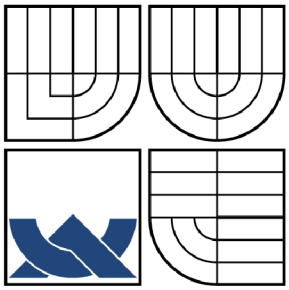

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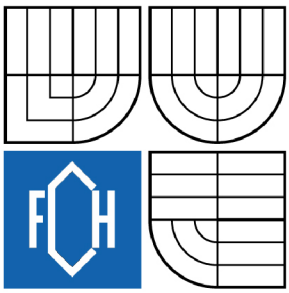

FAKULTA CHEMICKÁ

ÚSTAV CHEMIE POTRAVIN A BIOTECHNOLOGIÍ

FACULTY OF CHEMISTRY

INSTITUTE OF FOOD SCIENCE AND BIOTECHNOLOGY

FERMENTATION OF DIFFERENT CEREALS BY THE PROBIOTIC BACTERIA LACTOBACILLUS PLANTARUM 299V.

FERMENTANCE VYBRANÝCH CEREÁLIÍ POMOCÍ BAKTERIÍ LACTOBACILLUS PLANTARUM 299V .

DIPLOMOVÁ PRÁCE

DIPLOMA THESIS

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Zadání diplomové práce

Číslo diplomové práce	**FCH-DIP0157/2007** Akademický rok: **2008/2009**
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Název diplomové práce:

Fermentance vybraných cereálií pomocí bakterií Lactobacillus plantarum 299v .

Zadání diplomové práce:

Vybrané cereálie rýže a ječmen budou testovány jako substrát pro probiotické bakterie Lactobacillus plantarum 299v . V průběhu fermentace bude sledováno pH, CFU a produkt bude sledován i z hygienického hlediska. Výsledky fermentace budou aplikovány při výrobě produktu ProViva s přídavkem ovocné šťávy. Inovovaný výrobek bude posuzován i z hlediska trvanlivosti a chuti. Bude provedena i studie přežívání probiotických kultur v gastrointestinálním traktu dobrovolníků.

Termín odevzdání diplomové práce: 16.5.2008

Diplomová práce se odevzdává ve třech exemplářích na sekretariát ústavu a v elektronické formě vedoucímu diplomové práce. Toto zadání je přílohou diplomové práce.

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V Brně, dne 1.9.2007

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Abstract

The number of humans suffering from various infectious, inflammatory and allergic diseases as well as the occurence of lactose intolerance and high blood cholesterol levels has an increasing tendency. Some of those health disorders are caused by a disbalanced intestinal microbial flora.

Probiotics are though to contribute to intestinal microbial balance (Parker, 1974) between the beneficial and adverse bacteria. Therefore, the therapy based on administrating probiotics to humans has attracted research interest. Depending on what desired effect-health disorder should be aimed to leads to the choice of an appropriate probiotic bacterium. *Lactobacillus plantarum* 299v has proved its beneficial effects on a man and also has met the safety requirements to be recognized as a probiotic bacteria (Probi AB, Sweden). Therefore *Lactobacillus plantarum* 299v is sold on a marketplace as probiotics in many functional foods, probiotic drink ProViva is one example of them.

The aim of this work was to study the fermentation process in oats, barley and soya-based gruels by the strain *Lactobacillus plantarum* 299v with the focus on the soya and barley substrates. The objectives were to investigate the growth and metabolic activity of *Lactobacillus plantarum* 299v in association with cereal substrates and further in the mixture of the fermented cereal component with commercial fruit juices.

To optimise the fermentation process, several aspects have to be considered. The major role in the designing of the novel fermented food product belongs to the processing and composition of the raw material, the growth capacity and productivity of the bacterial culture and the stability of the final product during storage (De Vuyst, 2000). These parametres are important from the producers point of view. Apart from that, there are also consumers whose product acceptance is based mainly on the sensory characteristics of the final probiotic product, i.e. aroma and taste. The presence and accessibility of different nutrients, which were present in the fermentation media as an outcome of different cereals used, probably resulted in variations of the metabolic pathways which in turn might lead to the distinct differences in the organoleptic properties of the final product.

Abstrakt

Počet obyvatel trpících různými infekčními, zánětlivými a alergickými nemocemi stejně jako výskyt laktózové nesnášenlivosti a vysoké hodnoty krevního cholesterolu, má narůstající tendenci. Některé z těchto zdravotních problémů jsou způsobeny nevyváženou střevní mikroflorou.

Probiotika jsou pak chápána (nejen) jako potravní komponenty, které přispívají k ustanovení mikrobiální rovnováhy (Parker, 1974) mezi zdraví prospěšnými a škodlivými bakteriemi. Z tohoto důvodu, terapie založená na podávání probiotik pacientům přitáhla zájem ze strany vědců. Vhodný probiotický kmen se pak volí v závislosti na požadovaném zdravotním účinku (příp. zdravotním problému, který má být probiotickou terapií léčen).

Lactobacillus plantarum 299v již prokázal své blahodárné účinky na lidech a zároveň byla i potvrzena jeho zdravotní bezpečnost, díky čemuž může tato bakterie být kategorizována jako probiotický kmen (Probi AB, Sweden). I díky tomu je *Lactobacillus plantarum* 299v ve značné oblibě přidáván do mnoha fukčních potravin a prodáván na trhu pod různými jmény, probiotický nápoj ProViva je jedním takovým příkladem.

Cílem této práce bylo studovat fermentační proces na žitném, ječmenném a sojovém substrátu pomocí kmene *Lactobacillus plantarum* 299v, přičemž zvýšená pozornost byla věnována právě soji a ječmeni jako potenciálně novým substrátům pro výše uvedenou bakterii. Hlavními záměry bylo zkoumání růstu a metabolické aktivity bakterie *Lactobacillus plantarum* 299v v asociaci s různými cereálními substráty, a později bylo studováno totéž také ve směsi fermentované cereální komponenty s běžně dostupným ovocným džusem.

K tomu, aby se dosáhlo optimálních podmínek fermentace, je třeba vzít v úvahu několik aspektů. Hlavní role při konceptování nového fermentovaného produktu patří především zpracování a taktéž kompozici surového materiálu, růstové kapacitě a produktivitě bakteriální kultury a stabilitě

finálního produktu během skladování (De Vuyst, 2000). Tyto parametry jsou důležité hlavně ze strany výrobců. Krom toho jsou tu ale i zákazníci, pro něž je přijatelnost produktu založena z velké části na organoleptických vlastnostech finálního probiotického produktu, tj. aromatu a chuti. Přítomnost a dostupnost různých jednotlivých nutrientů, která byla obsažena ve fermentačním médiu výsledkem rozdílných použitých cereálních substrátů, pravděpodobně vyústila v odlišnosti metabolických drah, což pak později mohlo způsobit rozdíly v organoleptických vlastnostech finálního produktu.

Key words

probiotics, *Lactobacillus plantarum* 299v, probiotic drinks, fermentation, functional food, gastrointestinal disorders

Klíčová slova

probiotika, *Lactobacillus plantarum* 299v, probiotické nápoje, fermentace, funkční potraviny, poruchy zažívacího traktu

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Declaration

I declare that the diploma thesis has been worked out by myself and that all the quotations from the used literary sources are accurate and complete. The content of the diploma thesis is the property of the Faculty of Chemistry of Brno University of Technology and all commercial uses are allowed only if approved by both the supervisor and the dean of the Faculty of Chemistry, BUT.

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student's signature

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Contents

1. Introduction

The interest of modern consumers in their personal health is increasing as well as the awareness of the potential beneficial effects of functional food. Furthermore, the incidence rate of new patients suffering from various disorders related to their GI tract is also growing rapidly. At present, the correlation between the food consumed and its impact on human healthy intestinal microflora is discussed with a increasing frequency. Many clinical studies are also beeing performed in this, up to now, obscure area in order to formulate new product categories; improve and/or develop new manufacturing processes; select new probiotic strains; etc. Many previous studies have already shown that giving food products containing specific bacterial strains to patients with recurrent episodes of *Clostridium difficile* associated diarrhea (RCDAD) can be an attractive therapy by inhibiting mechanisms, such as changes in pH and producing inhibitory compounds such as short-chain fatty acids, H_2S, and bacteriocins, and providing a competition for nutrients and for binding sites on the gut epithelium which in turn leads to the alleviation of RCDAD symptoms (May et.al., 1994; Hogenauer et.al., 1998; Hove et.al., 1996). The probiotics using therapy might be administered also to the patients suffering from others gastro-intestinal (GIT) disorders. Nonetheless, probiotics can be given to a generally healthy population to keep their bacterial intestinal balance.

The balanced gut microbiota is considered to be the key target for functional food since it is a major prerequisite of our well being. Thus, functional food consumed is then expected to be healthy or even able to prevent illnesses. Also, a new trend in consuming of probiotic drinks has appeared recently which in turn results in research efforts focused to developing new non-dairy probiotic products. Researches to formulate novel non-dairy probiotic drinks (Mattila-Sandholm et al., 2002) suggested that fruit juice could serve as a good medium for functional ingredients like probiotics (Tuorila & Cardello, 2002). Furthermore, the fruit juice-based products don't contain any dairy allergens (e.g., lactose) what could be a potentional limitation from consuming probiotic products by a certain part of the population. For global food producers, this trend represents both a marketing and industrial challenge which requires further researches in the key area of new raw materials and inovated technologies as well as focus on specific groups of consumers such as the elderly, people with a certain type of food associated allergies, babies etc.

To be delivered to consumers, new probiotic products have to meet certain requirements:

- be able to be processed under industrial conditions
- survive and retain their functionality in suitable and declared counts during the product's shelf-life

Additionally, what's probably the most important from the consumer's point of view, the incorporation of probiotics shouldn't influence the texture and aroma of the final product with unpleasant products of their metabolism i.e. they should remain viable but not growing. These and many other demands need to be fulfilled in order to develop a product that is primairly functional, but also attractive to consumers.

2. Study background

2.1. Probiotics-definition

Basically, probiotics are a microbial culture proving beneficial potential which improves the balance of the gut microbiota composition of the host and thus preventing and correcting the microbial dysfunctions. Probiotics are thus defined as 'mono or mixed cultures of live micro-organisms which when applied to animal or man in adequate amounts, beneficially affect the host health by improving the properties of the indigenous microflora' could have therapeutic effects in gastrointestinal diseases such as Crohn's disease, Clostridium difficile associated diarrhea (RCDAD), ulcerative colitis and irritable bowel syndrome (Havenaar et.al.,1992; Goossens et.al.,2003). It is also important to note that probiotics are functionally defined bacteria that are not linked to their taxonomic classification (G. Mölin, 2007).

The term probiotic was introduced as early as 1965 by Lilly and Stillwell to describe compounds produced by one microorganism that stimulated the growth of other microorganisms. Since then, the definition has changed gradually in many ways depending on the understanding of the mechanism by which they influence human health and well being of humans.

At present, the commonly used definition is still that of Fuller (1989): "Probiotics are live microbial food supplements which beneficially affect the host animal by improving its intestinal microbial balance." This definition is still (more or less) valid, however the current conceptions are based mostly on specific affects of clearly defined strains. That basically means that the immune-enhancing as well as anti-inflammatory activities of specific strains are taken into account and new conceptions are thus expressed in terms of demonstrated clinical effects mediated either through probiotic effects on the intestinal immune system or via modulation of the gut microbiota at specific locations (Isolauri et.al., 2004).

First, it was thought that probiotics needed to be viable to be effective. Yet, for specific strains, it can be concluded that even non-viable probiotics or certain cell envelopes can stimulate the immune system. The health effects of non-viable probiotics have been recently summarized by Ouwehand and Salminen (Ouwehand & Salminen, 1998).

Currently, many improved trials focusing on characterization and validation of the composition of the bacterial genera species and strains, as well as the effects of different probiotics (viable, non-viable or cell components), have brought a lot of new and interesting results and information. As a consequence of that there is a need for a new definition for probiotics that would better characterize both the specific strains and components used for probiotic purposes. So, a new definition should thus include that probiotics do not necessarily need to be viable, as non-viable forms of probiotics have also been shown to have health effects. It should also be mentioned that several other applications besides the probiotics containing food have also been found to have beneficial health effects (Salminen & Ouwehand, 1999.). However, further rigorous scientific efforts are required to characterize the immunomodulatory potential of specific probiotic strains for these targets (Isolauri et.al.,2004).

2.2. Probiotic strains used in Functional Food

Throughout the past two decades, microorganisms promoting health effects on humans have been increasingly included into commercial dairy products in a response to the consumer demand for healthy food options that improve overall health, intestinal function, and digestion (Menrad, 2002). In most cases, monocultures of *Lactobacilli*, *Bifidobacteria* or *Saccharomyces* spp. are frequently used in the current functional food products. However, the general term of probiotics includes also the multispecies functional products which are recently of an increased occurence on the marketplace.

Nonetheless, in the functional food production, the members of the genera *Bifidobacterium* and *Lactobacillus* are of a particular interest. The inclusion of intestinal species of lactobacilli in probiotic products dates back to 1930s (Rettger et al., 1935). On the contrary, the inclusion of bifidobacteria in yoghurt is of more current occurence reflecting the realisation of their presence in the human GI tract among other numerous species (Gerald W. Tannock, 1998).

Based on the sequencing of cloned 16S rDNA, there were more than 130 species and subspecies of the genera *Lactobacillus* and more than 30 species of the genera *Bifidobacterium* identified. Interestingly, the probiotic potential of different bacterial strains, even within the same species, differs; different strains of the same species are always unique and may have differing areas of adherence (site-specific) and specific immunological effects. Also the actions on a healthy versus an inflamed mucosal milieu may be distinct from each other (Isolauri et.al., 2004).

Table 2.1: List of probiotic species used in functional food (Adapted from FAO/WHO, 2001; Španová, 2008).

Bifidobacterium species	*Lactobacillus* species
B. bifidum	*Lb.paracasei*
B.animalis subsp. *animalis*	*Lb.rhamnosus*
B.animalis subsp. *lactis*	*Lb.plantarum*
B.adolescentis	*Lb.johnsonii*
B.breve	*Lb.delbrüeckii* subsp. *bulgaricus*
	Lb.delbrüeckii subsp. *lactis*
	Lb.salivarius
	Lb.fermentum
	Lb.helveticus
	Lb.crispatus

In addition, there exists a striking difference within probiotic strains reflecting their nutritional requirements (Mölin, 2002). Basically, *Lactobacillus* need to be supplemented with amino acids and with a spectrum of different growth factors while *Bifidobacterium* can synthetize all what it needs out of salts and an energy source.

Also from the phylogenetic point of view, the genus Lactobacillus is very different from the genus *Bifidobacterium* and most probably performs different effects in human's GI tract. Another difference is obvious from their locations in the GI tract, which they are preferably associated to. *Lactobacillus* seem to be, to a high degree, associated to a mucosa while *Bifidobacterium* are more adopted to a leftovers in the colonic content (Mölin, 2007).

And finally, *Lactobacillus* exhibit better survival in food products in comparison to that of *Bifidobacterium* what is crucial from the commercial aspect.

2.3. Probiotics-requirements

To be considered as a potential probiotic strain there are some requirements that should be fulfiled in order to get desired beneficial effect on the gut health. Thus, the selection of probiotic strains, which are convenient for its incorporation into probiotic products, is greatly dependent on an understanding of colonization attributes, biochemical activities and immunostimulatory properties of bacteria.

The ability to adhere to intestinal surfaces is frequently reported to be the main criterion for selection of probiotic strains (Tuomola et al. 2000). Basically, mucus is the first barrier that digested bacteria confront in the gut. Therefore, the adhesion of bacteria to that matrix is considered to be a prerequisite for the gut colonization (Ouwehand et al. 1999). Another important fact necessary to point out is that intestinal mucus is continuously renewed, so bacteria that aren't able to adhere to the epithelium are eliminated. Though the exact mechanism of binding to the intestinal mucus is still largely unknown, there are several studies indicating that the adhesion may be an important strain characteristic for the treatment of rotavirus diarrhoea (Arthur C. Ouwehand, Pirkka V. Kirjavainen, Colette Shortt, Seppo Salminen, 1999).

As emerged from the study performed by Tallon et al. (1996), the mechanism of how some probiotic strains, particularly *Lactobacillus plantarum* strains, are attached to the intestinal matrix demonstrated strain-dependent and mannose-specific adhesion of some *Lactobacillus plantarum* strains to HT-29 cells (Adlerberth et al. 1996). As a result, strain 299v within 31 tested strains showed significant adhesion abilities whatever the adhesion test used. In conclusion, the combination of the results obtained contributed to the selection of several strains as probiotic candidates.

Another important requirement for probiotics is safety. Basically, every viable microorganism capable of growing and multiplying under the host's body enviroment can under certain conditions cause an infection. The likelihood of that is relativelly small for healthy individuals but some specific problems can be rarely observed especially in immunosupressive or in any other way disbalanced or very sensitive humans. However, usually an ingestion of probiotic bacteria results in symbiosis between the host and the microbe.

The safety of non-viable or inactivated microbial preparations have also been examined with the result of a very few, if any, adverse effects (Salminen et al., 1999). It can be concluded that even though current probiotics are considered safe for food use, non-viable probiotics and microbial cell wall components are the least likely to cause safety concerns (Ouwehand & Salminen, 1998; Kirjavainen et. al., 1999). Moreover, some studies have shown that even non-viable bacteria can have beneficial effects such as enhancing the immune system and some others (Ouwehand &Salminen, 1998; Salminen, Oewehand, Benno, & Lee, 1999)

The summary of the main prerequisities for efficient function of probiotics includes the following:

1. The strain has to be identified and characterised (healthy human GI-tract).

2. Safety requirements have to be fulfilled:
   - Adverse effect and pathogenic potential are not accepted.
   - Introduction in the GIT should not be dangerous for the consumer.
   - The strain should not be able to spread antibiotic resistance or to be a vector for mobile genes.
3. The strain must manifest some beneficial effects, especially on the gut, through clinical trials such as randomised, double-blind and placebo-controlled studies.
4. Functional aspects include: To be regarded as a probiotic, the product should contain a certain amount of living microorganisms. However, the beneficial effect of non-viable bacteria was alreday mentioned thus for certain strains it might be sufficient to grow to high cell counts in the probiotic product, but don't necessarily need to retain good viability during storage. The bacteria should survive during the storage prior to intake but also during its passage through the GI tract.

   The other functional properties include immunomodulation, antagonistic (especially againts *Helicobacter pylori*, *Salmonella* sp., *Listeria monocytogenes* and *Clostridium dificille*) and antimutagenic properties (Mattila-Sandholm et al., 1999; Saarela, Mogensen, Fonden, Matto, & Mattila- Sandholm, 2000).
5. Other properties for an efficient probiotic product would be that once the microbes have reached the intestines, they should be able to adhere to the mucosa and to multiply though they may never become the permanent member of the microflora. (Mölin, 2007; L. D Vuyst et. al., 1998).

2.4. Probiotics in humans

At present, scientific efforts aim at the specification of normal bacterial flora in each individual, assesing the species composition as well as the concentrations of different bacteria in each part of the GI tract. The understanding of host-microbe interactions within the gut, microbe–microbe interactions within the microbiota and the combined health effects of these interactions is the most targeted area in the current research (Isolauri et al., 2004).

