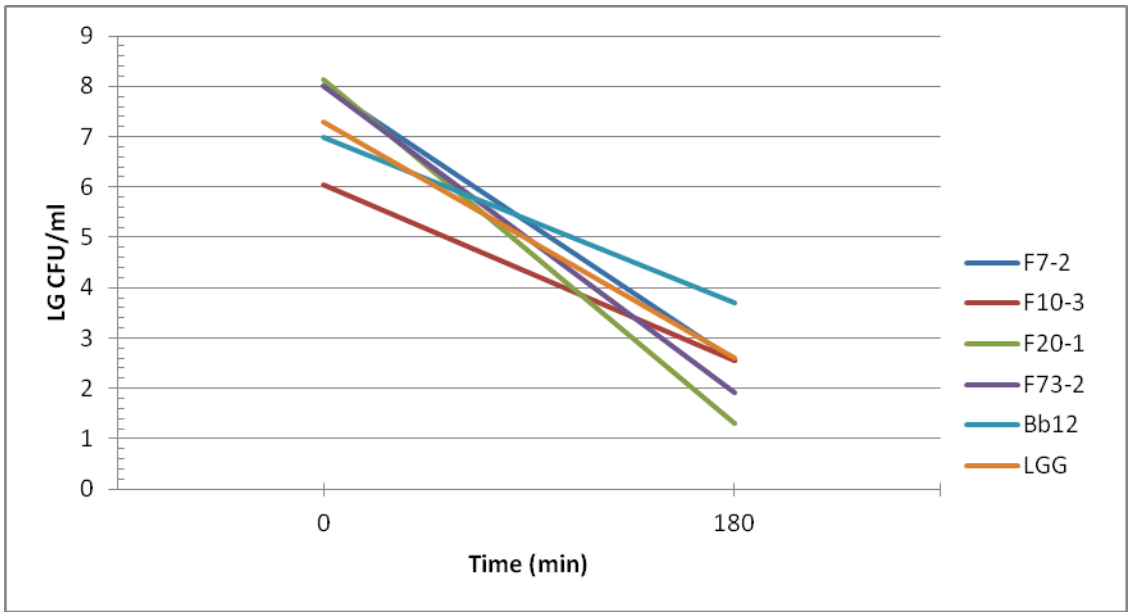

Figure 23 Results of bile juice study for *Lactobacillus kunkeei* strains as a function of time.

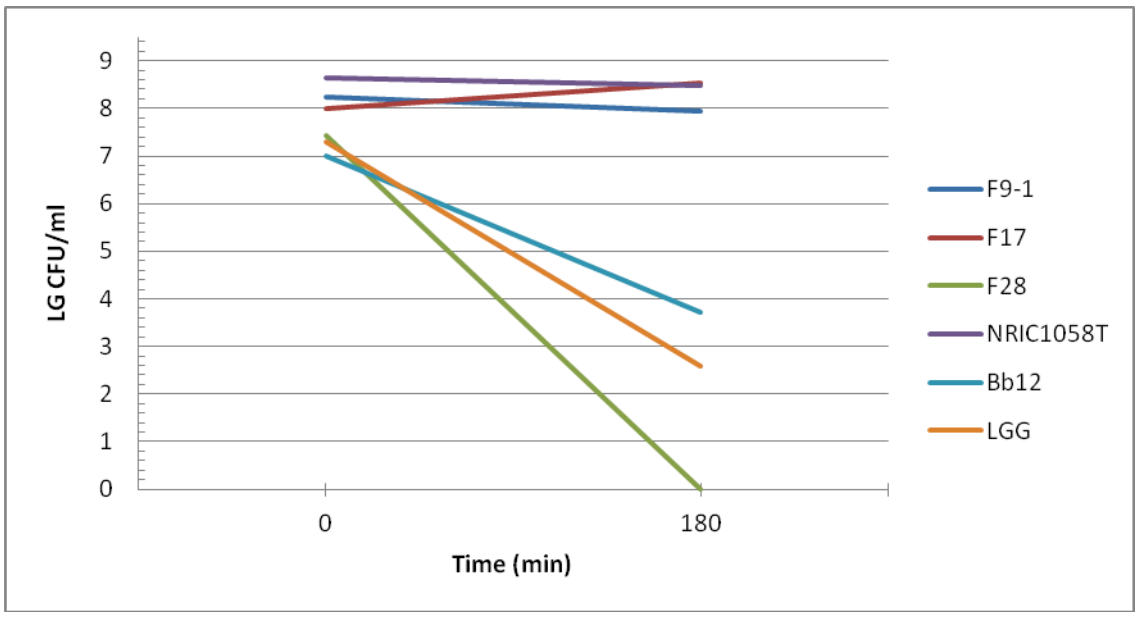

Figure 24 Results of bile juice study for *Lactobacillus florum* and *Fructobacillus fructosus* strains as a function of time.