The so called normal bacterial flora is thought to response for the human defense, and the sites of highest bacterial concentration are also the sites most frequently affected by inflammation in patients with inflammatory bowel disease (IBD) and intestinal cancer (Mölin, 2007). There is reported up to 500 different bacterial species found in the large intestine of an adult man (Erika Isolauri et al., 2004.). However, many of these species are present in relatively low numbers.

As a method of evaluation of the bacteria present it is often used the direct 16S rDNA gene identification in mucosa biopsies taken from different sites of the GI tract. On basis of that method , the GI flora has been reported to by dominated by the genera such as *Bacteroides*, *Eubacterium*, *Clostridium*, *Ruminococcus*, *Peptococcus*, *Peptostreptococcus*, *Bifidobacterium* and *Fusobacterium* reaching the major concentration of $10^{11}-10^{12}$ colony - forming units (CFU)/g in the colon (Molin, 2007). However, the direct 16S rDNA analysis only reveals species that are part of the dominating flora (by statistical reasons).

Probiotics are thought to contribute to intestinal microbial balance (Parker, 1974) between beneficial and adverse bacteria. As a beneficial are identified genera such as *Lactobacillus* and *Bifidobacterium*, while members of the family *Enterobacteriaceae* (for ex., E. Coli) and *Clostridium* group XI (for ex., Clostridium dificcile) are though to be able to cause problems if they are allowed to reach high concentrations (Molin, 2007). Therefore, it is obvious that the targeted site of probiotic bacteria is colon and the ability of the probiotic strain to attach the intestinal epithelium is generally considered to be the main selection criteria from the aspect of the colonization (Beachey, 1981); the mechanism of the adhesion was already shortly discussed above. However, colonization of the human's gut is only a transient state what is supported by continuously decreasing number of probiotic bacteria detected in faeces within the range from a couple of days to a few weeks after cessation of suplemented probiotics (Johansson, Molin, Jeppson, Nobaek, Ahrne H & Bengmark, 1993; Link-Amster, Rochat, Saudan, Mignot & Aeschlimann, 1994; Saxelin, 1997; Spanhaak, Havenaar & Schaafsma, 1998).

Probiotics are though to contribute to intestinal microbial balance (Parker, 1974) between the beneficial and adverse bacteria. Genera such as *Lactobacillus* and *Bifidobacterium* were identifed as beneficial while members of the family *Enterobacteriaceae* (for ex., E. Coli) and *Clostridium* group XI (for ex., *Clostridium dificcile*) are though to be able to cause problems if they are allowed to reach high concentrations (Molin, 2007). For the beneficial effect to be manifested the probiotic bacteria have to reach the colon. Thus the ability of the probiotic strain to attach to the intestinal epithelium is generally considered to be the main selection criteria from the aspect of the colonization (Beachey, 1981); the mechanism of the adhesion was already shortly discussed above.

It was also observed that the number of probiotic bacteria colonizing the host's guts is correlated inversely with the age of the subject, what may explain very low adhesion to the intestinal mucus that was isolated from the elderly (Ouwehand, Isolauri, Kirjavainen & Salminen, 1999).

2.5. Probiotics - mechanism

Some possible ways of how the beneficial effect of probiotics can be manifested in humans have been discussed although the exact mechanisms are still unknown and are under current research. There have been identified several factors, including for example antibiotic treatment, which can lead to the alteration of intestinal bacterial composition what in turn results in the unbalanced microflora. Such a deviant intestinal flora is then believed to cause infectious and antibiotic associated diarrhoea, food allergy, atopic eczema, inflammatory bowel diseases and arthritis (Isolauri, E. et.al., 2002). One conception of potencial mechanism of probiotics can therefore be based on normalization of the gut microbiota by stabilization of the gut microbial enviroment and intestine's permeability barrier. The beneficial effect proved by probiotic was assigned to intestinal microflora changes only. However, the major part of these desired effects is mediated through enhancing of the immune system as well as anti-inflammatory activities as some probiotic strains have been observed to establish a healthy homeostasis by optimizing the balance of pro- and anti-inflammatory cytokines and other mediators. (Neish A.S. et al., 2000).

One of the degradation products of peptidoglycan, which is the main cell wall component of the *Lactobacilli*, is the muramyl peptide which can be detected in the systemic

issue of the host animal. This component was shown to have a pharmacological activity, including strong adjuvant effects (Tannock, 1991). The immunomodulatory effect is principally mediated by the activation of the lymphoid cells of the gut-associated lymphoid tissue (Madara, 1997). These cells are then affected by probiotics through the interactions between the lymphoid tissue and the intact microorganism, its fragments or metabolites produced *in situ*.

Furthermore, the antioxidative effect of probiotics when combined with components containing high concentrations of polyphenols can be speculated. Since some fruits are a good source of polyphenols, this beneficial effect might be observed when consuming probiotic juices. It has been recently shown, in a model with mice, that the oxidative stress was significantly suppressed by a combination of *Lb. plantarum* 299v and fruit, while no significant effects were recorded when applying each component alone (Hakansson et al. 2006). The mechanism of that effect is being explained by the metabolism of polyphenols by intestinal bacterial flora and some probiotic strains as they have the ability to convert the original non-absorbable polyphenols to such compounds (forms) that can be absorbed and transported to the liver, where they excercise both the metabolic effects with anti-inflammatory consequences and antioxidative capacity per se. Polyphenols (for example anthocyanins) in their original form are difficult to break down and therefore they cannot be absorbed.

However, when exposed to the intestinal microflora or to some strains of *Lb. plantarum*, which in contrast to the other *Lactobacillus* species is able to break down polyphenols, active compounds such as phenyl valeric, phenyl propionic, phenyl acetic and benzoic acid derivatives with anti-oxidative capacity are formed (Molin, G., 2007). This potential way of action is important especially from the aspect of the protection of the body from the damaging effects of reactive oxygen species, that are believed as a cause of many inflammatory diseases as well as a causative agent of cancer.

Probiotics as a members of the lactic acid bacteria (LAB) group have been shown to inhibit the growth of pathogenic bacteria by the secretion of inhibitory substances (organic acids, bacteriocins) and by competitive adhesion to the epithelium. When talking about widely produced organic compounds, it is possible to mention lactic and acetic acid although hydrogen peroxide and carbon dioxide are also shown to be greatly produced by LAB. On condition that probiotic bacteria survive the passage through the intestinal tract and remain metabolically active, it is very likely that some of these compounds are released into the intestine (Ouwehand, A.C., Kirjavainen, P.V, Shortt, C., Salminen, S., 1999). The production of short-chain fatty acids (which are other active compounds produced by probiotics), such as butyric acid, may also improve the mucosal nutrition and integrity.

Another mechanism by which the bacterial balance in the intestine can be regulated is by the production of bacteriocins, which are principally substances produced by one microbial strain with inhibitory effects to another one (antagonistic effect). From a biochemical point of view bacteriocins can be defined as peptides, proteins or protein-carbohydrates complexes with a relatively narrow range of action produced by many microbial cells including lactobacilli (Tannock 1981). Probably the best studied bacteriocin of which there is reference of, is lactin produced by *Lb. johnsonii* (Fremaux et al., 1993, Klaenhammer et al., 1994). To summ up all the possible beneficial effects of probiotics mentioned above, there is a schematical picture showing different actions of both viable and non-viable probiotics (Salminen 1999).

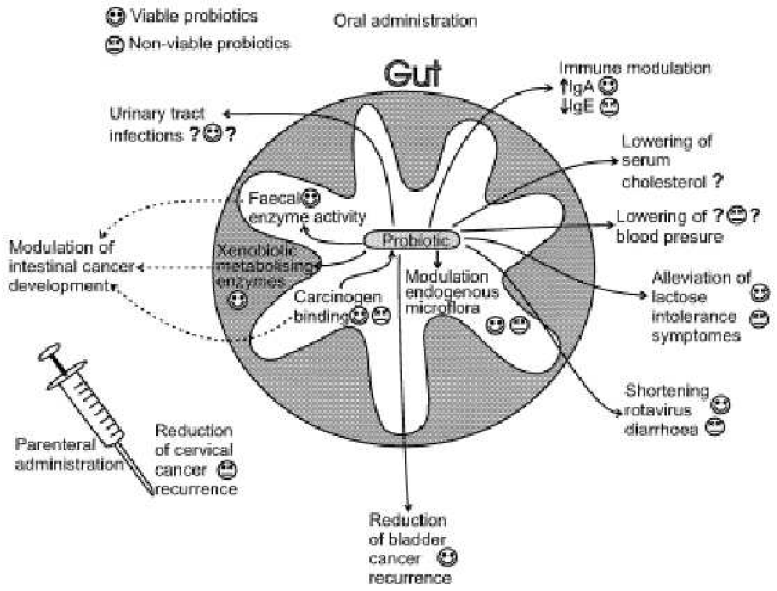

Figure 2.1: Proposed mechanisms of viable and non-viable probiotic health effects. S. Salminen et al. / Trends in Food Science & Technology 10 (1999) 107-110.

2.6. Probiotics - effects

To be recognized as a scientifically valid product, a beneficial impact on human well being is the prerequisite for probiotics. Although up to now plenty of evidence exists on possitive probiotic effects on the human host, further studies evaluating the impact of the probiotic strains on the intestinal mucosa need to be carried. The main research interest is attracted mainly by the urgent need for the evidence of probiotic activities in the average (generally healthy) population, since the major part of the beneficial probiotic effects have been demonstrated in diseased human population (Salminen et al., 1998). To obtain the most representable results the long duration (in the range of months), experiments should be performed better than those of shorter periods since some temporal variation in the intestinal microflora can occur and thus influence the outcome.

The recent reviews have documented such strains with clinical effects and properties (e.g. Lee & Salminen, 1995; Salminen, Isolauri & Salminen, 1996; Salminen et al., 1998). However there is still need to take into account many strain to strain variations in both technological properties and their effects on human health (Mattila-Sandholm et al., 1999). Therapy based on administrating probiotics to humans suffering from various infectious, inflammatory and allergic disease has attracted research interest. For therapeutic purposes probiotics are restricted by many selection criteria as mentioned above. Depending on what desired effect-health disorder should be aimed to leads to the choice of an appropriate probiotic bacterium.

Generally, probiotics are understand as „beneficial for the gut“ what is mainly proved for:

1. Rotavirus diarrhoea
2. *Clostridium difficille* diarrhoea

3. Antibiotic associated diarrhoea, whose adverse affects have been caused mainly by the unbalanced microbial composition in the gut and the affects on the immune system.

The most fully documented disease resulting in alteration of the gut microbiota is acute infectious diarrhoea in childhood and also rotavirus diarrhoea. By administrating *Lactobacillus rhamnosus* GG the duration of rotavirus-type diarrhoea has been shown to be shortened as documented in several studies around the world and also in a recent multi-center study in Europe (Guandalini, 1998; Valio, Finland). Some specific probiotic strains may also alleviate symptoms of lactose intolerance and there is also evidence of down-regulation of immune changes related to milk hypersensitivity (Pelto, Isolauri & Salminen, 1998).

Table 2.2: Proposed health benefits of probiotics (Adapted from Gibson & Roberfroid; 1995).

Probiotics - main beneficial effects on human health
Increase of the lactose tolerance and digestion
Positive influence in the intestinal microflora
Reduction of intestinal pH
Improvement of the intestinal functioning
Reduction of cholesterol level
Reduction of ammonia and other toxic compounds
Production of the B vitamins (folic acid)
Restoration of the normal intestinal microflora after antibiotic therapy
Treatment and prevention of acute diarrhoea by rotaviruses
Stimulation of the immune response
Preventing of the chronic diseases

2.6.1. *Lactobacillus plantarum*- effects

Lactobacillus plantarum 299v has been shown to possess anti-microbial activity against strains of potentially pathogenic species as *Listeria monocytogenes*, *Bacillus cereus*, *Escherichia coli*, *Yersinia enterocolitica*, *Citrobacter freundii*, *Enterobacter cloacae* and *Enterococcus faecalis*, in vitro (Jacobsen et al. 1999). When healthy volunteers consumed a mixture of lactobacilli strains, including *Lactobacillus plantarum* 299v, the level of lactobacilli in the intestinal mucosa increased, there was also a decrease in the level of Gram-negative anaerobes (Johansson et al. 1993), *Enterobacteriaceae* (Johansson et al. 1993) and sulphite-reducing clostridia (Johansson et al. 1993).

Decreased presence of entero-pathogens: In a study in Tanzania, *Lactobacillus plantarum* 299v had been used as a starter culture for producing a cereal based lactic acid fermented beverage called Togwa. The product was given to children younger than 5 years once a day for 13 days. The presence of faecal entero-pathogens such as *Campylobacter*, entero-pathogenic *Escherichia coli*, *Salmonella* and *Shigella* was evaluated. The proportion of children with isolated faecal entero-pathogens decreased significantly (P¡0.001) during the study period (Kingamkono et al. 1999). Spontaneously fermented togwa is frequently dominated by *Lactobacillus plantarum* (Mugula 2001).

Increased concentrations of carboxylic acids: *Lb. plantarum* 299v administrated in ProViva extregistered has, in a double blind placebo controlled study, been shown to increase the total level of carboxylic acids in faeces of health volunteers (mainly acetic acid and propionic acid; Johansson et al. 1998). Furthermore, the subjects administrated ProViva extregistered experienced a decreased flatulence during the treatment period (Johansson et al. 1998).

The carboxylic acids in the colon content are a major energy source to the human mucosa cells, i.e. an increased level of short-chain fatty acids in the lumen is beneficial for the condition of the mucosa. Moreover, absorbed short-chain fatty acids can have positive effects on the lipid metabolism in the liver (short-chain fatty acids that are absorbed from the colon are going directly to the liver). The observed increase in the level of short-chain fatty acids (Johansson et al. 1998) can be due to a changed composition of the colonic microflora, i.e. *Lb. plantarum* support an increased number of bacterial taxa able to produce acetic acid and propionic acid. A supplementary explanation might be that *Lb. plantarum* 299v increased the amount of mucin in the colon, i.e. *Lb. plantarum* 299v has been shown to stimulate the epithelial mucin production in vitro (Mack et al. 1999). Thus, the increased amount of mucin leads to a higher amount of fermentable material in the colon; material that can be converted to short-chain fatty acids.

2.7. Probiotic products

Probiotics may be found in the so called lactic acid fermented products what includes not only dairy-based but also, in recent, more popular non-dairy products. However, probiotics don't have to be necesarilly related only to a food matrices. Also a medical use in different forms such as pills, powders, syrups etc. is another common application of probiotics.

Lactic acid fermentation is an ancient and simple technique, whose prime purpose was to preserve the product and improved the eating quality of the product. This technique of preservation and refining food materials has been known to man for thousands of years. Interestingly, the lactic acid fermentation procedure is well-spread throughout the world, and almost every country has its own traditional lactic acid fermented products. Nigerian Ogi, Ethiopian Kocho or Tanzanian Togwa are such examples of the original fermentation procedure. These traditional cereal-based lactic acid fermented beverages are mostly used as a refreshment or a weaning food to children (Molin, G., 2007). However, as food production has progressed, there has been a need for optimisation of the product qualities. Therefore there is an urgent development of selected starter cultures suited for different types of food and feed products. The summary of the bacterial strains used in functional food is given in the Table 1. Nowdays, lactic acid fermentation is widely used in the preparation of products such as yoghurt, various types of fermented milk products, cheese, salted gherkins, sauerkraut, green olives in brine, sourdough etc.

The number of people suffering from lactose intolerance and high cholesterol level has an increasing tendency. The consumption of the probiotic products by consumers is hence limited by the amount of fat and presence of lactose, what are two important drawbacks of the fermented dairy products. Consequently, intensive scientific research aims to develop new types of functional foods focusing mainly on cereal, fruit and vegetable beverages (Flavera C. Prado , Jose L. Parada , Ashok Pandey, Carlos R. Soccol, 2007).

As an example of such a product, symbiotic functional drinks made from oat can be mentioned (Angelov, Gotcheva, Kuncheva, and Hristozova, 2006). Oat, as well as barley, is high in beta-glucans, which is the soluble fraction of dietary fibre. To clarify the relation between the content of beta-glucans and their influence on human's health, some experimental studies have been performed. As a result, the combined effect of the reduction of LDL-cholesterol and the overall reduced cardiovascular disease risk have been reported (Stark & Madar, 1994; Wrick, 1994).

Another illustration of a fermented non-dairy beverage is based on soybeans that have been shown to be suitable for the growth of lactic acid bacteria and bifidobacteria (Chou & Hou, 2000; Matsuyama et al., 1992). These soya-based products has attracted relativelly great research attention, mainly because of the high protein quality of soybeans. It was also proposed in several studies that further suplementation with inulin and oligofructose could improve product's properties (Fuchs, Borsato, Bona, & Hauly, 2005; Shimakawa, Matsubara, Yuki, Ikeda, & Ishikawa, 2003; Wang, Yu, & Chou, 2002).

2.7.1. ProViva®

Fruit and vegetable's drinks fortified with the probiotic and prebiotic components are of an increasing popularity since they have been suggested as an ideal medium for the functional health ingredients. The reason for that is the inherent content of beneficial nutrients, the pleasant taste profile and because they are perceived as refreshing and healthy (Tuorila & Cardello, 2002). The high content of minerals, vitamins, dietary fibres and antioxidants and no content of milk allergens makes fruits and vegetables to be the ideal medium for the functional beverages that can be used by more population groups (Luckow & Delahunty, 2004).

As a great demonstration of that type of product, it can be mentioned the patented fruit-based probiotic juice invented by swedish company Skanemejerier together with Probi, a company with a longstanding experience in the development of probiotic strains and nutritional products. Pro Viva is a brand name for a group of non-dairy and recently also dairy probiotic drinks sold in Sweden. Freely translated from latin, Pro Viva means „for life". These beverages have been on the Swedish market since 1994 and sales have exhibited an ongoing and very positive development (+28% during 2000). ProViva®is one of the few foodstuff products to have such strong growth on the Swedish market.

All of the offerings in the ProViva extregistered line use the lactic acid bacteria *Lb. plantarum* 299v which have healthy effects on humans. As the company says, this strain has been scientifically tested on humans and shown to be safe and effective. The holder of the rights to the strain *Lb. plantarum* 299v is the company Probi AB (Lund, Sweden). The original ProViva®fruit drink comes in 1-liter bottles and is offered in several servings. The lactic acid fermented component of the drink ProViva®is an oatmeal beverage (resulting in about $1x10^9$ colony forming units [CFU] per ml).This fermented oatmeal formula was originally brought forward as a new concept for enteral feeding. The lactic acid fermented formula (about 5%) is then mixed with drink of rose hip, strawberry, blueberry, black currant or tropical fruits where the *Lb. plantarum* 299v concentration is about 5 x 107 CFU/ml of fruit drink (Molin, G., 2007). Apart from the original version, there is also ProViva®Shot! on the marketplace. Basically, the Shot is a concentrated form of Pro Viva containing five times as many bacteria as the equivalent quantity of ProViva®fruit

drink. This means around 250 million beneficial bacteria per milliliter. The mechanism of the adhesion was already shortly discussed above.

Figure 2.2: ProViva®drink, www.natlivs.se

3. Experimental part

3.1. Aim of the study

The original fermentation process with oats has been shown a great stability and a high quality of the final product (see the part 2.7.1). However, the increasing demands from the consumers side require to introduce innovated products on the market. Before marketing and producing the product in the large scale there is a need to determine the crucial steps of the procedure in a laboratory scale. The suitable fermentation process and the choice of the right bacterial strain are under the main scope in the development of the new probiotic drink.

The aim of this work was to study the fermentation process in oat, barley and soya-based gruels by the strain *Lactobacillus plantarum* 299v. In other words, the objectives were to investigate the growth and metabolic activity of *Lactobacillus plantarum* 299v in the association with different cereal substrates. Furthermore, the correlation of the survival of *Lactobacillus plantarum* 299v and the composition of different types of fruit juices was speculated in the process of the design of a novel probiotic drink.

The first part of this study provides the overall view of the fermentation process in an oat, barley and a soya medium. The second part brings the information of the prepared probiotic drink (made only of the soya and barley medium) when stored under consumer's conditions. In order to evaluate the fermentation progress, measurements of following parametres were performed: pH, viable count and content of pathogens. For a better understandig of the metabolic pathways of bacteria during the fermentation and further in the fruit drink, the growth curves of *Lb. plantarum* in the soy- and barley-medium were designed. The newly designed product went through the same testing assays, moreover the taste and aroma were analysed by the group of volunteers.

The probiotic strain, *Lactobacillus plantarum* 299v, has been already proved successful by many clinical studies, hence the main focus in this study is given to the fermentation itself. To optimise the fermentation process, several aspects have to be considered. The major role in the design of the novel fermented food product belongs to the composition and processing of the raw material, the growth capacity and productivity of the bacterial culture and stability of the final product during storage (De Vuyst, 2000). These parametres are important from the producers point of view. Apart from that, there are also consumers whose product acceptance is based mainly on the sensory characteristics of the product.

Although the benefical effect of the oatmeal-based ProViva®drinks has been already reported in some clinical studies, some consumers could find the aromas and flavors, resulting from the functional ingredience characteristics in those products as an undesirable taste. Therefore the preference of such products could be lower. In this study the sensory impact of soya and barley-based formula on the taste and aroma of the final fruit drink has been studied in order to determine which taste would be preferred by consumers.

3.2. Material

3.2.1. Oat flour

General description of oat flour

Low production costs, high nutritional quality of oat, that is rich in fat and protein, and high content of beta glucans, which are believed to be especially effective in lowering the amount of serum cholesterol in man (Kritevsky 1978, Klopfenstein 1988), are the main advantages of oat and oat based nutritive solutions.

Originaly the oatmeal was processed as following: steaming of the oat kernels at 100-110°C for about 60 minutes, storage for 1-60 days, which was followed by the second steam treatment in the temperature range 100-110°C for 40 minutes. After that the oat groats are rolled out to flakes, dried and finally milled to the oatmeal.

The consequence of that steaming procedure is an inactivation of the endogenous enzymes of the oat kernel which are responsible for rancidity i.e. lipoperoxidases, lipoxygenases and lipases. Apart from those, enzymes such as amylases, proteinases and beta-glucanases are also inactivated. Thus, in order to substitute some endogenous enzymes, certain amounts of the malted barley flour were added to the mixture of oatmeal and water.

The oat flour for this study was provided by the swedish dairy company Skanemejerier.

Nutritional values

From the nutritional point of view the average nutrient contents of oatmeal used in the experiments were: 16% protein, 8.5% fat, 1.9% ash and 10.3% fibre. The protein content is higher than in any other grain (Lockhart and Hurt, 1986) and also the presence of some essential amino acids i.e. lysine contributes to oatmeal to be of high nutritive values and thus suitable as a nutritive solution. The amino acid content in oats also meets the relatively high nutritional requirements of the *Lb. plantarum* strain as they require suplementation with a wide spectrum of different growth factors for growth. The total lipid content is also relatively high compared to that of the other grains. The main components of fat in oats are linoleic acid, oleic acid and palmitic acid (Bodin, 1987). Oats contain realatively high amounts of phospholipids as well, which have been proven to have a protective function on the intestinal mucosa (Bodin, 1987). The mineral content of oats includes phosphorus, calcium, iron, zinc and manganese and is also high in some vitamins such as thyamine (Fröhlich and Nyman, 1988).

Health effects of oat on human

Oats were found to be rich in different types of fibers. Among them especially water-soluble beta- glucans have been shown advantageous in both reducing the serum cholesterol level (Petterson and Aman, 1991) and for their ability to enhance the overall production of short chain fatty acids(SCFAs) in the colon, especially of butyrate (Berggren et al., 1993). Although the solubility of beta-glucans is significantly reduced due to the thermal treatment of the oatmeal this is increased by the addition of malted barley flour.

However, like other cereals, oat is rich in phytic acid resulting in the decreased bioavailability of minerals (Lorri, 1993). One way how to decrease phytic acid content in flour is lactic acid fermentation (Lorri, 1993).

3.2.2. Barley flour

General description of barley flour

Grain of barley, Hordeum vulgare, is one of the hardest cereals used mainly in the form of malted barley in the production of beer. Barley belongs to the family Poaceae (the family Poaceae-the grasses) that is the most important for humans from the existentional aspect. Besides barley, the same family includes oat; rye; rice; wheat; sorghum etc. Recently, the interest of using barley in the food production is of increasing rate as barley has been shown of high nutritional values.

Barley flour is ground pearl barley, where the bran has been removed, and then steamed and polished. Since barley is weak in glutens in the yeast-bread production it is mixed with gluten-containing flour.

The barley flour used in this study was produced by Salta Kvarn, Järna and then bought as a 1 kg package in a common supermarket store (www.saltakvarn.se).

Nutritional values

Barley flour is an excellent source of many health beneficial (promoting) components. Moreover, when added to baked goods as a cooking ingredient it gives slightly nutty taste and an unique flavor. From the nutritionl point of view barley is complex cereal that is high in carbohydrates with moderate amounts of protein, containing both soluble and insoluble dietary fibre and is a source of certain vitamins such as niacin, vitamin B6 and folate and minerals such as copper, zinc and iron. Barley is very high in dietary fibre, particularly in the soluble portion where beta -glucan is the major component of soluble fiber at level of about 22% of the total dietary fiber, followed by pentosan (19.7%), Klason lignin (7.8%) and resistant starch (6.3%) (Bhatty, 1999). For more information on the nutritional values and amino acid profiles see the Tables 1-2.

Health effects of barley on human

Some health promoting effects of barley are contributed to low GI (glycemic index) in the combination with the right amount of the so-called indigestible carbohydrates. Dietary fibre and waxy starch,which is basically starch containing almost 100% amylopectin, are such components occuring in barley and rye (Anne Nilsson, Department for Applied Nutrition and Food Hygiene, Lund ,Sweden). Beta-glucan, found in the soluble fibre part of barley, has been shown as an effective agent in the lowering of the serum cholesterol level. The same component has been indicated in an oat as well. Also high concentration of tocotrienols and tocopherols (tocols), an oil components, that are known as natural antioxidants may reduce the risk of cancer and heart disease as they help to inactivate free radicals. Lutein and zeaxanthin are another important components in barley promoting the health of human eyes.

3.2.3. Soya flour

General description of soya flour

Soybean serves as a source of a low cost and high quality protein which nutrition contribution is enhanced by the excess of lysine , which is limiting in most staple sources. However, the availability of the nutrient varies with the form of soy food. Soybean is used in a variety of foods, and can be easily incorporated in the diet to promote desirable health effects (USDA, 1986; FNB, 1989; Messina et al., 1994; Klein et al., 1996). In spite that soyabeans are of good nutritional value, functional health benefits and unique chemical composition, in India only a small production part is used to direct conversion to food products.

The soya flour used in this study was produced by Salta Kvarn, Järna and then bought as a 300 g package in a common supermarket store (www.saltakvarn.se).

Nutritional values

The roasted soybeans are ground into a fine powder that is rich in high-quality proteins and other nutrients. Soy protein is considered to be an equivalent to a protein of an animal origin. Moreover, soybean contains enough essential amino acids to meet human nutritional requirements. Generally, beans lack sulphur containing amino acids. Also soybeans don't posses high amounts of them, but they are still in higher quantities in soybeans than in other beans (USDA, 1986).

The carbohydrate fraction makes almost up to 30 per cent of the weight, with 14 per cent of sugars and 13 per cent of fiber. However, the sugars in soy are often indegistible by humans (Slavin, 1991). Soy beans are an excellent source of fiber which is often separated and used as an additive to enhance the fiber content of other food products. For more information on the nutritional values and amino acid profiles see the Tables 1-2. Soybeans contain high levels of zinc, calcium, managanese and iron. Nonetheless, the availability of these elements is influenced by the presence of phytic acid. Phytic acid is known to bind metals, particularly iron and zinc, forming insoluble complexes and therefore decreasing their absorption. However, this negative effect might be supressed in fermented foods because of the lower phytate concentration. Soya flour is also rich in fat so two kinds of soya flour are available on the market - the full-fat or the defetted variant, which contains about 9 grams of total fat per 100 g of flour. The greater part of soybean oil is represented by omega-6 fatty acids forming the ratio omega-3 : omega-6 1:7 respectivelly. However, soya flour does not contain longer-chain fatty acids such as EPA (eicosapentaenoic acid, 20:5n-3) or DHA (docosahexaenoic acid, 22:6n-3) that are related to beneficial health effects in humans.

Health effects of soya on human

In medical research it has been shown the strong connection between soyafoods and the prevention of heart disease by lowering of blood cholesterol levels, osteoporosis, kidney diseases, cancer and relief of menopausal symptoms. However, not only the beneficial effects but also some disadvantageous properties of soyabeans are needed to take into account, especially the content of antinutrients such as trypsin inhibitor, phytic acid and flatulents should be those of increased interest.

3.2.4. Malted barley flour

Grains of malted barley have been milled to a flour. For my study malted barley flour was provided by Skanemejerier and used in all experiments. The main purpose for addition of malted barley flour, reffered to hereafter as MBF, is to lower the viscosity of the lactic acid fermented product as it is rich in extractable enzymes such as proteases, beta-glucanases and lipases. In the food processing industry MBF is mainly used as a source of amylases (Reed, 1975).

Without the suplemented malt barley, thick porridge would be formed due to the high content of beta-glucans in oats and other types of cereals. The decrease in viscosity is presumably due to degradation of starch since malt is rich in amylases but also the increased solubility of beta-glucans caused by the presence of beta-glucanase is observed. These effects of MBF enzymes in combination with a heat treatment, followed by the decrease in pH, contributes to decrease the viscosity enough so that the final fermented product could be administrated even through a thin tube as an enteral feeding (Molin, G., 2007).

3.2.5. *Lactobacillus plantarum* (299v)

The genera *Lactobacillus* belongs to the group of lactic acid bacteria (LAB) that includes bacteria producing predominantly lactic acid from sugar by their metabolic pathways (Bottazzi, 1988; Stiles and Holzapfel, 1997; Axelsson, 1998). Lactate formed in the fermentation media serves then as a preservative, acidulant and flavourant in many food processing applications.

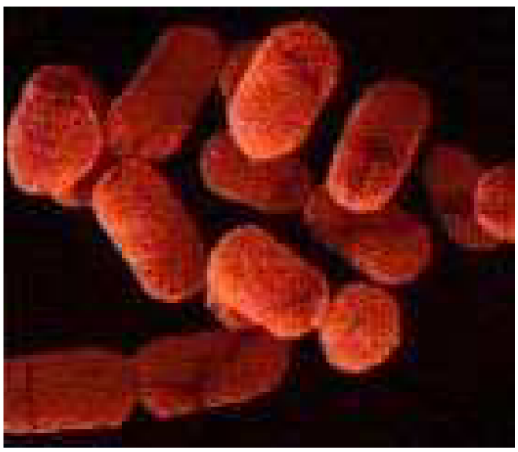

Figure 3.1: *Lactobacillus plantarum* 299v, www.teknologiportalen.dk

Lb. plantarum strains have been isolated from most of the traditional habitats of lactobacilli such as vegetables and other plant materials, fish, meat, faeces as well as from human and animal mucous membranes. The wide distribution of *Lb. plantarum* might be due to the fact that it is less fastidous than most other lacotbacilli, both in its requirements of amino acids and vitamins (Kandler and Weiss, 1986). Another explanation for the frequent isolation of L.plantarum could be that it is able to ferment a broad spectrum of carbohydrates, enabling the species to grow in many different niches. There is a common opinion that strains suitable as probiotics should be host specific i.e. strains used in humans should originate from the human intestinal flora (Salminen et al., 1993). *Lb.*

plantarum 299v strain has been isolated from the colon of healthy human proving its ability to survive the GI enviroment (Hakansson, et. al., 2006; Nobaek et. al., 2000) which is one of the basics (pre)requirements when selecting new probiotic strain. *Lb. plantarum* 299v and genomically related strains have also a pronounced ability to attach to human epithelial cells by a mannose-specific mechanism (Adlerberth et al., 1996) which is another important prerequisite when speaking about probiotics. The tolerance of *Lb. plantarum* to acidic enviroment of the GI has been already described by Giraud. The mechanism is explained by the ability of *Lb. plantarum* to maintain a proton (pH) and charge gradient between the inside and outside of the cells even in the presence of large amounts of lactate and protons (Giraud et. al., 1998).

Lb. plantarum follows the facultatively homofermentative metabolic pathway utilizing glucose and similar amounts of fructose, maltose and sucrose producing mainly lactic acid but also small amounts of acetic acid under aerobic conditions (in this reaction CO_2 and H_2O_2 are also produced). The preference of *Lb. plantarum* towards glucose has been suggested by Samuel et al. (1980) and Gobbetti et al. (1994). However, each bacterial strain has its own preference of sugar consumed during the exponential phase. The facultatively homofermentative lactobacilli are able to ferment pentoses to equimolar amounts of lactate and acetate.The lactic acid produced is a mixture of both the isomers - L+ and D- lactic acid (Kandler and Weiss, 1986). Moreover, acetate and formate was shown to be produced anaerobically from L-lactate by *Lb. plantarum* during sugar limitation (Lindgren et.al., 1990).

As stated above, *Lactobacilli* require suplementation with a wide spectrum of different growth factors such as different amino acids, vitamins, minerals, fermentable sugars etc. Enviromental factors such as pH, temperature and accumulation of metabolic end-products also play important role in the bacterial growth (Mercier et al. 1992; Gänzle et al. 1998; Lejeune et al. 1998). Thus, the substrate composition and availability of essential nutrients are factors influencing the performance of the fermentation process. Members of the *Lb. plantarum* grow in the presence of oxygen, and the species have enzymatic pathways for the oxidation of both pyruvate, lactate and NADH. O_2 then reduced to H_2O_2. Through the action of intracellular peroxidase, H_2O_2 can be further reduced to water (Daeschel and Nes, 1994). To impede the toxic effects of superoxide, *Lb. plantarum* has developed a unique system. In the presence of manganese (which can be accumulated at high levels within the bacterial cells), oxygen radicals are converted into H_2O_2. (Archibald, 1984). Plant materials have been suggested as a source for accumulation of intracellular manganese by *Lb. plantarum* (Daeschel and Nes, 1994). The presence of manganese is also regarded as a growth stimulating factor (Bruyneel et.al., 1990).

Citrate can be converted by *Lb. plantarum* to yield acetoin an diacetyl (Hansen et.al., 1989). This is a problem in fruit juices where the presence of diacetyle is not desirable (Daeschel and Nes, 1994). Furthermore, *Lb. plantarum* has enzymatic pathways for the conversion of malate, tartarate and citrate to lactate and/or acetate (Vescovo et. al., 1993). Proteolysis is rarely seen within *Lb. plantarum.* However, some amino acids can be deaminated e.g. serine to pyruvate and arginine to ornithine (Vescovo et. al., 1993).

In this study the probiotic strain *Lb. plantarum* 299v is used only. This pure culture was obtained from the company Probi AB, Lund, Sweden. The bacterial culture is cultivated in the laboratory of the company following an established procedure. The quality standards of the bacteria are proved by the output check in the laboratory. To assure the purity and high viability of the bacteria after the fermentation process they were deeply

frozen. In this cryophylised state the bacteria were delivered in the concentration of of 10^{10} cfu/ml and kept in the laboratory freezer at the temperature of – 80°C. Under this condition bacteria can be stored for couple of weeks without any change on probiotic properties. However, the manufacturing process of the bacteria is a trade secret and thus any detailed information on the production and control can't be provided within this study.

3.2.6. Fruit juices

Historically, fruit juice was recommended by pedetricians as a source of vitamin C and an extra source of water for healthy infants and young children as their diets expanded to include solid foods with higher renal solute. Fruit juice is marketed as a healthy, natural source of vitamins and, in some instances, calcium.

Water is the predominant component of fruit juice. Carbohydrates, including sucrose, fructose, glucose, and sorbitol, are the next most prevalent nutrient in juice. The carbohydrate concentration varies from 11 g/100 mL (0.44 kcal/mL) to more than 16 g/100 mL (0.64 kcal/mL). Juice contains a small amount of protein and minerals. Juices fortified with calcium have approximately the same calcium content as milk but lack other nutrients present in milk. Some juices have high contents of potassium, vitamin A, and vitamin C. In addition, some juices and juice drinks are fortified with vitamin C. The vitamin C and flavonoids in juice may have beneficial long-term health effects, such as decreasing the risk of cancer and heart disease. Drinks that contain ascorbic acid consumed simultaneously with food can increase iron absorption by twofold. This may be important for children who consume diets with low iron bioavailability. Juice contains no fat or cholesterol, and unless the pulp is included, it contains no fiber. The fluoride concentration of juice and juice drinks varies. One study found fluoride ion concentrations ranged from 0.02 to 2.8 parts per million. The fluoride content of concentrated juice varies with the fluoride content of the water used to reconstitute the juice. In general, fruit juices are rich sources of variety biologically active components. Many of them are believed to promote human health and prevent some chronical diseases by their antioxidant, anticarcinogenic, antiinflammatory, antibacterial, antihypertensive etc. capacities (R. Puupponen-Pimia, L. Nohynek, C. Meier, M. Kahkonen, M. Heinonen, A. Hopia, K.-M. Oksman-Caldentey; Helsinky, Finland 2000). On the other hand, their effect on probiotics is poorly studied and subsequent research should focus on possible inhibitory properties of those compounds.

To maintain the desired viable cell counts in fruit juices is more complex than in the dairy products because of the damaging acidic environment in juices. In order to enhance the bacterial survival, various protective mechanisms have been developed, namely, microencapsulation, addition of prebiotics etc. However, many clinical studies have already reffered *Lb. plantarum* strain to be able to survive a treatment with hydrochloric acid at pH 2.5 for 1h (D. Haller, H. Colbus, M.G. Ganzle, P. Scherenbacher, C. Bode, W. P. Hammes, Stuttgart, Germany; 2001). Another study (Jacobsen et al., 1999) demonstrates that *Lb. plantarum* is usually in stationary growth phase in fermented food. Since, *Lb. plantarum* in stationary phase has been found to be especially resistant to ecological stress, it is therefore able to survive under acidic conditions in juice. Also studies by Giraud et al. (1991) and Passos et al. (1993) recorded higher cell populations of *Lb. plantarum* compared with the other microbes because of the ability of *Lb. plantarum* to grow until

the pH value dropped to 3.4. However, the low pH is not the only limiting factor influencing the survival and growth of bacteria in the juice. Other physical-chemical properties have to be taken into consideration when evaluating the optimal growth conditions. In general, these factors could be described as environmental stress.