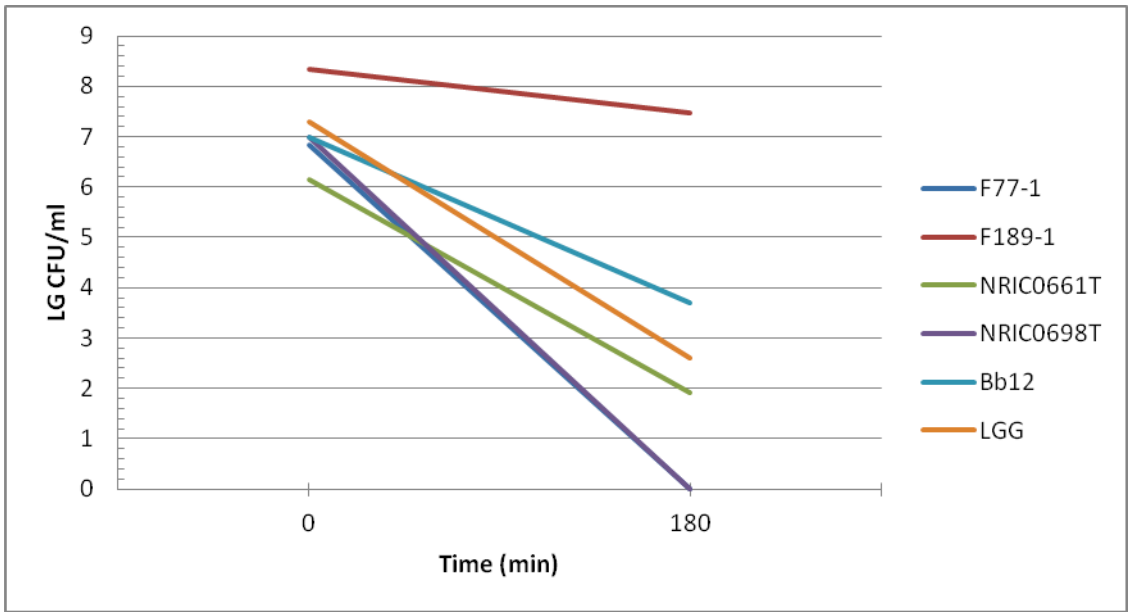

Figure 25 Results of bile juice study for *Fructobacillus pseudoficulneus* and *F. ficulneus* strains as a function of time.

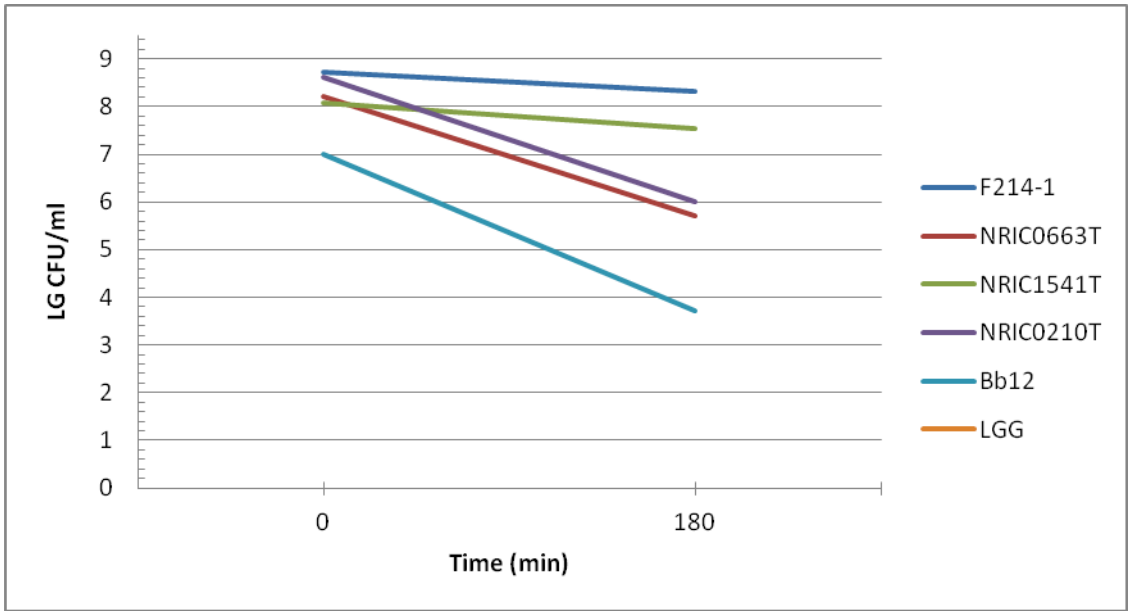

Figure 26 Results of the bile juice study for *F. tropaeoli, F. durionis, Leuconostoc mesenteroide* subsp. *Mesenteroides* and *L. fallax* strains as a function of time.

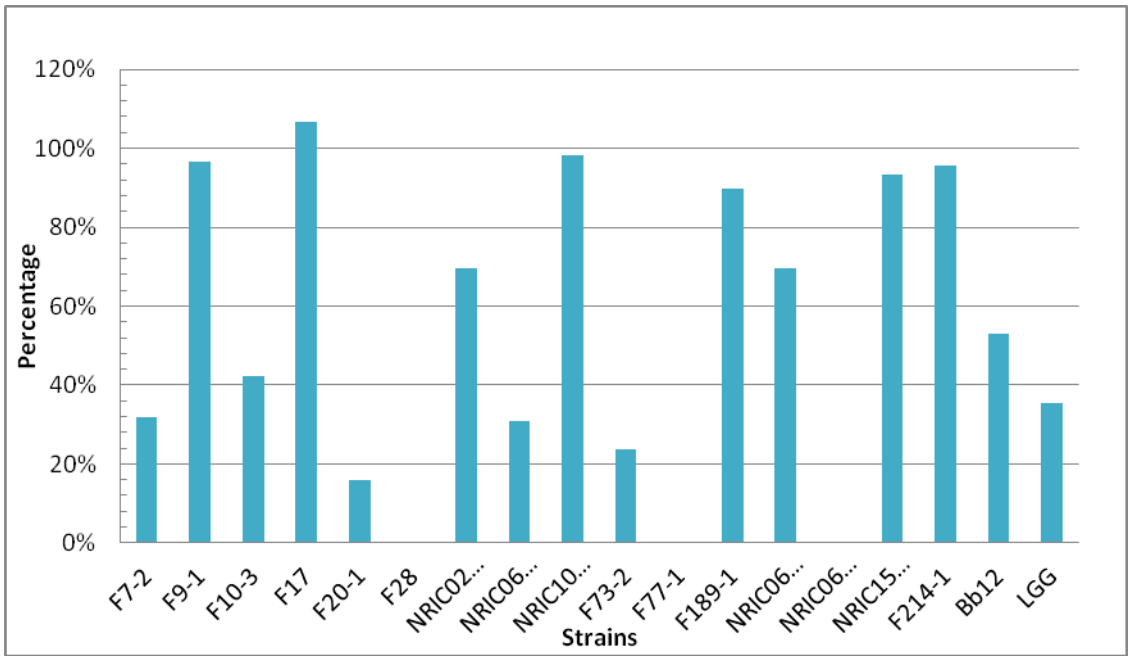

Figure 27 Results of bile juice study as a percentages of survived bacteria.

6.7 Discussion

Four different kinds of methods were tested in this study. The methods are used to verify if bacteria are able to survive food processing, storage and human digestion. These methods are heat shock, acid tolerance, simulated gastric juice tolerance, and bile juice tolerance. Each test was performed to sixteen fructophilic lactic acid bacterial strains and to two well known probiotic strain, *Bifidobacterium animalis* subsp. *lactis Bb12* and *Lactobacillus rhamnosus GG*. Over all, no fructophilic LAB strains survived every study as good as it is required for example in probiotic yogurts. However, some strains, NRIC1058T, F189-1, NRIC 1541T and F214-1, survived the simulated gastric juice and bile juice studies so well that they should be consider for a follow-up in different conditions such as higher pH and lower temperatures.

It was disclosed as a result of the heat shock study that strains NRIC 1058T and F9-1 survived well even after 20 minutes. No other strain survived as well, but it could be that the strains would survive in those temperatures if the heating time was shorter and product cooled quickly afterwards as in the pasteurisation.

Fructophilic lactic acid bacteria do not survive when pH is as low as 3.3, which was the only pH point that was studied. pH 3.3 is very low even for lactic acid bacteria and bifidobacteria even though they are commonly used in yoghurts and other dairy products. It is common that the pH of yoghurts containing probiotics is 4.0 – 4.5 (Vinderola *et al.*, 2000; Shah *et al.*, 2000) and in probiotic juices the pH variation can be wider from 3.5 to near 7 (Yoon *et al.*, 2004; Pereira *et al.*, 2012), whereas the pH of normal fruit juice can be between 3.5 and 4.5 (Mortazavian *et al.*, 2012). It cannot be said how the strains would survive in higher pH and how long they would survive, but it is sure they would survive somehow in normal juice and well in juice whose pH would be at the same level as that of probiotic juices.

The strain F214-1 is a good option, if some alternative conditions are used. As it is mentioned above the pH of probiotic juices can be much higher than 3.3, which was used in the acid tolerance study. It can be as high as near 7, which is neutral. The strain should survive in that kind of condition because it is isolated from the flower *Tropaeolaum majus*, whose condition is assumed to be near neutral. F214-1 could also survive in baby foods, for example in milk based fruit puree or in fruit puree which contains oatmeal. The pH of this kind of baby food is between 4.0 and 5.0 and are not sterilized but pasteurized. Baby foods are made from real fruit so they also contain fructose which is preferred by *F. tropaeoli*.

Different results were obtained for the reference strains, *Bifidobacterium animalis* subsp. *animalis* and *Lactobacillus rhamnosus GG*, from the acid and gastric juice study than was expected, because they were both commanly used and well known probiotic strains. Hence it was assumed that they would survive both best. However the reference strains did not survive best from any study. The reference strain *Lactobacillus rhamnosus GG* did not tolerance acid and gastric juice. Tolerance can be depending on metabolizable sugar, which was in all cases fructose. It is studied that the amount of glucose influences to survive of lactobacilli in the acid environments (Corcoran *et al.*, 2005). It can be that fructose does not have same influence to *Lactobacillus rhamnosus GG* strain

than glucose has. According the study of Buruleanu *Bifidobacterium animalis* subsp. *lactis Bb12* cells should be about 6.75 lg CFU/ml, pH 2.0, after one hour (Buruleanu, 2011). It is about 2 lg units more than in this study. Difference is able to be caused by different solutions, because in the study of Buruleanu was used solution were was 3 g/L of pepsin and added it to saline (Buruleanu, 2011), when in this study were 3 g/L of pepsin and 125 mM of NaCl, 7 mM of KCl and 45 mM of $NaHCO_3$.