In this study five different fruit juices were tested in order to get the information of how the different chemical composition of the juice might influence the survival of the bacteria and the organoleptic properties of the final drink. Apart from the juice components mentioned above there are more active compounds found within the juice depending on what kind of fruits were used for the preparation. By using various fruit mixtures, several chemical component's profile were created within the probiotic drinks. Organic acids, phenolic compounds and tannins are those of increased interest in this work. Part called „The presence of tannins in pomegranates" was included because of the further speculation on potentially damaging effect of pomegranates on bacteria.

Phenolic substances

In general, berries and red fruits are the rich source of two biologically active non-nutrients, phenolic substances, i.e. flavonoids (kaempferol, quercetin, myricetin) and phenolic acids (Shahidi & Naczk, 1995). To be more precise, the polyphenol group includes anthocyanins, flavonols (catechins), flavan 3-ols, gallic, ellagic and benzoic and cinnamic acid derivatives (caffeic, p-coumaric and ferulic derivatives) (Macheix J.J., Fleuriet A., Billot J.; 1990).Those components have demonstrated an antioxidative (Frankel,Kanner, German, Parks & Kinsella, 1993; Rice-Evans, Miller & Paganga, 1996) and anticarcinogen (Strube, Dragsted & Larsen, 1993) effect on human. Moreover, the antimicrobial activity has been measured. The phenolic profile in selected kinds of fruits is summarized in the Table 3 and Table 4.

The data obtained for intestinal pathogens, such as Salmonella, and selected Gram-positive and Gram-negative bacterial species, including some probiotic strains, suggested the inhibitory properties of phenolics with a different sensitivity within different bacterial species (Puupponen-Pimia, R., Nohynek, L., Meier, C., Kahkonen, M., Heinonen, M., Hopia, A., Oksman-Caldentey, K.-M., 2000). This observation is important mainly from the aspect of the interaction between the plant phenolics and probiotics, which is found in the studied probiotic juice. In other words, the knowledge of such interaction is important for the design of health-promoting functional foods containing both plant material and probiotic bacteria. Many reports have been written about the phenolic profile in different fruit and berry samples. Polyphenols such as anthocyanins, flavonols, flavan 3-ols and benzoic and cinnamic acid derivatives have been found the most occurring substances in the berries and red fruit (Macheix J.J., Fleuriet A., Billot J., 1990). According to a study carried out by Hakkinen (1999), the main phenolics found were flavonols, with the highest concentration in cranberry (74%) and lingonberry (68%). Ellagic acid was the main phenolic compound in strawberry, cloudberry, red raspberry and Arctic bramble (51 to 88%). The concentration of hydroxycinnamic acids was found high in blueberries and bilberry (63 to 75%) and hydroxybenzoic acids dominated in white currant (54%). However, this study excluded anthocyanins and catechins from the analysis.

According to another study (R. Puupponen-Pimia, L. Nohynek, C. Meier, M. Kahkonen, M. Heinonen, A. Hopia, K.-M. Oksman-Caldentey, 2000) anthocyanins have been found to be predominant in bilberries and blackcurrant. The antimicrobial activity of phe-

nolics has shown strong inhibitory effect on Gram negative pathogenic bacterial strains. LAB were in general more resistant to the phenolic compounds but sensitivity differed significantly among the tested organisms. However, myricetin (found for instance in cranberry and blueberry) was the only substance with the inhibitory effect on the growth of all LAB strains of human origin. Raspberry, cloudberry and blueberry at high concentrations also posses antimicrobial activity against some LAB. The phenolic acids (cinnamic acid, 3-coumaric acid, caffeic acid, ferulic acid and chlorogenic acid) showed activity only against Gram-negative bacteria at high concentrations (500 μg well $^{-1}$). In conclusion, from the study emerged, the berry extracts are active only against Gram negative bacteria. This may reflect the differences in the composition of the cell surface between Gram positive and Gram negative bacteria.

Tannins and organic acids

Tannins are phenolic compounds of high molecular weight with the ability to precipitate proteins. The natural sources are plants and food of plant origin, particularly fruits, cereal grains and some beverages (tea, wine, cocoa). Basically, tannins are divided into two groups: hydrolysable tannins derived from either gallic acid (gallotannins) or galloyl residues (ellagitannins) and proanthocyanidins. Both structure and the molecular weight give to tannins different properties in contrary to the low-molecular-weight phenolics. Santos-Buelga (Spain, 1999) analyzed the content of proanthocyanidins in different foodstuff. According to that study, the following substrates have been reported to contain proanthocyanidins: sorghum, barley, lentils, pears, grapes, apples, cherries, red raspberry, strawberry, blueberry and many more. The presence of ellagitannins has been recorded mainly in nuts and fruit, specifically in cashew nuts, pistachio, mango, pomegranate, red and black currant, grapes, apricot, cherries, raspberry, strawberry etc. (Haslam, E., Cambridge, 1989; Swain, T., Oxford, 1962).

The presence of organic and phenolic acids in pomegranates

A lot of studies have been carried out with varying results on sugar, organic and phenolic acids concentration in pomegranates. With the development in the analytical instrumentation, recent studies describe the pomegranate chemical composition in more detail (Artik et.al., 1998; Melgarejo et al., 2000). Some results taken from the study accomplished by E. Poyrazoglu (Turkey, 2001) reported the presence of 6 organic acids including mainly citric, but also L-malic, quinic, succinic, tartaric and oxalic acid. Also, 10 phenolic acids were detected in the pomegranate juice: gallic acid, protocatechuic acid, catechin, chlorogenic acid, p-coumaric acid, ferulic acid, o-coumaric acid, phloridzin, caffeic acid and quercetin. Moreover, the presence of tannins in the pomegranate juice was detected, but there were no details on their composition (M. Clifford, A. Scalbert, 2000). The summary of organic and phenolic acids present in pomegranates is given in the Tables 5 and 6.

3.3. The working procedure of the preparation of a novel probiotic drink

The preparation of a novel drink basically consists of three individual steps: 1) preparation of the cereal soups; 2) inoculation and fermentation of prepared cereal soups by a pure culture of *Lb. plantarum* 299v; 3) preparation of the final novel drink by mixing of the fermented cereal soup with a different types of fruit juices. These three steps are described in the following parts 3.3.1 – 3.3.3.

3.3.1. The formula and the preparation of cereal soups

The formula

The formula of the base for the probiotic fruit juice drink (the soy- or barley-meal soup base) was the same as that for the oatmeal-based drinks marketed in Sweden since 1994. The main ingredients such as oat (soya, barley) flour were mixed together with destiled water and with a certain amount of malted barley flour (Nord Malt AB, Soderhamn, Sweden) in order that the soup-base shall have a high-dry matter content (18.5%) since MBF serves as a source of enzymes, for instance, amylases, proteinases and beta-glucanases.

Table 3.1: The composition of formula for preparation of the cereal soups (Data provided by prof. Siv Ahrne, LTH, Department of Applied Nutrition)

The original formula used in all experiments is as following:	
oat/soya/barley flour	111 gr
water	600 gr
malted barley flour	4.6 gr

Preparation of the cereal soups

Oat or soya or barley flour and destilled water were mixed with 5% (w/w flour) MBF. The cereal soup-base (an oat, barley or soy) was then by continuous stirring in a stainless pot heated to the temperetaure of 50°C, then left for 30 minutes at the room temperature and again heated up to the temperature of 95°C. The final cereal soup was then transferred to sterilized beakers and cooled to 37°C, which is the inoculation temperature for the bacteria of human origin. All of the procedures with different substrates (oat -, barley - and soya - flour) were standardized in order to get reproducible results and therefore any possible deviation in the further fermentation process could be attributed only to the differences in metabolical pathways rather than to the influence of process parameters.

3.3.2. The fermentation

The fermentation process

Prior to the fermentation the concentrated suspense of *Lactobacillus plantarum* 299v was left to thawn for about 20 minutes on a crushed ice to avoid a potential temperature shock. The prepared soup-bases were inoculated with the liquid bacterial suspenses in

the concentration of 10^{10} cfu/ml and fermented with continuous stirring of the mixture under aerobical conditions. For the inoculation 1 ml of the bacterial suspense was used. The fermentation temperature was set at 37°C, which is usually the optimum for lactic acid bacteria of human origin. The fermentation time was optimised individually for each cereal soup according to the rate of pH drop. This aspect is further discussed in the result section. For the fermentation process it was used sterile glass fermentation tank in the volume of 1 litre. To maintain the constant temperature during the fermentation a water bath with water circulation was used.

The fermentation parameters

During the fermentation parametres such as pH, bacterial count and the count of pathogens were measured. Moreover, in order to gather a deeper knowledge of Lactobacillus plantarum metabolism, its growth curve was designed by the method of Rogosa plate counting. Samples were taken with a sterile pipette in a time intervals that were optimised in accordance to the fermentation differences between individual cereal-soup bases. In order to avoid any bacterial contamination, which could further distort the results, all of the operations were done in a sterile room. For more detailed description of the controlled parameters see the section 3.6.

3.3.3. „Novel“ probiotic fruit juice

Preparation of the drinks

The fermented formula was mixed with a comercial fruit juice in the both concentration ratios 1:19 giving the classical drink and 1:3 for the drink-shots. Five different commercial juices were used in this test (the type of juice and its composition are summarized in the Table 9). In this trial oat - based soup was left beyond further examination and the main focus was given to new types of cereals. Therefore only barley- and soya-based soups were tested as a potencial substrate for „novel“ probiotic drinks. For the preparation of those drinks only sterile disposable pipettes of corresponding volume were used. The prepared novel drinks were transfered into small glass flasks which were steriled prior to their usage.

Parameters controlled within the tested drinks during their storage

Both types of drinks (classical drink and drink-shot) were stored in the fridge at the temperature of 4°C for 2 weeks. Samples for the analysis of pH and bacterial count were taken with sterile pipette. The changes in pH have been regularly controled in 2 days intervals. After 2 weeks, the final count of *Lb. plantarum* as well as the occurence of pathogens were examined.

3.4. Methods of evaluation

3.4.1. pH measuring

The fermentation progress is characterised by the conversion of fermentable sugars to acids, that is, by the drop in pH which is further measured. Therefore this method has been performed in order to detect the rate at which acids are produced.

In this trial all types of fermented soup-bases (oat-, barley-, soy-) as well as mixtures of fruit juice with fermented soup-bases were investigated. The list of investigated samples is given in the Tables 7 and 8. First, pH values were taken during the fermentation process approximately every 2 hours until the pH reached the value of about 4 or slightly less than that. As the second step, after the fermentation process was terminated, the fermented soup-base was mixed with a comercial fruit juice. Each time a new type of fresh fruit juice was used (see the Table 9) and was mixed together with both fermented soya- and barley-soup in two concentrations of the fermented component, i.e. classical drink and shot drink (see the part 3.5). Those juice mixtures have been stored in the fridge in order to get the information of viability and activity of bacteria (how bacteria are surviving and/or growing) under normal storage conditions. At the begining of every 2 weeks storage period, the initial pH of fruit juice and fermented soup was measured. The storage conditions were performed in the laboratory fridge under the temperature of 4°C. In the second part of pH measurements, pH was taken four times a week. Concurrently, the progress of pH was measured within the fermented soups, which were as well stored in the fridge under the same conditions.

Limitations for the pH measuring

All the measurements have been performed under the same conditions (room temperature, atmospheric pressure) using the laboratory pH meter (ORION, model 420). The set of buffer standards (pH 5, 7, 10) were used to calibrate the pH meter prior to every measuring of the pH. The pH of samples was measured after the temperature of the sample equalized to the temperature of a room.

3.4.2. Bacterial enumeration

The fermentation samples, that are summarized in the Tables 7 and 8, were decimally diluted in a sterile dilution solution up to the concentration of 10-7. For this step 1 ml of a sample was taken using pre-calibrated pipette with disposable sterile tips and then mixed thoroughly with a vortex. Afterwards, 0.1 ml from each concentration has been plated on sterile Rogosa agar which is a specific growing medium for Lactobacilli using pre-calibrated pipette and then incubated anaerobically at 37°C for three days. After the incubation time bacterial colonies on plates were counted and the viable bacterial count was expressed as a colony forming units (CFU)/ml of fermented solution. The same procedure was also used when the fermented soup was mixed with fruit juice in the concentration of either 5 or 25% depending on the type of the final product (classical drink or shot-drinks). The scheme of the serial dilutions is shown in the Figure 4. The bacterial enumeration in the cereal soups is a median value of four repetitions (samples taken from four fermentations), the same for the probiotic drinks was done in duplicates and the final count is a median value of the results obtained.

Limitations for the bacterial enumeration

For the statistical reasons were counted only the final plates in the series that had between 30 and 300 colonies. Fewer than 30 is not acceptable since too few may not be representable of the sample. More than 300 colonies are likely to form colonies too close to each other to be distinguished as distinct CFUs (colony forming units). The assumption

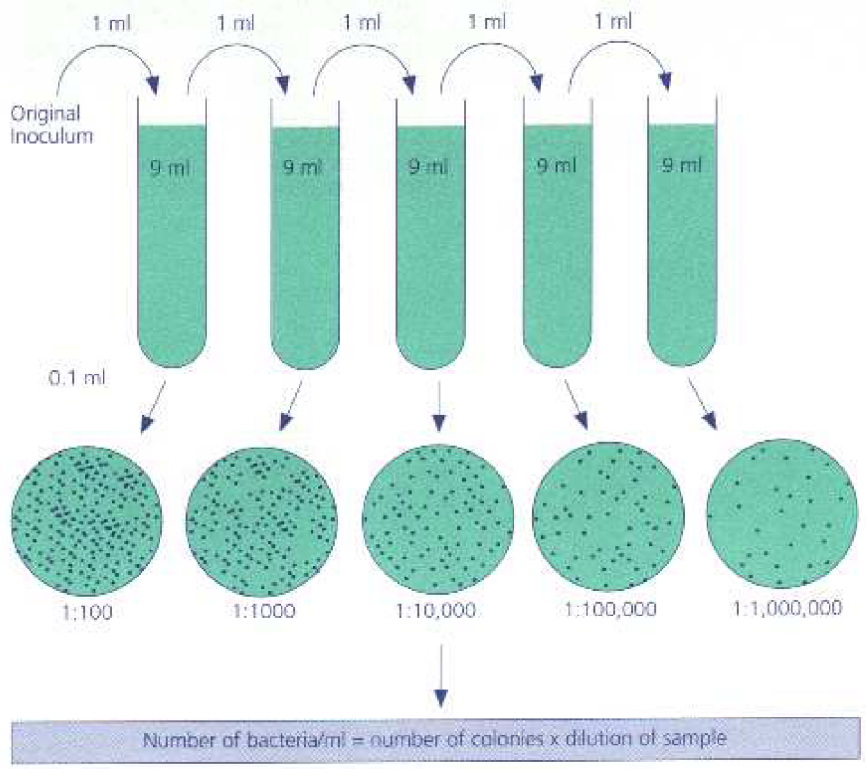

Figure 3.2: Serial dilution, www.sigmaaldrich.com/img/assets/4261/micro_7.gif

is that each viable bacterial cell is separate from all others and will develop into single discrete colony. Thus, the number of colonies should give the number of bacteria that can grow under the incubation conditions employed.

Preparation of dilution solution

The amounts of the following components were weighed out into the sterile flask: natrium chloride (NaCl) – 8,5 g; bacteriological pepton – 1 g; Tween 80 – 1 g; cysteinium chloride – 0,2 g. One litre of distilled water was added and the mixture was vigorously stirred in order to dissolve all the components. 10 ml of this solution was distributed to sterile tubes, closed and sterilized again. After that tubes were cooled down and stored in the laboratory fridge (4°C).

Preparation of Rogosa agar

82 grams of pre-prepared mixture for Rogosa agar was suspended in 1 litre of distilled water and brought to the boil to dissolve completely. 1.32 ml of glacial acetic acid was added and mixed thoroughly. The agar solution was heated up to 90 – 100 °C for 2-3 minutes with frequent agitation. By that prepared agar was cooled down to 40 – 50 °C and distributed to sterile petri dishes. After this procedure petri dishes with agar were left to solidify in a sterile room and then stored in the laboratory fridge.

Typical formula for Rogosa agar (g/l)

pH 5.4 ± 0.2 at 25 °C tryptone 10.0; yeast extract 5.0; glucose 20.0; sorbitan mono-oleate 1.0; potassium dihydrogen phosphate 6.0; ammonium citrate 2.0; sodium acetate 17.0; magnesium sulphate 0.575; manganous sulphate 0.12; ferrous sulphate 0.034; agar 20.0

3.4.3. Pathogenic bacteria enumeration

The sample preparation followed the same sequention of steps as was used in the bacterial enumeration assay but instead of Rogosa the VBRD agar was used. VBRD is the specific medium for the indication of faecal contamination, or in other words, this medium supports the growth of pathogenic bacteria. This trial has been done for both fermentation media and for the juice drinks. Plates were inoculated (Figure 5.) and aerobically incubated for one day at 37°C. The bacterial enumeration in the cereal soups is a median value of four repetitions (samples taken from four fermentations), the same for the probiotic drinks was done in duplicates and the final count is a median value of the results obtained.

Figure 3.3: Inoculation of the VBRD agar

Limitations for the pathogenic bacteria enumeration

The same limitations as for the bacterial enumeration. For more information see the section 3.6.2.1

Preparation of crystal-violet neutral-red bile glucose agar (VBRD agar)

39.5 grams of pre-prepared mixture for VBRD agar was suspended in 1 litre of distilled water and heated up in a boiling water bath with frequent stirring until completely dissolved. Afterwards the solution was not boiled for more than 2 minutes. By that prepared agar was cooled down to 40 – 50 °C and distributed to sterile petri dishes. After this

procedure petri dishes with agar were left to solidify in a sterile room and then stored in the laboratory fridge.

Typical formula for VBRD agar (g/l)

pH 7.4 ± 0.2 at 25 °C peptone from gelatine 7.0; yeast extract 3.0; sodium chloride 5.0; D(+) glucose 10.0; bile salt mixture 1.5; neutral red 0.03; crystal violet 0.002; agar agar 13.0

3.4.4. The growth curve of Lactobacillus plantarum in the association with a soy- and barley- substrates

This trial was included into the study in order to get the information of how the time dependancy of separate phases of growth of Lactobacillus plantarum differ between soya-based and barley-based soup. By this experiment we will obtain the knowledge of the number of bacteria on time dependancy and thus the information about the phase of growth in which the bacteria were when mixed with a fruit juice. As explained later in the relation with an enviromental stress, this information might be of a high importance when considering the further survival of bacteria in the novel probiotic juice.

The fermentation followed the same procedure steps as it was stated in the part 3.4.1. Contrarily to the preparation of fermented soups for a further use in the probiotic drinks this fermentation was left to run for 34 hours. This time was considered to be long enough to be able to distinguish different phases of growth. The control of pH value was beyond the scope of this experiment as it was already part of a previous measurement.To estimate the phases of growth the fermentation samples were plated on the Rogosa agar aproximatelly every two hours. After that the incubation of plates was the same as mentioned in the part 3.6.2.