REFERENCES

Adams, M.R. & Moss, M.O. 2000. Food Microbiology, Second Edition. The Royal Society of Chemistry, United Kingdom.

Advanced Aquarists. 2002. Chemistry and the Aquarium: Calcium Carbonate as a Supplement. Can be found from Internet: http://www.advancedaquarist.com/2002/7/chemistry

Arboleya, S., Ruas-Madiedo, P., Margolles, A., Solís, G., Salminen, S., de los Reyes-Gavilán, C. & Gueimonde, M. 2010. Characterization an *in vitro* properties of potentially probiotic *Bifidobacterium* strains isolated from breast-milk. International Journal of Food Microbiology, 149: 28 – 36.

Antila, M.A., Karppinen, M., Leskelä, M., Mölsä, H., Pohjakallio, M. 2008. Tekniikan kemia. 10th edition, p. 159. Edita Publishing Oy, Helsinki, Finland.

Axelsson, L. 1998., Lactic Acid Bacteria: Classification and Physiology. At book: Salminen, S. & von Wright Atte. 1998. Lactic Acid Bacteria: Microbiology and Functiona Aspects. 2., Revised and Expanded. New York: Marcel Dekker, Inc.

Barrangou, R., Lahtinen, S.J., Ibrahim, F. & Ouwehand, A.C. Genus ıLactobacillus. At book: . At book: Lahtinen, S., Ouwehand, A.C., Salminen, S. &von Wright, A. 2012. Lactic acid bacteria: microbiological and functional aspects. 4th edition. Boca Raton: CRC Press, Taylor & Francis Group. Pages: 77-80.

Biavat, B., Vescovo, M., Torriani, S. and Bottazzi, V. Bifidobacteria: history, ecology, physiology and applications.

Buruleanu, L. 2011. Acid and Bile Tolerance of Probiotic Bacteria Used for Lactic Acid Fermentation of Vegetable Juices. Journal of Science and Arts, **1(18):** 57-62.

Chaplin, M. 2012. Water Structure and Science, Water Activity. Can be found from Internet: http://www.lsbu.ac.uk/water/activity.html [Referred online 18.6.2012].

Corcoran, B.M., Stanton, C., Fitzgerald, G.F. & Ross, R.P. 2005. Survive of Probiotic Lactobacilli in Acidic Enviroments Is Enchanced in the Presence of Metabolizable Sugar. Applied and Anviromental Microbiology, 71: 3060-3067.

Decagon Devices, Inc. 2011. Water activity. Can be found from Internet: http://www.wateractivity.org/ [Referred online 18.6.2012].

Endo, A.; Futagawa-Endo, Y. & Dick, L.M.T. 2009. Isolation and characterization on fructophilic lactic acid bacteria from fructose-rich niches. Systematic and Applied Microbiology, **32** (2009): 593 – 600.

Endo, A., Futagawa-Endo, Y., Sakamoto, M., Kitahara, M., Dicks, L.M.T. 2010. *Lactobacillus florum* sp. Nov., a fructophilic species isolated from flowers. Journal of Systematic and Evolutionary Microbiology, 60: pages 2478-2482.

Fulle, B.J. 2004. Cryoprotectants: The Essential Antifreezes to Protect Life in the Frozen State. United Kingdom, University Department of Surgery, Royal Free & University College Medical School.

Goodsell, D. (2004). Can be found from Internet: http://www.rcsb.org/pdb/101/motm.do?momID=57 [Refered online, 2.3.2012].

Gueimonde, M., de Los Reyes-Gavilán, C.G. & Sánchez, B. Stability of Lactic Acid Bacteria in Foods and Supplements. At book: Lahtinen, S., Ouwehand, A.C., Salminen, S. &von Wright, A. 2012. Lactic acid bacteria: microbiological and functional aspects. 4. Boca Raton: CRC Press, Taylor & Francis Group. Pages: 361-383.

He, H., Chen, Y., Zhang, Y. & Wei, C. 2011. Bacteria associated with gut lumen of *Camponotus japonicas* mayr. China: Northweat A&F University.

Kekkonen, R., Kumpu, M.,Myllyluoma, E., Saxelin, M. 2009. LGG® Summatim, health effects of LGG®. 3rd edition. Finland, Valio Ltd, R&D and Strategy.

Koch, H. & Schmid-Hempel, P. 2011. Bacterial communities in central European bumblebees: Low diversity and high specificity. Switzerland: Institute of Integravite Biology.

Lichestein, H.C. & Soule, M.H. 1943. Studies of the effect of sodium azide on microbic growth and respiration: 1. The Action of Sodium Azide on Microcic Growth. USA, Hygienic Laboratory, University of Michigan.