Tasting assay

The potentionaly new probiotic drinks were also examined from the organoleptic aspect. The fermented formula was made by the traditional way and mixed with different types of fruit juice in the concentration of 5% giving the final drinks. The original Pro Viva drink was prepared as well for more objective comparison. Following juice flavors were used: black currant, rosehip, pear/kiwi, mango and blueberry. Two groups of volunteers participated in this trial. One group was formed out of 10 Skanemejerier employees, who previously participated in the same type of tasting with the original Pro Viva drink. Consequently, they could better recognize the difference between the novel drink and the clasical one. The second group were random people with no or just a little experience with probiotic drinks. This type of group was included in the study in order to get the information of taste preferences between ordinary and trained customers. Those two groups were then asked to taste 15 drink samples (the list of tested samples is summarised in the Table 30) and to assess their overall impression of both oat-, soya- and barley-based juices and also to rate their acceptance of the sensory characteristics on the scale from 1 (the least acceptable) to 9 (the most acceptable).

4. Results

4.1. Drop of the pH value in the cereal soups

To control the fermentation progress the pH value was measured during its course. The presence of fermentable (utilizable) sugars in the fermentation media resulted in the drop of pH due to the accumulation of lactic acid and also acetic acid as a minor product. (D. Charalampopoulos, S.S. Pandiella, C. Webb; Manchester, UK, 2001). All the tested samples are summarized in the Table 7. The results of changes in pH are shown in the Table 10.

4.1.1. Evaluation of pH drops in the cereal soups

Time taken to reach pH 4 or lower in the soup-bases varried from 7 to 24 hours depending on the flour used for the soup-base formula.

The barley soup

The initial pH in the barley soup varied between pH 5.35 and 5.46. After 8 hours of fermentation the soup has reached the final pH values between 3.51 and 3.63. Further decrease in pH was observed after 20 hours of fermentation when pH reached 3.25.

The soya soup

The initial pH in the soya soup varied between 6.1 and 6.3 and time taken to reach the pH value 4 was far longer than that for barley soup. The fermentation time was about 24 hours in contrary to 7 hours for the barley medium. A pH value taken after 8 hours of fermentation was between 4.85 and 5.

The oat soup

An oat soup, which was taken as a control study, had the initial pH between 6.14 and 6.22. Time to reach the pH value 4 was almost the same as for the soya soup. After 22-23 hours the pH was in the range 3.85-4.

4.1.2. Conclusion

From the aspect of the pH drop, all the studied soups have showed high stability of the fermentation process. The shortest fermentation time was found in the barley medium. The soya and oat media showed similarities in the fermentation progress. If the economical aspect was another controlled parameter, the barley soup would be a potentially suitable substrate as it has demonstrated the shortest fermentation time.

4.2. Counts of *Lb. plantarum* cells in the soup-bases

The bacterial count that was measured by the number of colonies growing on the Rogosa agar gives us the overall information of how parametres such as time and the chemical

composition of the growing medium (i.e. soya- , barley- or oat-based soups) might influence the survival of the bacteria. The flourishing and results are given in the Tables 11 - 14.

4.2.1. Observing and counting of colonies of *Lb.plantarum*

After the cultivation time petri plates were taken out of the incubator. The Figure 4 shows what was observed on the plates as a result of the presence of *Lb. plantarum* in the tested medium. The white colonies occured in the dilution concentrations from 10^{-1} up to 10^{-7} . According to the limitations mentioned in the part 3.6.2.1, all of the petri dishes containing between 30 and 300 colonies were selected and the number of colonies was then manually counted. In the most cases these numbers corresponded to the both dilution concentrations 10^{-5} and 10^{-6}.

The amount of bacteria per milliliter of sample was counted using following formula: number of colonies (CFUs)/ (dilution x amount plated) = # of bacteria/ml

Figure 4.1: Picture of colonies of *Lactobacillus plantarum* 299v growing on Roggosa agar

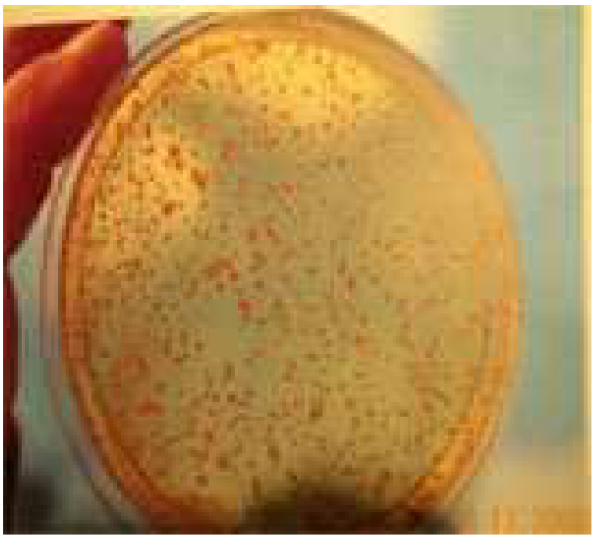

Figure 4.2: Petri plate with *Lb. plantarum* colonies after the cultivation

4.2.2. Evaluation of the counts of *Lb. plantarum* cells in the soup-bases

The bacterial counts at the beginning of fermentation were found more or less the same in all the tested soups reaching the value about 2.10^7 (cfu/ml) as for the inoculation was always used the same amount of bacterial suspense of a constant concentration of bacteria. The highest number in the cell population have been found in the oat soup with about 2.10^9 (cfu/ml) after 24 hours of fermentation. The results for the barley and soya soups varied in the range 2.10^8 1.10^9 (cfu/ml). These figures comprise the count of bacteria presented in the medium when the pH reached value 4 or lower. This is basically 8 hours for the barley and 22-24 hours for the soya and oat medium.

Another interesting finding reflected an influence of the fermentation time on the bacterial count which is analysed in the discussion section. It was observed that when the fermentation was left to run either a longer or shorter time than the optimal, bacteria reached lower numbers in the medium. This was obvious for the soya medium where bacteria decreased their numbers approximately by one decimal order when the fermentation time was 34 hours instead of 17 (see the Tables 16 - 17). A similar pattern was recorded also with the barley medium but the bacterial count reduction wasn't that significant.

4.2.3. Conclusion

As a conclusion it can be stated that in general the number of bacteria (cfu/ml) increased during the fermentation but the final counts varied depending on the fermentation media used. Also the fermentation time has been observed as another important factor influencing the final bacterial count.

The type of cereal substrates is then thought to be an important aspect that could affect the shape of the growth curves. In other words, the type of cereal substrate seems to have an influence on the duration of the fermenation cycle which in turn might further influence the bacterial counts in the drinks. Considering that, the drop in the CFU value in the juice drink might reflect the reality that bacteria were either in the declining phase of growth or in their exponential phase in which they tend to be more sensitive to an enviromental stress (as explained in the discussion part 5.5). For more details about the growth phases see the results in the part 4.5. There is also a need to note that the highest bacterial count doesn't correspond with the pH value. This observation is obvious on the example of fermented soya soup as the highest count of bacteria was reached at pH higher than 4. Another important aspect linked to the counts of bacteria is the possibility of forming of viable but non-cultivable colonies (VBNC). This important factor that might lower the final bacterial counts is further speculated in the discussion section.

4.3. pH changes and counts of *Lb. plantarum* cells in the mixture with juices

The same testing procedure, i.e. the pH measurements and the viability test, was also applied on the novel probiotic drink which was the mixture of either barley or soya soup with different types of comercial fruit juices. The final count of bacteria, expressed as a CFU/ml, gives us the information about the metabolical activity and survival of the

bacteria when mixed with another type of substrate – fruit juice. Moreover, the storage time, as an important factor in the design of novel probiotic product, was evaluated. The results are given in the Tables 18 – 32.

4.3.1. Evaluation of pH changes and counts of *Lb. plantarum* cells in the mixture with fruit juices

Remarkable results were observed for the drink with juice number 2 (see Tables 19 and 25 - 26). While the viable count in the mixture with juice 1 (see Tables 18 and 23 – 24) after 2 weeks of storage was about 4.108 cfu/ml for the drink on the soya basis and uncountable in a drink on a barley basis, there was a significant reduction in the bacterial count in the barley-based drink with juice 1. For the soya-based juice the change was not so significant but for barley-based juice this change was represented by the lowering of the bacterial count by 2-3 orders to the final count of about 2.108 cfu/ml. Also the progress of pH values was found unchanged for the soya-based juices but different for barley-based drink with juice 2 indicating reduced or altered bacterial metabolical activity in that medium.

Both, soya and barley stored soups followed similar ways of the pH progress with two increases on the 8 and 15 day. An exception of that was the soya soup (from now on reffered to an exceptional soya medium) in which the fermentation was untimely terminated at the pH value 4.68 what was after 17 hours of the fermentation. In that medium there was only one pH increase on the 15 day. The other fermented soya soups had the initial pH of about 4.3 when got stored. From the viability aspect, there were recorded more significant differences between the soya and barley medium. In the barley soup bacteria were not only surviving but they also exhibit a small increase in their counts, whereas in the soya soup the lowering of bacterial count was observed. Even though, there was slightly higher initial bacterial count in the „exceptional“ soya medium, the final count was found almost the same as for the another soya soups.

Generaly, higher bacterial counts after 2 weeks of storage were recorded in the soya-based drinks. This could, after all, be attributed to the fact that there was a higher initial concentration of bacteria in the soya soup. During the storage, the number of bacteria was slowly decreasing. This was even more noticeable again in the exceptional soya medium. In contrary, in the barley-based drinks the initial bacterial concentration was lower, but the decrease was less significant and in some cases bacteria were even growing. Nevertheless, the bacterial count was still slightly higher in soya-based than in barley-based drink.

Comparison with the control study performed without fruit juice

In parallel with the juice assay there was also just a soup-base assay in order to compare the viability and metabolical activity in the medium with and without juice. As emerged from the results (see the Tables 18 - 30), the stored soup-base demonstrated relatively stable pattern of the bacterial activities. In contrary to that, there was, to a high extent, dependency of the drink on the juice vs. cereal soup combination. This fact was then reflected in the pH changes and viable counts of bacteria. In the drink, the first drop in pH occured within a few days after the drink was prepared. In the soup-base bacteria followed the same pH course and also the number of bacteria reached the similar values.

4.3.2. Conclusion

The viability test showed a striking difference in the bacterial counts after two weeks of a storage in the fridge. It is very likely that the different chemical composition of tested fruit juices was the reason for this phenomena. It was detected by a simple comparison of viable counts in different tested drinks that some fruit juices are more suitable as a growing medium and some are suitable less. Therefore further research aiming the correlation bacteria-medium compounds needs to be carried out.

It seemed that also the pH fluctuations were significantly influenced by the medium and no uniforme pH pattern was recorded. The pH value in the drink was either decreasing for a certain time or started to increase. However, the changes in pH during the storage indicated that bacteria were still metabolically active. The reason for that could be attributed to the diverse ways of metabolism. It will be briefly discussed later, how different factors, for instance chemical composition, are related to the bacterial metabolism.

Importantly, although the declining course in most cases was observed, the final bacterial count after two weeks of storage was still high enough reaching the concentration of about 2.10^7 cfu/ml. This finding might propose both soya and barley soup as an optional growing medium for *Lactobacillus plantarum* 299v.

4.4. Test for the pathogenic risk

All samples (this means samples taken during the fermentation as well as the stored samples) were investigated from the pathogenic aspect in order to detect any serious health risk for consumers. Results are summarized in the Tables 15 and 33.

4.4.1. Evaluation of the pathogenic risk

In all cases there was no growth of pathogenic microorganisms observed, that means that no pathogenic bacteria were present in any of the tested products.

4.4.2. Conclusion

This basic and overall trial showed that there is no potential health risk for consumers. However, there is a need for more complex and specific evaluation in order to exclude any possible health risk related to the consumption of such products.

4.5. The growth curve of *Lb. plantarum* 299v with the soy- and barley-substrate

In orded to investigate the metabolical differences of *Lb. plantarum* on new cereal substrates (soy and barley) the growth curve was designed by the method of counting colonies cultivated on Rogosa agar. Another important information obtained from the growth curve is the metabolical state in which the bacteria were when mixed with a fruit juice, which might be a factor influencing the survival of the bacteria when expossed to an enviromental stress which was represented by the change of a growing enviroment from a cereal soup to a fruit juice.

4.5.1. Evaluation of the growth curve in the association with soy substrate

According to the results of the bacterial counts (Table 16) taken approximately every two hours during the course of fermentation one can distinguish several individual phases of bacterial growth (see Graph 1). In the first 4-5 hours after the start of fermentation almost no growth of bacteria was recorded, therefore this time could be referred to a lag phase. At the 6th hour a doubling of the bacterial count was recorded referring to a phase of faster growth. This phase then switched to an exponential phase which was observed from the 7th to 17th hour of the fermentation. At the end of this phase the bacteria reached their maximum counts and started to slacken their growth rate. The time between the 17th and 24th hour could correspond to a stationary phase which was the time when bacteria maintained their counts on the same level. After this period the declining phase of growth occured when bacteria started to lower their counts. First it was only a slow decrease in counts which passed to an increased death rate of the bacteria after 30 hours of the fermentation. This is probably a consequence of accumulated lactic acid and a depletion of nutrients in the fermentation medium.

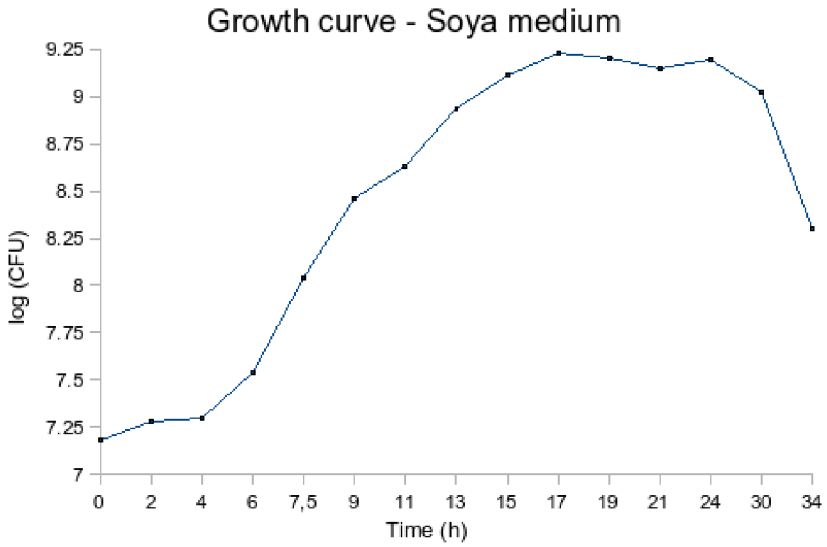

Figure 4.3: The growth curve of *Lactobacillus plantarum* 299v in the association with a soya substrate

4.5.2. Evaluation of the growth curve in the association with barley substrate

From the results of bacterial counts (see the Table 17) was designed the growth curve of *Lb. plantarum* on a barley substrate (see the Graph 2). In the barley medium the lag phase was recorded in the first 2 hours of the fermentation which was subsequently followed by the phase of faster growth which was probably of a short time period as there is no obvious part of the growth curve that could be identified with this phase. The bacteria then switched to an exponential phase which was observed in the time interval from the 2,5 - 3rd to 13 - 14th hour of the fermentation. A stationary phase was found

to be the longest phase of the bacterial growth in the barley medium with its 17 hours of duration. Approximately this phase was observed from the 13 - 14th to 30th hour of fermentation. Then a declining phase of growth has started.

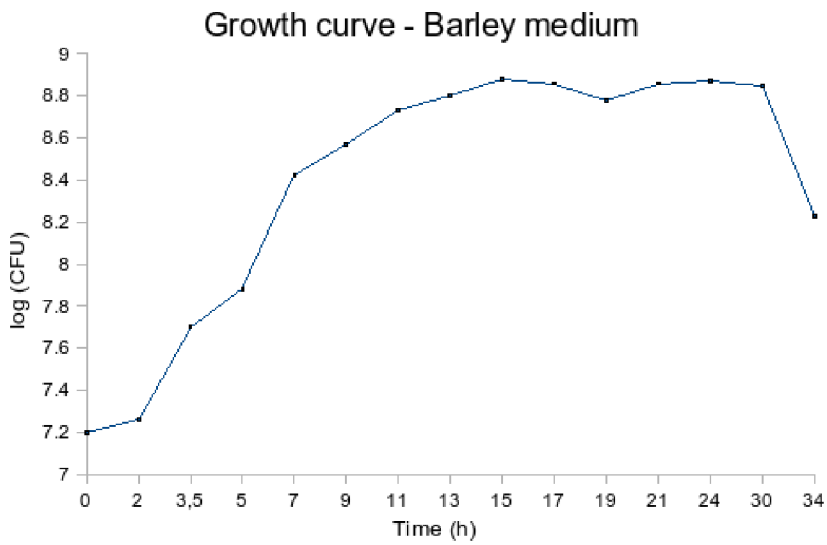

Figure 4.4: The growth curve of *Lactobacillus plantarum* 299v in the association with a barley substrate

4.5.3. Conclusion

From the obtained growth curves of *Lb. plantarum* on two different substrates emerged some metabolical variations that were reflected in the time dependant records of bacterial counts. Also the pH progress seems to be directly linked to the bacterial phase of growth as one can see that by a simple comparison of the pH progress (see the Table 10) and the individual growth phases in the time dependancy. The pH drop in a barley medium is faster as the exponential phase occures already in 2-3 hours of the fermentation. Likewise, in a soya medium the pH drop is more gradual as the bacteria come to the exponential phase later. There were several differences recorded between the two growing media used. The lag phase was found to be shorter with a barley medium lasting around 2 hours compared to 5 hours for a soya medium. The duration of an exponential phase was more or less the same for both media lasting 10-11 hours. Another difference was recorded within the stationary phase with 17 hours lasting for a barley medium and 7 hours for a soya medium. Also a declining phase differed in the time of commencement: this occured after 30 hours of fermentation in a barley medium while this was observed some 6 hours before in a soya medium. On the contrary, the bacterial counts in a soya medium reached higher values during the course of the fermentation which is an important aspect to consider for a designing of a probiotic drink. The metabolical differences between the soya and barley medium might reflect the variability and accessibility of nutrients and growing factors what is speculated later. The information given by the growth curves also indicates the metabolical state in which the bacteria were when used for the preparation of probiotic drinks. In most of the experiments the barley soup was used for further experiments after 8-9 hours of the fermentation while the fermentation of the soya soup

was mostly terminated after 21-22 hours with the exception when the fermented soya medium was mixed with a juice after 17 hours. Considering these facts, we can see that in a barley medium the bacteria were most likely in their exponential phase when they changed their growing enviroment while in a soya medium bacteria probably already reached their stationary state. Again with the exception of the soya medium where the bacteria still probably were in the exponential phase.

4.6. Tasting trial

To asses the organoleptic properties of the newly prepared probiotic drink the tasting trial was a method of my choice. For this assay to perform, two different groups (trained group and a group random people) of evaluators were asked to participate. The overall impression of 15 tasted samples was then evaluated and summarized in the Tables 35 – 36.

4.6.1. Evaluation of the tasting trial - group 1

Between the two testing groups there was found a relatively significant difference in the taste preferences.The volunteers of group 1 generally expressed more or less uniforme rankings of the tested drinks (see the Table 34). The oat-based drinks were assessed with the highest acceptance score in all cases. In contrary to that, soya-based drinks were accepted the least indicating some unpleasant aromas related to the fermented soya component. Importantly, some barley-based drinks had comparable results to that of oat. Some individuals even rated barley higher than oat but soya in this group never reached the value higher than 6. The most preferable drink combinations were oat- and barley-based formulas with black currant and blueberry juice. Also soya-based drink obtained higher ratings when mixed with those juice flavours.

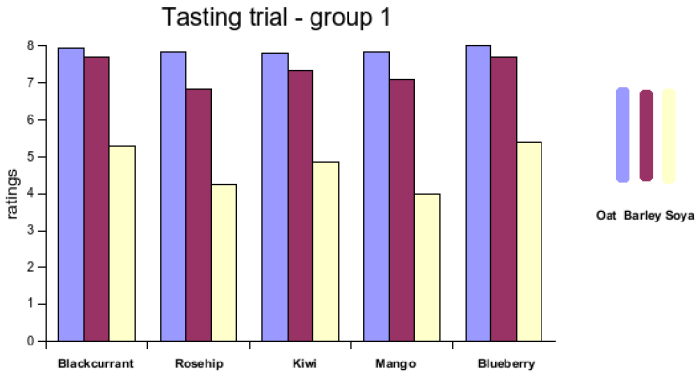

Figure 4.5: Evaluation of the tasting trial - group 1

4.6.2. Evaluation of the tasting trial - group 2

The group 2 had generaly lower ratings and also the taste preferences were distinctly different from the group 1 (see the Table 36). Also the perception of tastes within the

members of the group was found more subjective. Basically, there was no clear unity from the taste aspect among volunteers of the group 2, what was probably influenced by only a little experience with probiotic juices. Oat-based drinks didn't obtain so high median values and moreover rosehip and blueberry barley-based combinations were described as more tasty. The combination pear/kiwi with barley soup was found of the same taste as with oat soup (again expressed as a median value). Furthermore, the values obtained for oat-, barley- and soya-based drinks in the mixtures with rosehip and mango were more equal than it was found in the group 1.

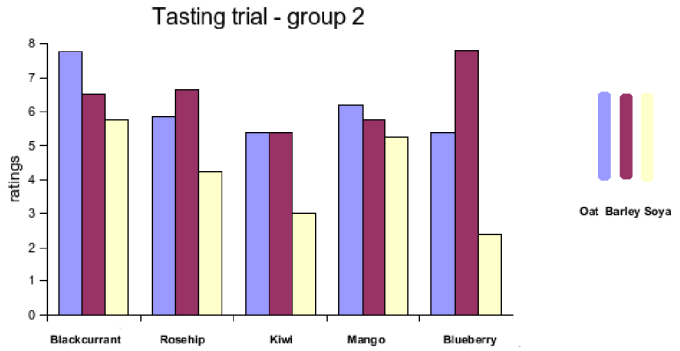

Figure 4.6: Evaluation of the tasting trial - group 2

4.6.3. Conclusion

The two participating groups differed in their ratings which could be attributed to distinct food habits and overall personal backgrounds. In general, from the tasting assay which was carried out with 2 different groups of volunteers, emerged that soya-based drinks had a specific taste and aroma which were perceived to have an unpleasant, and for some individuals even disapetizing, characteristics.

Despite the diversity between two participated groups, group 2 has exhibited some similarities with the group 1. At first, the increased popularity of black currant - and blueberry - probiotic combinations was recorded within asked volunteers and secondly, soya-based drinks were again perceived as the least tasty mixtures. In two cases the median value was 3 and even lower. For some individuals the taste of soya was so unpleasant that they rated some drinks on soya basis with the number 1 on the rating scale. This was observed for the soya mixture with blueberry juice which was the most tasty combination obtained for soya in the group 1.

5. Discussion

5.1. The influence of MBF on the fermentation process

From the previous studies, dealing with the fermentation process in the barley medium with and without added malted barley flour (MBF), has emerged that addition of MBF resulted in the shorter fermentation times that is characterized by a certain drop in pH (Marklinder, 1991). In this study the pH value was set at pH 4. The shortage of the fermentation time might be explained by the higher availability of certain amino acids due to the presence of, in the malted barley flour contained, hydrolyzing enzymes. Not only amino acids but also higher amounts of fermentable sugars and the presence of another supporting (protective) compounds can lead to the high cell growth and acidification rates. As a consequence, this can result in the enhanced viability of probiotic strain. This phenomenon is mainly due to the prevention of growth of the undesirable microorganisms present in the raw material as a result of a pH drop (Marklinder and Lönner 1992).

5.2. The influence of chemical composition of different cereal substrates on the fermentation process

Although the highest acidification rate was found in the barley medium, the viability test showed higher bacterial counts in the oat and soya medium. The higher rate of acidification might be caused by the presence of easily fermentable sugars in the barley medium while soya and oat medium may either lack these sugars or have much lower amounts. Nonetheless, there are other substrates in soya and oat that can be fermented by *Lb. plantarum*. For example, soya flour is rich in wide spectrum of amino acids that can be utilized in the lactose metabolism. Even though amino acids are not preferred as a primary carbon and energy source they can still be fermented after the metabolism is adapted on new substrates. This adaptation time is probably why the accumulation of acids occurred first in the barley medium and then in the soya and oat medium. The different types of potential substrates and the possible ways of their metabolism are discussed later. While the drop in pH in the soya and oat medium continued even after 24 hours, there was only a little change in the barley medium. This finding may indicate that after the depletion of sugars, there are not so many fermentable substrates left in the medium. Furthermore, the changes in pH during the 2 weeks storage in the fridge demonstrated the fermentation differences between the soya and barley medium. Also the comparison of the fermented medium and juice drinks was interesting from the pH and bacterial count aspect. In the fermented formula there was more or less the same fermentation pattern recorded while the bacteria in juice drinks followed a unique way of consuming and releasing compounds from and into the medium. The variations in the uptake and production resulted in the diversity of the pH changes. The observed difference between the fermented medium with and without fruit juice might indicate the presence of some active substances in the fruit juice. This could probably result in the variations of the acidification rates and the bacterial counts after the addition of different types of commercial fruit juices.To understand more the fluctuations of the pH in the

juice drinks, the ability of *Lb. plantarum* to ferment substrates others than sugars is one aspect that is needed to consider. Another aspect is the further lactate and pyruvate metabolism which is discussed later.

5.3. The presence of other fermentable substrates in the growing medium and its influence on the acidification rate

Polyols, organic acids and amino acids, if present in the fermentation medium, have been reported as other suitable substrates for *Lb. plantarum* as it has its enzymatic pool to utilize a wide spectrum of substrates (see the section 2.2.5). Organic acids are utilisable subtrates that are very available in fruit juices as fruit is a natural source of these compounds. Apples, pears, cherries, wine grapes, pomegranates, lemons are just a small demonstration of the variability of fruit containing organic acids. These can be found under the name citrate, malate, fumarate, tartrate and more.

5.3.1. Organic acids

As indicated above, *Lb. plantarum* changes its metabolical activities according to the actual conditions in the fermentation medium. Some possible mechanisms of the metabolism of organic acids have been already explained in the recent studies (S.-Q. Liu, 2002). One of them describes the citrate catabolism via the pathway: citrate → acetate + oxaloacetate → pyruvate + CO_2 → lactate (Hugenholtz, 1993; Sarantinopoulos et al., 2001). Besides citrate, malate is another common organic acid being fermented by most LAB in the process known as a malolactic fermentation. During that, malate is directly converted into lactate and CO_2. Tartarate as a major organic acid in wine can be stechiometrically decomposed by certain strains of *Lb. plantarum* producing the mixture of lactate and acetate (Radler, 1975). However, it is not the aim of this study to provide the deep overwiev of all metabolic pathways, but to give a brief description of how pyruvate and lactate can be formed from other substrates different from sugar.

5.3.2. Polyols

Since polyols are not generally fermentable by most of LAB it is interesting phenomena that some *Lb. plantarum* strains have the ability to utilize some hexitols (six-carbon polyols) as a carbon and energy source. A good demonstration of that is mannitol of which utilization appears to be a common feature of *Lb. plantarum.* This has been proven by many studies performed on food matrices such as cheese (Jordan and Cogan, 1994), fermented vegetables (Chen et al., 1983; Chen and McFeeters, 1986; McFeeters and Chen, 1986) and wine (Davis et al., 1988; Liu et al., 1995). There is another example of fermentable polyols sorbitol, known also as hexitol or glucitol worth metionning. (Carr and Davies, 1970; Carr and Whiting, 1971). These two examples of polyols stated above are commonly used in the food production as sweeteners. The general metabolic pathway of mannitol is as following: mannitol → mannitol-1-P → fructose-6-P → 2-pyruvate → 2-lactate

5.3.3. Amino acids

Amino acids also follow an uncommon metabolic pathway in order to produce pyruvate and lactate. Both the direct and undirect formation of pyruvate from amino acids seems to be typical property of LAB. In my study, the soya medium, with its high content of amino acids, might especially contribute to the different metabolisms of *Lb. plantarum*. Nonetheless, all of these substrate transformations are both species- and strain-specific (Christensen et al., 1999; Weimer et al., 1999; Yvon and Rijnen, 2001).

5.4. The influence of lactate and acetate metabolism on the pH diversity

Another optional explanation of the diversity in the pH pathways might be given by the further lactate and acetate metabolism. During the fermentation, the accumulation of lactate and acetate resulted in the drop of pH in the soup-bases. However, increases of pH were also observed during the experiment indicating that there were more metabolic pathways than just that described for homofermentative LAB. One possible explanation could be a further conversion of lactate resulting in an increase of pH. Unfortunately, only a little attention has been given to a lactate metabolism by LAB in contrary to the conversion of carbohydrates to lactate which has been well studied (S.-Q. Liu, New Zeland, 2002). Lactate, the main end-product of LAB fermentation, and its metabolic precursor, pyruvate, might be under specific circumstances further converted to other important compounds that are of significance in food and beverage fermentations. For this reaction to occur, LDH (lactate dehydrogenase) must be present to catalyse the conversion of pyruvate to lactate in LAB. Lactate is then the predominant fermentation end-product. Nonetheless, under aerobic conditions both lactate oxidase or NAD+ - independent LDH catalyse the catabolism of lactate to pyruvate which is further catabolised to acetate and carbon dioxide (Murphy et. al., 1985; Kandler 1983). However, pyruvate can also be catabolised by some LAB to other compounds such as formate, ethanol, acetaldehyde, diacetyl, acetoin and 2,3-butanediol (S.-Q. Liu, New Zeland, 2002).

5.5. Enviromental stress and its impact on the survival of Lactobacillus plantarum

There is an evidence of a “suicidal” behaving of bacteria exposed to the stress. This stress is described as an environmental condition different from the normal growth parameters. Nonetheless, not all stresses are lethal for bacteria and the bacterial response differs greatly depending on its growth phase or metabolical state. The higher sensitivity of the exponentially growing bacteria compared to those in stationary phase is explained by the excessive production of a free radicals having the lethal effect on a bacteria (T. G. Aldsworth, R. L. Sharman and C. E. R. Dodd, 1997). Indeed, that are these compounds responsible for the bacterial death, rather than the stress per se. In my study, bacteria first growed on the soya or barley substrate. Afterwards, this fermented medium was mixed with the fruit juice. Thus the stress conditions could be represented by the simple change of enviroment, presence of some potentional inhibitors, low pH in the juice and

low temperature (the fridge storage) etc. If the bacteria were in their exponential phase of growth when mixed with the juice, it might resulted in their increased susceptibility to the enviromental stress and subsequently to their higher death rates. For the second drink, the fermentation of the soya formula was terminated after 17 hours instead of 22, which was the usual fermentation time. So accordingly to the obtained growth curve it is very likely that bacteria were still in the exponential phase of growth. Therefore the decrease of bacteria in the soya-based drink and in soya formula might be contributed to suicidal behaving of *Lb. plantarum*. There were also some deviations observed in the barley-based drink. From the growth curve obtained for a barley medium bacteria were very probably in their exponential phase so this could explain some of those variations in counts. However, it is just an optional explanation.

5.6. The impact of antimicrobial substances on the survival of *Lb. plantarum*

Among the other important points, the presence of phenolic acids should be mentioned the first. Within my study there was no research dealing with the chemical compounds in the fruit juice. Thus, I can only speculate of how the bacterial growth was influenced by the presence of phenolic acids or other potential inhibitory components, such as phytic acid. Some of those possible mechanisms are stated in the following section.

The behavior of L.plantarum in the mixture of the juice with cereal base is not easily predictable. This is probably due to the variations in the juice composition, to the presence and concentration of antimicrobial components or contrarily to some growth stimulators, juice-base interferences, intervarietal differences within the same kind of fruit, etc. For instance, the chemical composition of fruits differs depending on the cultivar, growing region, climate, maturity, cultural practice and storage. All of these factors lead to the significant variations in organic acids, phenolic compounds, sugars, water-soluble vitamins and minerals composition (Cemeroglu et al., 1988, 1992; Unal et al., 1995; Melgarejo et al., 2000).

5.6.1. Phenolic and organic acids

The results obtained for the bacterial count and the pH progress in the probiotic drink show a striking difference between the different juice-formula combinations. It was already mentioned that the possible cause of this phenomena is the presence of the substances potentially limiting the bacterial growth. By the simple comparison of juices' content, basically made of mixtures of various kinds of fruit juices, it was suggested which components could have the damaging effect on the number of living bacteria. Moreover, the information about the chemical composition of the fruits that are the most suspected of having inhibitory activity is summed up in the Tables 3 - 6. This gives the basic knowledge of the chemical environment in the medium. However, these values are strongly influenced by many factors, so these figures are presented only for a better understanding. For instance, in the juice 2 the main focus was given to the pomegranate fruit, which represented 20% of the juice. In the experiment with this juice there were two interesting observations recorded. Firstly, the number of bacteria in the barley-based juice was significantly reduced compared to the other experiments and secondly, in the soya-based juice,

there was nearly no reduction of the bacteria content. The reasons, why pomegranate was deducted as the growth inhibitor were the high content of phenolic and organic acids and also the fact, that there was none or much lower presence of pomegranate in other juices used. It is only a hypothesis that one of those compounds could cause the reduction in the bacterial count in the juice containing barley soup as a probiotic component. The explanation, why the count in the soya-based juice remained on the nearly same value, could be attributed to the prebiotic effect of the soya. The protective effect of soya proteins on the bacterial growth is another speculation, although the proteins and peptides have been recognized as having a potentially prebiotic characteristic (Crittenden, 1999; Ziemer & Gibson, 1998)

5.6.2. Complex phenolic polymers

Although *Lb. plantarum* belongs to the Gram positive group (the link between an antimicrobial activity of phenolics and their inhibitory effect on bacterial strains is explained in the part 3.2.6.1), more complicated inhibitory effects might inhibit the growth of the bacteria. The presence of more complex phenolic polymers such as ellagitannins, tannins and proanthocyanidins could be a demonstration of that. The synergy of various phenolic compounds is also evidently responsible for the bacterial inhibition. Moreover, there are other bioactive compounds in a plant tissues that might, either alone or in combination with phenols, contribute to the antimicrobial effect (R. Puupponen-Pimia, L. Nohynek, C. Meier, M. Kahkonen, M. Heinonen, A. Hopia, K.-M. Oksman-Caldentey; Helsinky, Finland 2000). Also the increased sensitivity of bacteria in the exponential phase of growing (as noted above) combined with the enviromental stress , which was the presence of antimicrobial components, might weighed in on the bacterial flourishing. Anyhow, different interactions and/or synergistic effect of the phenolic and other components related to the bacterial survival are beyond the scope of my study but on the other hand, it is necessary to mention their existence. Thus the attention is paid only to the possible antimicrobial mechanism exhibited by tannins and tannin-like compounds.

Tannins have been reported in general to be bacteriostatic and/or bacteriocidal (Hada et al. 1989; Toda et al. 1989; Chung et al. 1998). So hypothetically, tannins can also reduce the number of probiotic bacteria. This speculation is supported by a study of the effect of tannic acid on Lactobacillus hilgardii. The inhibition of the growth was detected after adding tannic acid into the growing media (A. Bossi, S. Rinalducci, L. Zolla, P. Antonioli, P.G. Righetti, G. Zapparoli; Verona, Italy, 2006). The study also explained the possible way of inhibition by tannin-protein interaction. Moreover, the correlation between concentration of polyphenols and the inhibitory effect was studied. The experimental approach used suggests the interference of tannins on cell protein expression but the exact mechanism is not elucidated (A. Bossi, S. Rinalducci, L. Zolla, P. Antonioli, P.G. Righetti, G. Zapparoli; Verona, Italy, 2006). Another proposed mechanism is mediated through the complexation of metal ions by tannins (Scalbert 1991). However, the mode of action of tannins probably depends on the individual micro-organism and furthermore *Lactobacillus plantarum* 299v seem to posses the protective mechanism againts tannins(S.Ahrne, 2008). Nonetheless, the combined effect of more stressing factors contributing to bacterial survival is not excluded.

5.6.3. Phytic acid

Phytic acid is another potentional inhibitory compound found in plants, particularly in cereals and legumes and to a lesser extent in fruits, vegetables and oilseeds (Shamsuddin, 1999). Basically, phytic acid is composed of an inositol sugar with six phosphate groups (Wodzinski & Ullah, 1996). Similarly to tannin, phytic acid is able to bind and prevent the absorption of proteins and can complex di- and trivalent cations such as Fe^{3+} , Mg^{2+} , Ca^{2+} , Zn^{2+} , Cu^{2+} and Mn^{2+} (Graf & Eaton, 1985; Wodzinski & Ullah, 1996). For that reason, phytic acid has been often regarded as an anti-nutrient although recently, an antioxidant activity (Graf et al., 1984) was described in some studies. Yet, from the aspect of the nutritional demands of Lactobacilli for metal ions the presence of phytic acids might result in the bacterial inhibition.

According to the study performed by T.Steer and G.Ribson (2002) *Bifidobacterium* spp. and *Lactobacillus* spp. were found unable to metabolize phytic acid. Moreover, the decrease in both named species occurred once phytic acid was introduced into the growing medium. In contrary to these results, another study (M.C.Palacios et al., 2006) observed some members of the same species to have phytate degrading enzymes i.e. phytase and phosphatase. In general, the enzyme activity againts sodium phytate was showed lower in *Lactobacilli* than that in *Bifidobacteria* (Haros, Bielecka & Sanz, 2005; Palacios et al., 2005). However, the phytase activity does not seem to be common metabolic property of lactobacilli and is therefore strain-dependent.