Leslie, S.B., Israeli, E., Lighthart, B., Crowe, J.H. & Crowe, L.M. 1995. Trahalose and Sucrose Protect Both Membranes and Proteins in Intact Bacteria during Drying. Applied and Enviromental Microbiology, U.S.A.

Lourence-Hattingh, A. & Viljoen, B. 2001. Review, Yogurt as Probiotic Carries Food. International Dairy Journal, **11**: 1-17.

Mattila-Sandholm, T., Myllärinen, P., Crittenden, R., Mogensen, G., Fondén, R. & Saarela, M. 2002. Technological challenges for future probiotic foods. International Dairy Journal, **12:** 173 – 182.

McDowall, J. (2004). Can be found from Internet: http://www.ebi.ac.uk/interpro/potm/2004_9/Page1.htm [Refered online, 2.3.2012].

Mortazavian, M.A. Mohammadi, R. & Sohrabvandi. 2012. Delivery of Probiotic Microorganisms into Gastrointestinal Tract by Food Products. At book: Brzozowski, T. New Advances in the Basic and Clinical Gastroenterology, pages 121-146. InTech.

Neogen Corporation. 2011a. Can be found from Internet: http://www.neogen.com/acumedia/pdf/prodinfo/7184_pi.pdf [Referred online, 5.3.2012].

Neogen Corporation. 2011b. Can be found from Internet: http://www.neogen.com/acumedia/pdf/ProdInfo/7163_PI.pdf [Referred online, 5.3.2012].

Nordberg, J & Arnér, E.S.J. 2001. Reactive Oxygen Species, Antioxidants, and the Mammalian Thioredoxin System. Medical Nobel Institute for Biochemistry, Sweden.

Patel, R.P., Patel, M.P. & Suthar, A.M. 2009. Spray Drying Technology: an overview. India, Indian Journal of Science and Technology, Vol. 2.

Pereira, A.L.F., Maciel, T.C. & Rodrigues, S. 2010. Probiotic beverage from cashew apple juice fermented with *Lactobacillus casei*. Food Research International, **44**: 1267-1283.

Protocols Online. 2010. Can be found from Internet: http://protocolsonline.com/featured-articles/phosphate-buffered-saline-pbs/ [Referred online, 12.3.2012].

Pundir, R.K. & Jain, P. 2010. Change in Microflora of Sauerkraut During Fermentation and Storage. India, World Journal of Dairy & Food Sciences **5 (2)**: 221-225.

RLG, Sigma-Aldrich. 2006. Can be found from Internet: http://www.sigmaaldrich.com/etc/medialib/docs/Sigma-Aldrich/Product_Information_Sheet/p8074pis.Par.0001.File.tmp/p8074pis.pdf [Referred online 12.3.2012].

Salminen, S., Deighton, M.A., Benno, Y. & Gorbach, S.L. 1998. Lactic acid bacteria in health and disease. At book: Salminen, S. & von Wright Atte. 1998. Lactic Acid Bacteria: Microbiology and Functiona Aspects. 2., Revised and Expanded. New York: Marcel Dekker, Inc.

Science Buddies, All About Agar. 2012. Can be found from Internet: http://www.sciencebuddies.org/science-fair-projects/project_ideas/MicroBio_Agar.shtml [Referred online, 12.3.2012].

Shah, N.P. & Ravula, R.R. 2000. Influence of water activity on fermentation, organic acid production and viability of yogurt and probiotic bacteria. Australian Journal of Dairy Technology, **55**: 127 – 132.

Sigma Aldrich. 2006. Can be found from Internet: http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Product_Information_Sheet/c7698pis.Par.0001.File.tmp/c7698pis.pdf [Referred online 25.5.2012].

Solano-Aguilar, G., Dawson, H., Restrepo, M., Andrews, K., Vinyard, B., Urban, J.F. Jr. 2008. Detection of *Bifidobacterium animalis* subsp. *lactis* (Bb12) in the Intestine after Feeding of Sows and Their Piglets. Applied Environmental microbiology, America.

Silva, J., Carvalho, A.S., Teixeira, P. & Gibbs, P.A. 2001. Bacteriocin Production by Spray-Dried Lactic Acid Bacteria. Portugal, Letters in Applied Microbiology, **4**. Pages: 77-81.

Stolaki, M., De Vos, W.M., Kleerebezem, M. & Zoetendal, E.G. Lactic Acid Bacteria in the Gut. At book: Lahtinen, S., Ouwehand, A.C., Salminen, S. &von Wright, A. 2012. Lactic acid bacteria: microbiological and functional aspects. 4. Boca Raton: CRC Press, Taylor & Francis Group. Pages: 385-401.

Sun, W. & Griffiths, M.W. 2000. Survival of bifidobacteria in yogurt and simulated gastric juice following immobilization in gella-xanthan beads. International Journal of Food Microbiology, **61**: 17-25.

Thaochan, N., Drew, R.A.I., Hughes, J.M., Vijaysegaran, S. & Chinajariyawong, A. 2010. Alimentary tract bacteria isolated and identified with API-20E and molecular cloning techinques from Australian tropical fruit flies, *Bactrocera cacuminata* and *B. tryoni*. U.S.A: University of Wisconsin Library.