5.7. Viable but non-cultivable colonies (VBNC)

Another important factor that is needed to mention in the relation to bacterial counts is the ability of bacteria to form a non-cultivable colonies. There is a need to differ between the terms 'cultivability' and 'viability'. While the former refers to the capacity of cells to grow the later describes the metabolic activity of cells. Cultivability is then determined by the counting plate method. However, the use of this traditional method does not seem to be suitable for the detection of viable microbial populations, due to the absence of a link between viability and cultivability. In this study the number of individual organisms in a sample was detected by forming colonies on an agar-based medium. However, viable but noncultivable cells may be present within the microbial population. This „noncultivable“ state of a bacteria might be caused by a complex of factors such as by using standard laboratory techniques (Kell et al.1998), but yet there is a lack of knowledge on the physiology and metabolical activity of *Lb. plantarum* (Ahrne, 2008). From the study performed by Bunthof et al. (1999, 2000) on the storage conditions and their influence on the cultivability of Rhodotulla glutinis emerged couple of findings. Some of them concerned the exposition of the yeast to a sublethal stress during the storage. The reserves of carbohydrates, the integrity and functionality of the cytoplasmatic membrane, the intracellular pH, the growth phase of yeast when got stored and many more were investigated and proved to have some influence on the cultivability of yeast. Yet, i can only speculate if some of those mentioned factors could play a role in the final bacterial counts in my study. However, if this was the case, than bacterial counts of the stored drinks were more likely to be affected rather than the bacterial counts in fermented soups. This could be attributed to a higher probability of a presence of some

risk factors in the stored drinks such as lack of some nutrients, disruption of the membrane integrity, presence of some specific chemical components, low pH etc.

5.8. The influence of bacterial metabolites on the organoleptic properties

As mentioned above, *Lb. plantarum* is able to metabolize a number of substrates including carbohydrates, organic acids, amino acids and polyols. Lactate is then the major end-product of the homofermentative metabolic pathway of *Lb. plantarum* contributing to sour taste of the fermented soups. The small amounts of acetic acid are produced due to the presence of oxygen (Murphy and Condon 1984; Bobillo andMarshall 1991). In general, lactic acid is described as 'acid-sour' while acetic acid as 'acid-sharp' (De Vuyst 2000). Nonetheless, other components such as lysine, histidine, arginine, proline and glutamic acid have been found increased in soups fermented by *Lb. plantarum* (I.Marklinder C. Löner, Lund, Sweden 1992). All together, these compounds are important for their contribution to sensory attributes.

In my study two groups of volunteers have participated. Volunteers from the group1 rated tasted drinks usually with values 4 – 6 while volunteers from the group 2 used the ratings in the range 1-7. However, in most cases soya-based drinks received much lower values and the values higher than 6 were rather exceptional. On this basis, the high amount of amino acids and products of their further metabolism in the soya formula might have the "negative" impact on sensory characteristics of the drink. But as was already mentioned, the amino acid metabolism is both species- and strain-specific and therefore deeper discussion can't be provided without the exact knowledge of *Lactobacillus plantarum* 299v metabolical pathways. Some juice flavours (black currant, blueberry) seemed to "cover" the undesired taste of some tested drinks, particularly those of soya what resulted in a bit higher ratings of such drinks. This might indicate the interaction between taste-giving compounds and compounds in juice with the binding capacity for these molecules in the fermented formula. As an example of such mechanism it might be the interaction between soya amino acids and tannins or phytate in the fruit juice, where the ability of these compounds to bind proteins was already discussed in the text above. The different taste preferences observed within trained and ordinary volunteers were strongly dependent on individual perception of tastes and also only a small sample of population was included in this trial. Therefore the data obtained don't have so significant statistical value and results might vary greatly if more numerous and heterogenous groups were involved.

6. Summary

The aim of this study was to clarify the fermentation process and approximately estimate the growth and acidification properties of *Lb. plantarum* first on new types of cereal substrates (soy and barley) and then in the mixture of the fermented cereal component with five different types of comercial juices. By that reason, the pH value, bacterial count and the number of pathogenic bacteria were measured. Moreover, for a better understandig of the metabolic pathways of bacteria during the fermentation and further in the fruit drink the growth curves of *Lb. plantarum* in the soy- and barley-medium were designed and analyzed. To evaluate those parameters several methods were involved including a detection of the changes of pH value using pH meter, counting bacterial and pathogenic bacteria colonies growing on Rogosa and VBRD agar respectivelly. Further in the study the sensory impact of soya- and barley-based components in the novel probiotic drink was studied through a tasting assay with two different panels (trained and untrained) of volunteers. As the last trial performed was the evaluation of shelf-life, resp. how bacteria were flourishing after 2 weeks of storage in a fridge, again including measurements of pH and both bacterial counts – *Lb. plantarum* and pathogenic bacteria.

The obtained results demonstrated that there were more than just one metabolical pathway. Even though the fermentation by itself followed more or less stable pattern, the tested probiotic drink showed considerable diversity of the investigated data. The fluctuations in the pH values and bacterial counts reflected the variations in the nutrient composition and/or different interactions within the drink. In this context various potential inhibitory or contrarily growth supporting mechanisms were speculated in the discussion part. Among the most likely aspects there is a mention about different metabolical pathways. Those are results of both – chemical components differences within the cereals as well as the presence of variety of chemical compounds in fruit juices. The other mentioned hypothetical aspects include an enviromental stress for bacteria and also the risk of non-cultivability of *Lb. plantarum*. As emerged from the tasting assay soya-based drinks were generally rated with much lower numbers than it was obtained for the oat- or barley based drinks. As a potential factor that might have such an impact on sensory characteristics of the final drink a different chemical components' profile of the cereals used was hypothetised.

7. Conclusion

In this study, five different fruit juices were mixed with barley and soya soup in order to estimate the behavior of bacteria in different growing enviroments. Interestingly, not all the juices used were found as a suitable medium for *Lb. plantarum*. The intensity of bacterial response expressed as a change in bacterial count varried with the type of inhibitory action. On the other hand, some juices exhibited a promoting effect on the bacterial growth. Nonetheless, in most cases the bacterial counts were slowly declining during the two weeks of storage with the final count reaching the concentration about 2.10^7 CFU/ml. Compared to the original Pro Viva®fruit drink, where the declared concentration of *Lb. plantarum* 299v is about 5. 10^7 CFU/ml, this count was still high enough. Moreover, the storage time was a bit longer considering that consumers commonly use a juice in 3 days.

To clarify the diversity of results, potentional ways of inhibition and uncommon metabolical pathways are speculated. In this context, the presence of organic acids, phenolic compounds and different sugars in fruit juice and also the availability and different sugar, amino acid, phytic acid and mineral composition in the barley and soya flour are taken into account. However, this study represents only the hypothetical model of the bacteria-medium relation. Nevertheless, the viable count of *Lb. plantarum* 299v in the fermented formula and in some of the stored drinks indicated the metabolical activity and the ability of the bacteria to grow on other substrates than just on an oat.

Another assayed aspect was the bacteriological safety which showed that there is no pathogenic risk for consumers. Therefore the novel probiotic drinks can be declared as safe. However, the more complex analysis would need to be performed in order to exclude all possible healthy risks for people.

The last part of my study deals with the taste of final product and its potential to succeed on the marketplace. For these reasons, novel soya- and barley-based fruit juices containing the probiotic cultures were compared with the original oat-based Pro Viva drink by the simple analysis of taste preferences within 2 groups of volunteers. In general, the trained panel of volunteers preferred oat-based drinks. However, some of the barley-based combinations were found almost of the same taste properties as the original oat-based drink. Also the ratings given by the members of first group to a certain drink were analogous. The other group of volunteers rated different drinks randomly according to their personal taste preferences. Similarly in both asked groups, the soya-based drinks were described to contain unpleasant aroma and taste characteristic of the functional component. To conclude this part, novel drinks on barley basis seem to be generally more accepted by consumers but a certain group of people would prefer soya-based drinks. With the respect on the eating habits in Asian countries, it might be interesting to include this population in the further tasting trial.

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8. Abbrevations

CFU	colony forming units
FAO	Food and Agriculture Organisation
GIT	gastrointestinal tract
LAB	lactic acid bacteria
Lb.	Lactobacillus
LDL	low-density lipoprotein
MBF	malted barley flour
USDA	United States Department of Agriculture
VRBD	crystal-violet neutral-red bile glucose agar
WHO	World Health Organisation

9. Appendixes

A. Details of flours used in the study
B. Results

A. Details of flours used in the study

Table 1.
The chemical composition of three types of flours used in the study

	oat	barley	soya
Total carbohydrates (g)	66.0	64.4	26.0
dietary fiber (g)	6.5	7.6	11.9
sugars (g)	~ 0.8	~ 1.0	~ 11.0
sucrose (g)	0.5	n	n
fructose (g)	n	n	n
glucose (g)	0.1	n	n
Total fat (g)	9.0	3.0	8.9
Total protein (g)	15.0	9.0	37.0
Vitamin K (mcg)	3.2	2.2	4.1
Thiamin (mg)	0.7	0.4	1.1
Riboflavin (mg)	0.1	0.1	0.3
Niacin (mg)	1.5	6.3	3.0
Vitamin B6 (mg)	0.1	0.4	1.1
Folate (mcg)	32.0	8.0	305.0
Vitamin B12 (mcg)	0.0	0	0.0
Pantothenic acid (mg)	0.2	0.1	1.5
Calcium (mg)	55.0	32.0	199.0
Iron (mg)	4.0	2.7	8.2
Magnesium (mg)	144.0	96.0	285.0
Sodium (mg)	19.0	2.0	1.0
Manganese (mg)	4.0	1.0	3.2
Selenium (mcg)	34.0	37.7	58.9
Zinc (mg)	3.2	2.0	4.1

n – data not known
source: http://www.nutritiondata.com; http://www.saltakvarn.se

Table 2.
Amino acid profile of three types of flours used in the study

Amino acid (mg)	oat*	barley	soya
Tryptofan	0,0017	175	717
Threonine	0,0036	356	2144
Isoleucine	0,0039	383	2395
Leucine	0,0079	713	4019
Lysine	0,0041	391	3286
Methionine	0,0021	202	666
Cystine	0,0034	232	795
Phenylalanine	0,0053	589	2576
Tyrosine	0,0023	301	1867
Valine	0,0053	515	2463
Arginine	0,0065	526	3830
Histidine	0,0023	236	1331
Alanine	0,0047	409	2326
Aspartic acid	0,0088	655	6207
Glutamic acid	0,0215	2741	9561
Glycine	0,0050	380	2283
Proline	0,0055	1247	2888
Serine	0,0055	443	2861
Hydroxyproline	~	~	~

~ values not found

* values expressed in percentage (%); N J. D. Hahn, T. K. Chung and D. H. Baker, University of Illinois, Urbana 61801, 2008.

http://www.nutritiondata.com

Table 3.

Phenolic composition of berry extract (expressed as mg g^{-1} phenolic extract)

	anthocyanins	flavonols	OH-cinnamates	flavon-3-ols	total
blackcurrant	106	10	7	7	410
redcurrant*	0.2	0.6	0.3	3	4.6
blueberry	260	6	23	5	360
cranberry	117	59	43	ND	330
cloudberry	0.9	8	10	1	470
lingonberry	23	10	6	6	280
raspberry	24	2	4	3	470
strawberry	34	3	8	1	460
cherry*	0.2	0.4	1.6	2.3	4.6

ND-not determined

* values expressed in g/l

R. Puupponen-Pimia, L. Nohynek et al., University of Helsinki, Department of Applied Chemistry and Microbiology, Food Chemistry Division, University of Helsinki, Finland, 2001.

Table 4.

Phenolic profiles in berries (expressed as percentages from the total content of all compounds studied)

	kaempferol	quercetin	myricetin	p-coumaric acid	caffeic acid	ferulic acid	p-hydroxy benzoic	ellagic acid	total
CB	1.4	59.2	13.5	2.1	3.3	18.4	0.4	1.8	100
LB	1.6	63	2.6	19.9	2.6	7.0	2.1	1.1	100
BLB	0.6	18.3	3.8	0.5	6.4	68.3	0.0	2.3	100
BIB	2.6	21.4	6.2	29.0	9.2	25.7	0.7	5.2	100
BC	5.9	29.8	15.5	24.4	16.4	3.1	2.6	2.3	100
RC	2.2	39.6	2.1	12.1	14.4	5.4	15.9	8.2	100
SB	3.1	6.0	1.6	34.3	0.0	0.0	4.0	50.9	100
RB	0.2	1.7	0.7	2.5	1.5	2.5	2.9	88	100
CLB	0.6	2.2	0.5	8.5	2.0	1.3	2.2	76.7	94.0 *

Note:

CB: cranberry; LB: lingonberry; BLB: blueberry; BIB: bilberry; BC: black currant; RC: red currant; SB: strawberry; RB: raspberry; CLB: cloudberry

* gallic acid 6%

source: Mar a J. Bermffldez-Soto, Francisco A. Toms-Barber; Research Group on Quality, Safety and Bioactivity of Plant Foods; Department of Food Science and Technology; Murcia, Spain, 2004.

S. Hakkinen et al.; Department of Clinical Nutrition, University of Kuopio, Finland, 1999.

Table 5.

Average content in organic acids (g /100 g) for different pomegrante cv group

Cultivar group	oxalic	citric	malic	lactic	fumaric	tartaric	acetic	total
average	0.034	0.282	0.139	tr	0.003	0.014	0.015	0.474
sweet	0.037	0.142	0.135	tr	0.005	0.014	na	0.317
soursweet	0.015	0.566	0.160	tr	tr	tr	0.06	0.786
sour	0.017	2.317	0.176	tr	tr	tr	0.0215	2.725

tr -traces

na – not applicable

source: Ender Poyrazoglu, Vural Gökmenw, Nevzat Artik; Department of Food Engineering, Ankara University, 2001.

Table 6.

Phenolic compound composition of pomegranate juices (Artik et.al., 1998; Melgarejo et al., 2000). Some results taken from the study accomplished by E. Poyrazoglu (Turkey, 2001)

	gal	pro	cat	chl	caf	p-cou	fer	o-cou	phl	que
Overall mean	4.55	0.84	3.72	1.24	0.78	0.06	0.01	0.17	0.99	2.5

Note: Phenolic compounds:
gal: gallic acid; pro: protocatechuic acid; cat: catechin; chl: chlorogenic acid; caf: caffeic acid;
p-cou: p-coumaric acid; fer: ferulic acid; o-cou: o-coumaric acid; phl: phloridzin; que: quercetin.

Source: Pablo Melgarejo, Domingo Manuel Salazar, Francisco Artes; Department of Vegetable Crops, University of Valencia, 1999.

B. Results

Table 7.
List of tested cereal soups.

Sample no.	Sample name:	Sample composition:
1.	Fermented oat soup	Oat flour, dist. water, *Lb. plantarum* 299v
2.	Fermented barley soup	Barley flour, dist. water, *Lb. plantarum* 299v
3.	Fermented soy soup	Soy flour, dist. water, *Lb. plantarum* 299v

note: For the original formula of the preparation of the cereal soups see Table in the part

Table 8.
List of tested drinks.

Sample no	Sample name	Sample composition
4.	Barley based drink - classical 5%	FBS (5%) + fruit juice (1, 2, 3, 4, 5)*
5.	Barley based drink - shot 25%	FBS (25%) + fruit juice (1, 2, 3, 4, 5)*
6.	Soya based drink - classical 5%	FSS (5%) + fruit juice (1, 2, 3, 4, 5)*
7.	Soya based drink - shot 25%	FSS (25%) + fruit juice (1, 2, 3, 4, 5)*

FBS – fermented barley soup; FSS – fermented soya soup
* the list of all tested fruit juices is given in the Table 9.

Table 9.
List of fruit juices and their composition used in the study

Juice no	Juice composition	Market name of the juice
1	apple juice, crushed mango 38%, orange juice 16%, passion fruit juice, mashed banana	Froosh
2	water, fruit juice from concentrate (pomegranate 14%, cranberry 10,5%, apple 6,5%), sugar, glucose – fructose syrup; acidifier: citric acid; vitamin C	Ocean Spray, Cranberry & Pomegranate
3	grape, raspberry, blackberry, cherry, red currant	Tropicana, Red Pleasure
4	apple juice, pear juice, raspberry juice	Kiviks
5	orange juice, apple juice, apricot puree, carrot puree, mashed banana	God Morgon, Frukt & Grönt

Table 10.
The pH progress measured in the cereal soups measured during the fermentation process (final pH values obtained as a mean value from 10 measurements).

Time (h)	pH		
	Oat soup	Barley soup	Soya soup
0	~ 6.20	~ 5.36	~ 6.20
2	6.05	5.30	6.10
4	5.80	4.60	5.80
6	4.95	3.90	5.30
8	4.70	~3.60	4.80
15	4.30	3.40	4.50
24	4.00	3.25	4.10

Table 11.

The initial viable counts of *Lb. plantarum* 299 v in the cereal soups measured at the beginning of the fermentation process at the concentration of $10^{-4.}$

Time (h)	**Viable count of *Lb.plantarum* 299 v**		
	Oat soup	Barley soup	Soya soup
0	189	157	166

Table 12.

The initial viable counts of *Lb. plantarum* 299 v expressed as CFU/ml.

Time (h)	**Viable count of *Lb. plantarum* 299 v (CFU/ml)**		
	Oat soup	Barley soup	Soya soup
0	~2.10^7	~$1,5.10^7$	~2.10^7

Table 13.

The viable count of *Lb. plantarum* 299 v in the cereal soups measured during the fermentation process

Time (h)	**Viable count of *Lb. plantarum* 299 v**					
	Oat soup		Barley soup		Soya soup	
	10^{-6}	10^{-7}	10^{-5}	10^{-6}	10^{-5}	10^{-6}
8	N	N	295	35	N	N
17	55	(5)	un.	**61**	un.	**163**
24	**221**	**18**	N	N	103	(7)

note: N – not measured; **highlighted** values are the highest bacterial counts obtained; un – uncountable; (values) weren't taken into account when counting the final CFU/ml

Table 14.

The viable count of *Lb.plantarum* 299 v expressed as CFU/ml

Time (h)	**Viable count of *Lb.plantarum* 299 v (CFU/ml)**		
	Oat soup	Barley soup	Soya soup
8	N	3.10^8	N
17	5.10^8	**6.10^8**	**$1{,}6.10^9$**
24	**2.10^9**	N	1.10^8

note: N – not measured; **highlighted** values are the highest bacterial counts obtained

Table 15.

The viable count of pathogens (CFU/ml) in the cereal soups measured during the fermentation process

Time (h)	**Viable count of pathogens (CFU/ml)**		
	Oat soup	Barley soup	Soya soup
8	0	0	0
17	N	N	N
24	0	0	0

N – not measured

Table 16.

The bacterial counts obtained for the growth curve of *Lb. plantarum* 299v growing on the soya medium

Time (h)	Viable count of *Lb. plantarum* 299v				CFU/ml	Log CFU
	10^{-4}	10^{-5}	10^{-6}	10^{-7}		
0	147	(13)	0	0	$1,5.10^{7}$	7,18
2	193	(18)	(2)	0	$1,9.10^{7}$	7,28
4	201	(19)	(1)	0	$2,0.10^{7}$	7,30
6	366	34	(2)	0	$3,5.10^{7}$	7,54
7,5	un.	113	(11)	(2)	$1,1.10^{8}$	8,04
9	un.	286	28	(4)	$2,9.10^{8}$	8,46
11	un.	480	43	(4)	$4,3.10^{8}$	8,63
13	un.	un.	85	(5)	$8,5.10^{8}$	8,93
15	un.	un.	130	(11)	$1,3.10^{9}$	9,11
17	un.	un.	189	(10)	$1,9.10^{9}$	9,23
19	un.	un.	158	(12)	$1,6.10^{9}$	9,20
21	un.	un.	144	(21)	$1,4.10^{9}$	9,15
24	un.	un.	155	(13)	$1,6.10^{9}$	9,19
30	un.	un.	123	(10)	$1,2.10^{9}$	9,08
34	un.	197	(21)	(1)	2.10^{8}	8,30

note: un. – uncountable; (values) weren't taken into account when counting the final CFU/ml

Table 17.

The bacterial counts obtained for the growth curve of *Lb. plantarum* 299v growing on the barley medium

Time (h)	Viable count of *Lb. plantarum* 299v				CFU/ml	Log CFU
	10^{-4}	10^{-5}	10^{-6}	10^{-7}		
0	155	(18)	0	0	$1,610^{7}$	7,20
2	175	(12)	(2)	0	$1,8.10^{7}$	7,26
3,5	~ 340	48	(1)	0	$4,8.10^{7}$	7,70
5	un.	75	(6)	(1)	$7,5.10^{7}$	7,88
7	un.	259	(21)	(2)	$2,6.10^{8}$	8,42
9	un.	~ 400	37	(4)	$3,7.10^{8}$	8,57
11	un.	un	43	(3)	$4,3.10^{8}$	8,63
13	un.	un.	62	(11)	$6,2.10^{8}$	8,80
15	un.	un.	76	(12)	$7,6.10^{8}$	8,88
17	un.	un.	72	(22)	$7,2.10^{8}$	8,86
19	un.	un.	60	(5)	$6,0.10^{8}$	8,78
21	un.	un.	73	(10)	$7,3.10^{8}$	8,86
24	un.	un.	75	(6)	$7,5.10^{8}$	8,87
30	un.	un.	71	(4)	$7,1.10^{8}$	8.85
34	un.	166	(19)	(3)	$1,7.10^{8}$	8,23

note: un. – uncountable; (values) weren't taken into account when counting the final CFU/ml

Table 18.

The progress of pH values during 2 weeks storage in the drinks with the juice 1 (initial pH of the juice before mixing with fermented soup: 3.28)

Time (days)	pH					
	bf (25%)+j	bf (5%)+j	bf	sf (25%)+j	sf (5%)+j	sf
0	3.24*	3.13*	3.63*	3.80*	3.66*	4.30*
2	3.19	3.10	3.56	3.83	3.69	4.33
4	3.13	3.08	3.50	3.84	3.71	4.34
6	3.11	3.07	3.53	3.77	3.68	4.30
8	3.11	3.07	3.52	3.78	3.65	4.25
10	3.08	3.02	3.45	3.72	3.61	4.20
12	3.06	3.01	3.43	3.70	3.60	4.18
15	3.15	3.10	3.50	3.76	3.68	4.20

* these pH values reffer to an initial pH of the drinks and fermented soups

bf (25%) +j – fermented barley soup added in the amount of 25 % and mixed with juice

bf (5%) +j – fermented barley soup added in the amount of 5 % and mixed with juice

bf – fermented barley soup

sf (25%) +j – fermented soya soup added in the amount of 25 % and mixed with juice

sf (5%) +j – fermented soya soup added in the amount of 5 % and mixed with juice

sf – fermented soya soup

Table 19.

The progress of pH values during 2 weeks storage in the drinks with the juice 2 (initial pH of the juice before mixing with fermented soup: 3.56)

Time (days)	pH					
	bf (25%)+j	bf (5%)+j	bf	sf (25%)+j	sf (5%)+j	sf
0	3.35*	3.30*	3.63*	3.87*	3.96*	4.68*
2	3.26	3.15	3.58	3.85	3.95	4.52
4	3.18	3.09	3.53	3.80	3.91	4.44
6	3.58	3.36	3.53	3.83	4.00	4.41
8	3.81	3.50	3.56	3.82	4.02	4.30
10	3.33	3.39	3.42	3.77	3.99	4.28
12	3.20	3.23	3.44	3.75	3.90	4.23
15	2.73	3.10	3.51	3.76	3.92	4.25

* these pH values reffer to an initial pH of the drinks and fermented soups

bf (25%) +j – fermented barley soup added in the amount of 25 % and mixed with juice

bf (5%) +j – fermented barley soup added in the amount of 5 % and mixed with juice

bf – fermented barley soup

sf (25%) +j – fermented soya soup added in the amount of 25 % and mixed with juice

sf (5%) +j – fermented soya soup added in the amount of 5 % and mixed with juice

sf – fermented soya soup

Table 20.

The progress of pH values during 2 weeks storage in the drinks with the juice 3 (initial pH of the juice before mixing with fermented soup: 3.50)

Time (days)	pH					
	bf (25%)+j	bf (5%)+j	bf	sf (25%)+j	sf (5%)+j	sf
0	3.33*	3.20*	3.68*	3.79*	3.63*	4.23*
2	3.58	3.49	3.60	3.84	3.65	4.29
4	3.67	3.65	3.59	3.86	3.66	4.35
6	3.69	3.60	3.62	3.83	3.62	4.31
8	3.66	3.61	3.62	3.84	3.59	4.30
10	3.57	3.51	3.51	3.79	3.60	4.18
12	3.52	3.45	3.47	3.76	3.54	4.15
15	3.58	3.51	3.56	3.83	3.60	4.21

* these pH values reffer to an initial pH of the drinks and fermented soups

bf (25%) +j – fermented barley soup added in the amount of 25 % and mixed with juice

bf (5%) +j – fermented barley soup added in the amount of 5 % and mixed with juice

bf – fermented barley soup

sf (25%) +j – fermented soya soup added in the amount of 25 % and mixed with juice

sf (5%) +j – fermented soya soup added in the amount of 5 % and mixed with juice

sf – fermented soya soup

Table 21.

The progress of pH values during 2 weeks storage in the drinks with the juice 4 (initial pH of the juice before mixing with fermented soup: 3.30)

Time (days)	pH					
	bf (25%)+j	bf (5%)+j	bf	sf (25%)+j	sf (5%)+j	sf
0	3.20*	3.15*	3.65*	3.67*	3.52*	4.25*
2	3.31	3.24	3.60	3.73	3.51	4.20
4	3.35	3.28	3.62	3.75	3.60	4.21
6	3.33	3.27	3.67	3.79	3.61	4.21
8	3.33	3.25	3.68	3.81	3.63	4.23
10	3.30	3.26	3.63	3.79	3.63	4.20
12	3.27	3.20	3.63	3.80	3.63	4.18
15	3.30	3.23	3.70	3.82	3.65	4.20

* these pH values reffer to an initial pH of the drinks and fermented soups

bf (25%) +j – fermented barley soup added in the amount of 25 % and mixed with juice

bf (5%) +j – fermented barley soup added in the amount of 5 % and mixed with juice

bf – fermented barley soup

sf (25%) +j – fermented soya soup added in the amount of 25 % and mixed with juice

sf (5%) +j – fermented soya soup added in the amount of 5 % and mixed with juice

sf – fermented soya soup

Table 22.

The progress of pH values during 2 weeks storage in the drinks with the juice 5 (initial pH of the juice before mixing with fermented soup: 3.53)

Time (days)	pH					
	bf (25%)+j	bf (5%)+j	bf	sf (25%)+j	sf (5%)+j	sf
0	3.21*	3.14*	3.61*	3.71*	3.62*	4.33*
2	3.20	3.15	3.53	3.70	3.62	4.36
4	3.18	3.11	3.48	3.66	3.58	4.40
6	3.19	3.13	3.49	3.67	3.58	4.43
8	3.23	3.15	3.53	3.68	3.60	4.48
10	3.19	3.16	3.49	3.61	3.58	4.42
12	3.20	3.14	3.45	3.59	3.55	4.39
15	3.22	3.13	3.49	3.61	3.57	4.39

* these pH values reffer to an initial pH of the drinks and fermented soups

bf (25%) +j – fermented barley soup added in the amount of 25 % and mixed with juice

bf (5%) +j – fermented barley soup added in the amount of 5 % and mixed with juice

bf – fermented barley soup

sf (25%) +j – fermented soya soup added in the amount of 25 % and mixed with juice

sf (5%) +j – fermented soya soup added in the amount of 5 % and mixed with juice

sf – fermented soya soup

Table 23.

The viable count in the drink with juice 1 during 2 weeks storage

Time (days)	Viable count of *Lb.plantarum* 299 v											
	bf (25%)+j		bf (5%)+j		bf		sf (25%)+j		sf (5%)+j		sf	
	10^{-5}	10^{-6}	10^{-5}	10^{-6}	10^{-5}	10^{-6}	10^{-5}	10^{-6}	10^{-5}	10^{-6}	10^{-6}	10^{-7}
0	62	(8)	11	(3)	214	37	163	(12)	32	(6)	63	(4)
15	**un.**	**un.**	163	(15)	278	33	208	(8)	35	(4)	45	(8)

note: **highlighted** values are the highest final bacterial counts obtained; un – uncountable; (values) weren't taken into account when counting the final CFU/ml

Table 24.

The viable count in the drink with juice 1 during 2 weeks storage expressed as CFU/ml.

Time (days)	Viable count of *Lb.plantarum* 299 v(CFU/ml)					
	bf (25%)+j	bf (5%)+j	bf	sf (25%)+j	sf (5%)+j	sf
0	6.10^{7} *	1.10^{7} *	2.10^{8} *	$1{,}6.10^{8}$ *	3.10^{7} *	6.10^{8} *
15	**uncount.**	$1{,}5.10^{8}$	3.10^{8}	2.10^{8}	$3{,}5.10^{7}$	$4{,}5.10^{8}$

* these pH values reffer to an initial bacterial counts of the drinks and fermented soups
uncount. - ucountable
bf (25%) +j – fermented barley soup added in the amount of 25 % and mixed with juice
bf (5%) +j – fermented barley soup added in the amount of 5 % and mixed with juice
bf – fermented barley soup
sf (25%) +j – fermented soya soup added in the amount of 25 % and mixed with juice
sf (5%) +j – fermented soya soup added in the amount of 5 % and mixed with juice
sf – fermented soya soup;
highlighted values are the highest final bacterial counts obtained

Table 25.

The viable count in the drink with juice 2 during 2 weeks storage

Time (days)	Viable count of *Lb.plantarum* 299 v											
	bf (25%)+j		bf (5%)+j		bf		sf (25%)+j		sf (5%)+j		sf	
	10^{-4}	10^{-5}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-5}	10^{-6}	10^{-4}	10^{-5}	10^{-5}	10^{-6}
0	330	37	un.	72	153	18	213	(8)	un.	42	un.	84
15	32	(2)	51	(3)	**218**	**25**	76	(3)	83	(5)	93	9

note: **highlighted** values are the highest final bacterial counts obtained; un – uncountable; (values) weren't taken into account when counting the final CFU/ml

Table 26.

The viable count in the drink with juice 2 during 2 weeks storage expressed as CFU/ml.

Time (days)	Viable count of *Lb.plantarum* 299 v(CFU/ml)					
	bf (25%)+j	bf (5%)+j	bf	sf (25%)+j	sf (5%)+j	sf
0	$3{,}5.10^{7}$ *	7.10^{6} *	$1{,}5.10^{8}$ *	2.10^{8} *	4.10^{7} *	8.10^{8} *
15	3.10^{6}	5.10^{5}	**2.10^{8}**	$7{,}5.10^{7}$	8.10^{6}	9.10^{7}

* these pH values reffer to an initial bacterial counts of the drinks and fermented soups
bf (25%) +j – fermented barley soup added in the amount of 25 % and mixed with juice
bf (5%) +j – fermented barley soup added in the amount of 5 % and mixed with juice
bf – fermented barley soup
sf (25%) +j – fermented soya soup added in the amount of 25 % and mixed with juice
sf (5%) +j – fermented soya soup added in the amount of 5 % and mixed with juice
sf – fermented soya soup;
highlighted values are the highest bacterial counts obtained

Table 27.
The viable count in the drink with juice 3 during 2 weeks storage.

Time (days)	Viable count of *Lb.plantarum* 299 v											
	bf (25%)+j		bf (5%)+j		bf		sf (25%)+j		sf (5%)+j		sf	
	10^{-5}	10^{-6}	10^{-4}	10^{-5}	10^{-5}	10^{-6}	10^{-5}	10^{-6}	10^{-4}	10^{-5}	10^{-5}	10^{-6}
0	57	(6)	158	20	223	21	111	(7)	253	23	un.	53
15	32	(2)	104	(4)	**324**	**37**	62	(8)	63	(14)	**332**	**34**

note: **highlighted** values are the highest bacterial counts obtained; un – uncountable; (values) weren't taken into account when counting the final CFU/ml

Table 28.
The viable count in the drink with juice 3 during 2 weeks storage expressed as CFU/ml.

Time (days)	Viable count of *Lb.plantarum* 299 v (CFU/ml)					
	bf (25%)+j	bf (5%)+j	bf	sf (25%)+j	sf (5%)+j	sf
0	$5{,}5.10^{7}$ *	$1{,}6.10^{7}$ *	2.10^{8} *	1.10^{8} *	$2{,}5.10^{7}$ *	5.10^{8} *
15	3.10^{7}	1.10^{7}	**$3{,}5.10^{8}$**	6.10^{7}	6.10^{6}	**$3{,}5.10^{8}$**

* these pH values reffer to an initial bactrial counts of the drinks and fermented soups
bf (25%) +j – fermented barley soup added in the amount of 25 % and mixed with juice
bf (5%) +j – fermented barley soup added in the amount of 5 % and mixed with juice
bf – fermented barley soup
sf (25%) +j – fermented soya soup added in the amount of 25 % and mixed with juice
sf (5%) +j – fermented soya soup added in the amount of 5 % and mixed with juice
sf – fermented soya soup;
highlighted values are the highest final bacterial counts obtained

Table 29.

The viable count in the drink with juice 4 during 2 weeks storage

Time (days)	Viable count of *Lb.plantarum* 299 v											
	bf (25%)+j		bf (5%)+j		bf		sf (25%)+j		sf (5%)+j		sf	
	10^{-4}	10^{-5}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-5}	10^{-6}	10^{-4}	10^{-5}	10^{-5}	10^{-6}
0	261	25	116	(15)	334	30	156	(13)	313	29	un.	73
15	279	31	153	(9)	**un.**	**62**	82	(3)	93	(6)	258	27

note: **highlighted** values are the highest final bacterial counts obtained; un – uncountable; (values) weren't taken into account when counting the final CFU/ml

Table 30.

The viable count in the drink with juice 4 during 2 weeks storage expressed as CFU/ml.

Time (days)	Viable count of *Lb.plantarum* 299 v (CFU/ml)					
	bf (25%)+j	bf (5%)+j	bf	sf (25%)+j	sf (5%)+j	sf
0	$2{,}5.10^{7}$ *	$1{,}1.10^{7}$ *	$3{,}3.10^{8}$ *	$1{,}5.10^{8}$ *	3.10^{7} *	7.10^{8} *
15	3.10^{7}	$1{,}5.10^{7}$	**6.10^{8}**	8.10^{7}	9.10^{6}	$2{,}6.10^{8}$

* these pH values reffer to an initial bacterial counts of the drinks and fermented soups
bf (25%) +j – fermented barley soup added in the amount of 25 % and mixed with juice
bf (5%) +j – fermented barley soup added in the amount of 5 % and mixed with juice
bf – fermented barley soup
sf (25%) +j – fermented soya soup added in the amount of 25 % and mixed with juice
sf (5%) +j – fermented soya soup added in the amount of 5 % and mixed with juice
sf – fermented soya soup;
highlighted values are the highest final bacterial counts obtained

Table 31.

The viable count in the drink with juice 5 during 2 weeks storage.

Time (days)	Viable count of *Lb.plantarum* 299 v											
	bf (25%)+j		bf (5%)+j		bf		sf (25%)+j		sf (5%)+j		sf	
	10^{-4}	10^{-5}	10^{-4}	10^{-5}	10^{-5}	10^{-6}	10^{-5}	10^{-6}	10^{-4}	10^{-5}	10^{-5}	10^{-6}
0	208	23	89	(16)	259	(13)	75	(17)	154	(18)	un.	42
15	92	(13)	52	(4)	**287**	**32**	33	(7)	78	(8)	207	(18)

note: **highlighted** values are the highest final bacterial counts obtained; un – uncountable; (values) weren't taken into account when counting the final CFU/ml

Table 32.

The viable count in the drink with juice 5 during 2 weeks storage expressed as CFU/ml.

Time (days)	Viable count of *Lb.plantarum* 299 v (CFU/ml)					
	bf (25%)+j	bf (5%)+j	bf	sf (25%)+j	sf (5%)+j	sf
0	2.10^{7} *	9.10^{6} *	$2{,}5.10^{8}$ *	$7{,}5.10^{7}$ *	$1{,}5.10^{7}$ *	$4{,}2.10^{8}$ *
15	9.10^{6}	5.10^{6}	**3.10^{8}**	3.10^{7}	8.10^{6}	2.10^{8}

* these pH values reffer to an initial bacterial counts of the drinks and fermented soups
bf (25%) +j – fermented barley soup added in the amount of 25 % and mixed with juice
bf (5%) +j – fermented barley soup added in the amount of 5 % and mixed with juice
bf – fermented barley soup
sf (25%) +j – fermented soya soup added in the amount of 25 % and mixed with juice
sf (5%) +j – fermented soya soup added in the amount of 5 % and mixed with juice
sf – fermented soya soup;
highlighted values are the highest final bacterial counts obtained

Table 33.
The viable count of pathogens – juices 1 to 5

Time (days)	Viable count of pathogens(CFU/ml)					
	bf (25%)+j	bf (5%)+j	bf	sf (25%)+j	sf (5%)+j	sf
0	0*	0*	0*	0*	0*	0*
15	0	0	0	0	0	0

* these pH values reffer to an initial bacterial counts of the drinks and fermented soups
bf (25%) +j – fermented barley soup added in the amount of 25 % and mixed with juice
bf (5%) +j – fermented barley soup added in the amount of 5 % and mixed with juice
bf – fermented barley soup
sf (25%) +j – fermented soya soup added in the amount of 25 % and mixed with juice
sf (5%) +j – fermented soya soup added in the amount of 5 % and mixed with juice
sf – fermented soya soup

Table 34.
The list of samples tested by both groups 1 and 2.

	blackcurrant	rosehip	pear/kiwi	mango	blueberry
oat	x	x	x	x	x
barley	x	x	x	x	x
soya	x	x	x	x	x

Table 35.
Tasting trial – group 1 (13 trained volunteers from Skånemejerier)

	blackcurrant	rosehip	pear/kiwi	mango	blueberry
oat	7.95	7.85	7.8	7.85	8
barley	7.7	6.85	7.35	7.1	7.7
soya	5.3	4.25	4.85	4	5.4

Note: the scale used in this trial was 1 for the unacceptable taste and 9 for the most acceptable taste; the values in the Table 23 are the median values from the values obtained.

Table 36.
Tasting trial – group 2 (10 untrained randomly chosen volunteers)

	blackcurrant	rosehip	pear/kiwi	mango	blueberry
oat	7.75	5.88	5.38	6.2	5.4
barley	6.5	6.63	5.38	5.75	7.8
soya	5.75	4.25	3	5.25	2.4

Note: the scale used in this trial was 1 for the unacceptable taste and 9 for the most acceptable taste; the values in the Table 23 are the median values from the values obtained.