Todar, K., 2008a. Can be find from Internet: http://textbookofbacteriology.net/lactics_2.html [Refered online, 27.2.2012].

Todar, K., 2008b. Can be find from Internet: http://textbookofbacteriology.net/metabolism_3.html [Refered online, 27.2.2012].

Todar, K., 2008c. Can be find from Internet: http://textbookofbacteriology.net/nutgro_3.html [Referred online, 27.2.2012].

Ventura, M., Canchaya, C., Tauch, A., Chandra, G., Fitzgerald, G.F., Chater, K.F. & van Sinderen, D. 2007. Genomics of Actinobacteria: Tracing the evolutionary history of an ancient phylum. USA, Microbiology and Molecular Biology Reviews.

Ventura, M., Turroni, F. & van Sindere, D. *Bifidobacteria:* General Overview on Ecology, Taxonomy, and Genomic. At book: Lahtinen, S., Ouwehand, A.C., Salminen, S. &von Wright, A. 2012. Lactic acid bacteria: microbiological and functional aspects. 4th edition. Boca Raton: CRC Press, Taylor & Francis Group. Pages: 147-150.

Vinderola, C.G., Bailo, N. & Reinheimer, J.A. 2000. Survival of probiotic microflora in Argentinian yoghurts during refrigerated storage. Food Research International, **33**: 97-102.

Yoon, K.J., Woodams, E.E. & Hand, Y.D. 2004. Probiotication of Tomato Juice by Lactic Acid Bacteria. The Journal of Microbiology, **42**: 315-318.

Strains used during the studies

Table 1 Strains used during the studies and where they are isolated from.

Strain	**Isolated from**
F7-2: *Lactobacillus kunkeei*	Azalea, flower
F10-3: *L. kunkeei*	Narcissus, flower
F20-1: *L. kunkeei*	Cosmos, flower
F73-2: *L. kunkeei*	Cosmos, flower
F9-1: *L. florum*	Peony, flower
F17: *L. florum*	Bietou, flower
F28: *Fructobacillus fructosus*	Azalea, flower
NRIC 1058T: *F. fructosus*	Flower
F77-1: *F. pseudoficulneus*	Banana, fruit
F189-1: *F. pseudoficulneus*	Fig, fruit
NRIC 0698T: *F. pseudoficulneus*	Ripe figs, fruit
NRIC 0661T: *F. ficulneus*	Ripe figs, fruit
F214-1: *F. tropaeoli*	*Tropaeolaum majus,* flower (Endo *et al.*, 2011)
NRIC 0663T: *F. durionis*	Fermentef condiment made of durian fruit
NRIC 1541T: *Leuconostoc mesenteroides* subsp. *Mesenteroides*	Unknown. *Leuconostoc mesenteroides* have been found from sauerkraut (Pundir & Jain, 2010).
NRIC 0210T *Leuconostoc fallax*	Sauerkraut
LGG: *Lactobacillus rhamnosus GG*	
BB12: *Bifidobacterium animalis* subsp. *lactis BB12*	

Results in the experiments CFU/ml

Table 2 Results of the heat shock study in 60 °C. Results are shown in CFU/ml at zero, ten and twenty minutes.

Strain	**0 min**	**10 min**	**20 min**
F7-2: *Lactobacillus kunkeei*	1,900,000,000	450	0
F10-3: *L. kunkeei*	2,300,000,000	5,900	0
F20-1: *L. kunkeei*	870,000,000	22,000	2,5000
F73-2: *L. kunkeei*	5,500,000,000	180,000	0
F9-1: *L. florum*	160,000,000	150,000,000	13,000,000
F17: *L. florum*	1,200,000,000	15,000	18,000
F28: *Fructobacillus fructosus*	3,400,000,000	0	0
NRIC 1058T: *F. fructosus*	1,100,000,000	660,000,000	610,000,000
F77-1: *F. pseudoficulneus*	700,000,000	260	0
F189-1: *F. pseudoficulneus*	830,000,000	23,000,000	170,000
NRIC 0698T: *F. pseudoficulneus*	2,000,000,000	0	0
NRIC 0661T: *F. ficulneus*	1,000,000,000	30	0
F214-1: *F. tropaeoli*	4,300,000,000	6,9000	34,000
NRIC 0663T: *F. durionis*	2,700,000,000	1,200,000	4,000
NRIC 1541T: *Leuconostoc mesenteroides* subsp. *Mesenteroides*	260,000,000	5,000	300
NRIC 0210T *Leuconostoc fallax*	810,000,000	0	0

Table 3 Results of the heat shock study in 70 °C. Results are shown in CFU/ml at zero, ten and twenty minutes.

Strain	**0 min**	**10 min**	**20 min**
F20-1: *L. kunkeei*	600,000,000	260	0
F9-1: *L. florum*	1,500,000,000	180,000	54,000
F17: *L. florum*	560,000,000	2,000	0
NRIC 1058T: *F. fructosus*	1,000,000,000	650,000	2,100
F189-1: *F. pseudoficulneus*	760,000,000	2,100	50
F214-1: *F. tropaeoli*	1700,000,000	120,000	200
NRIC 0663T: *F. durionis*	140,000,000	0	0
NRIC 1541T: *Leuconostoc mesenteroides* subsp. *Mesenteroides*	340,000,000	0	0

Table 4 Results of the heat shock study at the second time in 60 °C. Results are shown in CFU/ml at zero, ten and twenty minutes.

Strain	**0 min**	**10 min**	**20 min**
F9-1: *L. florum*	320,000,000	65,000,000	32,000,000
NRIC 1058T: *F. fructosus*	1,000,000,000	850,000,000	750,000,000
F189-1: *F. pseudoficulneus*	850,000,000	22,000,000	360,000
F214-1: *F. tropaeoli*	1,700,000,000	1,100,000	49,000

Table 5 Results of the heat shock study at the second time in 70 °C. Results are shown in CFU/ml at zero, ten and twenty minutes.

Strain	**0 min**	**10 min**	**20 min**
F9-1: *L. florum*	320,000,000	130,000,000	28,000
NRIC 1058T: *F. fructosus*	1,200,000,000	5,500,000	77,000
F189-1: *F. pseudoficulneus*	1,300,000,000	3,400,000	2,000
F214-1: *F. tropaeoli*	1,500,000,000	300	0

Table 6 Results of the acid tolerance study. Results are shown in CFU/ml at day zero, one, two, three, four, five and seven.

Strain	**0 d**	**1 d**	**2 d**	**3 d**	**4 d**	**5 d**	**7 d**
F7-2: *Lactobacillus kunkeei*	120,000,000	790			0		
F10-3: *L. kunkeei*	820,000,000	30		0			
F20-1: *L. kunkeei*	240,000,000	200			0		
F73-2: *L. kunkeei*	280,000,000	40		0			
F9-1: *L. florum*	670,000,000	310		0			
F17: *L. florum*	32,000,000	1,300,000		0			
F28: *Fructobacillus fructosus*	310,000,000	4,700			0		
NRIC 1058T: *F. fructosus*	210,000,000	12,000,000	10	0			
F77-1: *F. pseudoficulneus*	690,000,000	0	0				
F189-1: *F. pseudoficulneus*	120,000,000	350,000	10	0			
NRIC 0698T: *F. pseudoficulneus*	440,000,000	0	0				
NRIC 0661T: *F. ficulneus*	300,000,000	20	100				
F214-1: *F. tropaeoli*	610,000,000	25,000,000	2,300,000				390
NRIC 0663T: *F. durionis*	140,000,000	21,000	10				
NRIC 1541T: *Leuconostoc mesenteroides* subsp. *Mesenteroides*	100,000,000	0	0				
NRIC 0210T *Leuconostoc fallax*	220,000,000	0					
LGG: *Lactobacillus rhamnosus GG*	54,000,000			1,000,000		0	
BB12: *Bifidobacterium animalis* subsp. *lactis BB12*	45,000,000			1,200,000		0	

Table 7 Results of the simulated gastric juice study. Results are shown in CFU/ml at zero and ninety minutes.

Strain	**0 min**	**90 min**
F7-2: *Lactobacillus kunkeei*	2,000,000	0
F10-3: *L. kunkeei*	310,000,000	1,700
F20-1: *L. kunkeei*	140,000,000	0
F73-2: *L. kunkeei*	99,000,000	0
F9-1: *L. florum*	170,000,000	0
F17: *L. florum*	220,000,000	0
F28: *Fructobacillus fructosus*	27,000,000	3,100
NRIC 1058T: *F. fructosus*	620,000,000	2,000
F77-1: *F. pseudoficulneus*	100,000,000	0
F189-1: *F. pseudoficulneus*	330,000,000	4,500
NRIC 0698T: *F. pseudoficulneus*	510,000,000	0
NRIC 0661T: *F. ficulneus*	100,000,000	17,000
F214-1: *F. tropaeoli*	580,000,000	600,000
NRIC 0663T: *F. durionis*	280,000,000	0
NRIC 1541T: *Leuconostoc mesenteroides* subsp. *Mesenteroides*	27,000,000	1,000
NRIC 0210T *Leuconostoc fallax*	480,000,000	0
LGG: *Lactobacillus rhamnosus GG*	20,000,000	390
BB12: *Bifidobacterium animalis* subsp. *lactis BB12*	10,000,000	5,100

Table 8 Results of the bile juice study. Results are shown in CFU/ml at 0 and 180 minutes.

Strain	**0 min**	**180 min**
F7-2: *Lactobacillus kunkeei*	100,000,000	350
F10-3: *L. kunkeei*	1,100,000	360
F20-1: *L. kunkeei*	140,000,000	20
F73-2: *L. kunkeei*	99,000,000	80
F9-1: *L. florum*	170,000,000	90,000,000
F17: *L. florum*	100,000,000	350,000,000
F28: *Fructobacillus fructosus*	27,000,000	0
NRIC 1058T: *F. fructosus*	450,000,000	310,000,000
F77-1: *F. pseudoficulneus*	7,000,000	0
F189-1: *F. pseudoficulneus*	220,000,000	30,000,000
NRIC 0698T: *F. pseudoficulneus*	10,000,000	0
NRIC 0661T: *F. ficulneus*	1,400,000	80
F214-1: *F. tropaeoli*	520,000,000	210,000,000
NRIC 0663T: *F. durionis*	160,000,000	510,000
NRIC 1541T: *Leuconostoc mesenteroides* subsp. *Mesenteroides*	120,000,000	34,000,000
NRIC 0210T *Leuconostoc fallax*	400,000,000	1,000,000
LGG: *Lactobacillus rhamnosus GG*	20,000,000	390
BB12: *Bifidobacterium animalis* subsp. *lactis BB12*	10,000,000	5,100

Percentage of survived bacteria after each experiment

Table 9 Results of the heat shock study in 60 and 70 °C as a percentage of survivors.

	Heat shock in 60 °C		Heat shock in 70 °C	
Strain	**After 10 minutes**	**After 20 minutes**	**After 10 minutes**	**After 20 minutes**
F7-2: *Lactobacillus kunkeei*	29 %	0 %		
F10-3: *L. kunkeei*	40 %	0 %		
F20-1: *L. kunkeei*	49 %	49 %	27 %	0 %
F73-2: *L. kunkeei*	54 %	0 %		
F9-1: *L. florum*	89 %	77 %	68 %	52 %
F17: *L. florum*	46 %	47 %	38 %	0 %
F28: *Fructobacillus fructosus*	0 %	0 %		
NRIC 1058T: *F. fructosus*	98 %	97 %	65 %	37 %
F77-1: *F. pseudoficulneus*	27 %	0 %		
F189-1: *F. pseudoficulneus*	82 %	59 %	37 %	19 %
NRIC 0698T: *F. pseudoficulneus*	0 %	0 %		
NRIC 0661T: *F. ficulneus*	16 %	0 %		
F214-1: *F. tropaeoli*	61 %	47 %	55 %	25 %
NRIC 0663T: *F. durionis*	64 %	38 %	0 %	0 %
NRIC 1541T: *Leuconostoc mesenteroides* subsp. *Mesenteroides*	44 %	29 %	0 %	0 %
NRIC 0210T *Leuconostoc fallax*	0 %	0 %		

Table 10 Results of the acid tolerance study as a percentage of survivors.

	Acid tolerance					
Strain	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 7**
F7-2: *Lactobacillus kunkeei*	36 %			0 %		
F10-3: *L. kunkeei*	17 %		0 %			
F20-1: *L. kunkeei*	27 %			0 %		
F73-2: *L. kunkeei*	19 %		0 %			
F9-1: *L. florum*	28 %		0 %			
F17: *L. florum*	81 %		0 %			
F28: *Fructobacillus fructosus*	43 %			0 %		
NRIC 1058T: *F. fructosus*	85 %	12 %				0 %
F77-1: *F. pseudoficulneus*	0 %	0 %				
F189-1: *F. pseudoficulneus*	69 %	12 %				0 %
NRIC 0698T: *F. pseudoficulneus*	0 %					
NRIC 0661T: *F. ficulneus*	4 %	24 %				
F214-1: *F. tropaeoli*	84 %	72 %				29 %
NRIC 0663T: *F. durionis*	53 %					
NRIC 1541T: *Leuconostoc mesenteroides* subsp. *Mesenteroides*	0 %	0 %				
NRIC 0210T *Leuconostoc fallax*	0 %	0 %				
LGG: *Lactobacillus rhamnosus GG*			78 %		0 %	
BB12: *Bifidobacterium animalis* subsp. *lactis BB12*			79 %		0 %	

Table 11 Results of the simulated gastric juice and bile juice studies as a percentage of survivors.

	Gastric juice tolerance	**Bile juice tolerance**
Strain	**After 90 minutes**	**After 180 minutes**
F7-2: *Lactobacillus kunkeei*	0 %	32 %
F10-3: *L. kunkeei*	0 %	42 %
F20-1: *L. kunkeei*	0 %	16 %
F73-2: *L. kunkeei*	0 %	24 %
F9-1: *L. florum*	0 %	97 %
F17: *L. florum*	0 %	107 %
F28: *Fructobacillus fructosus*	47 %	0 %
NRIC 1058T: *F. fructosus*	38 %	98 %
F77-1: *F. pseudoficulneus*	0 %	0 %
F189-1: *F. pseudoficulneus*	43 %	90 %
NRIC 0698T: *F. pseudoficulneus*	0 %	0 %
NRIC 0661T: *F. ficulneus*	53 %	31 %
F214-1: *F. tropaeoli*	66 %	96 %
NRIC 0663T: *F. durionis*	0 %	70 %
NRIC 1541T: *Leuconostoc mesenteroides* subsp. *Mesenteroides*	40 %	93 %
NRIC 0210T *Leuconostoc fallax*	0 %	70 %
LGG: *Lactobacillus rhamnosus GG*	36 %	36 %
BB12: *Bifidobacterium animalis* subsp. *lactis BB12*	53 %	53 %