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Production of health rice-based drink: use of ultrasound-attenuated lactic acid bacteria and yeasts

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Ai miei genitori e a Mary

Ogni momento di ricerca è un momento d'incontro (Paulo Coelho).

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Abstract

"Production of health rice-based drink: use of ultrasound-attenuated lactic acid bacteria and yeasts"

The main goal of this PhD thesis was the optimization of a flow-chart for the production of a cereal-beverage inoculated with lactic acid bacteria and yeasts; moreover, a special focus was the use of a physical approach (homogenization and ultrasound) to avoid the post-acidification throughout the storage of the beverage at 4°C. The PhD project consists of 3 different steps:

Step 1: Screening on yeast metabolism by using a head-space gas analyzing approach and viability and acidification in an organic rice-drink. The final goal of this step was to choose the best yeast.

Step 2: Metabolism of lactic acid bacteria: cell viability and pH change in cereal-based media and rice drink. *Latcobacillus plantarum* strain 12 and *Lactobacillus reuteri* were used as targets.

Step 3: Attenuation of the best strains through homogenization and ultrasound and confirmation of the results on two commercially available probiotics (*Lactobacillus casei* LC01 and *Bifidobacterium animalis* subsp. *lactis* BB12). As an additional goal of this last step, the best strains were also combined with β-glucans, added as healthy compounds; thus, I studied the effects of this combination on the sensory scores.

Key words: filtered flour, carbon dioxide, head-space, cereals, rice drink, attenuated microorganisms, ultrasound, probiotics, lactic acid bacteria, yeasts, β-glucans.

Riassunto

“Produzione di una bevanda salutistica a base di riso: uso di batteri lattici e lieviti attenuati mediante ultrasuoni”

L'obiettivo principale di questa tesi di dottorato è stata l’ottimizzazione di un diagramma di flusso per la produzione di una bevanda di cereali inoculata con batteri lattici e lieviti; inoltre, particolare attenzione è stata rivolta all'uso di un approccio fisico (omogeneizzazione e ultrasuoni) per evitare la post-acidificazione durante la conservazione della bevanda a 4°C. Il progetto di dottorato consiste in 3 diverse fasi:

Fase 1: Screening sul metabolismo dei lieviti mediante valutazione della CO_2 prodotta nello spazio di testa e della vitalità e dell’acidificazione in una bevanda di riso biologica. L'obiettivo finale di questa fase è stato quello di scegliere il migliore lievito.

Fase 2: Metabolismo dei batteri lattici: studio della vitalità cellulare e del pH in sistemi modello a base di cereali e in una bevanda di riso. *Lactobacillus plantarum* strain 12 e *Lactobacillus reuteri* sono stati usati come target.

Fase 3: Attenuazione dei migliori ceppi mediante omogeneizzazione e ultrasuoni e conferma dei risultati su due probioici disponibili in commercio (*Lactobacillus casei* LC01 e *Bifidobacterium animalis* subsp. *lactis* BB12). Come ulteriore obiettivo di questo ultima fase, i migliori ceppi sono stati combinati con β-glucani, aggiunti come composti salutistici, valutandone gli effetti sulle caratteristiche sensoriali.

Parole chiavi: filtrati di farina, anidride carbonica, spazio di testa, cereali, bevanda di riso, attenuazione, ultrasuoni, probiotici, batteri lattici, lieviti, β-glucani.

Aims of PhD project

Increasing consumer awareness towards healthy diets and changing eating habits due to urbanization has created a huge market demand for new functional foods with a beneficial effect on health. Different cereals have complex nutrient composition and can considerably modify the properties of the food. Cereal-based beverages have a huge potential as functional food; they can serve as carriers for a range of functional compounds, for example antioxidants, dietary fiber, minerals, probiotics, and vitamins. Special attention should be given to the application of probiotics in cereal-based beverages, as cereal malt extracts represent excellent media for the growth of microorganisms. Consequently, when the production of a functional beverage is planned, raw materials and processing steps need to be carefully assessed to fulfill the demands of consumer with a special focus to taste, aroma, and appearance as well as ensuring that desired functional properties are available and active. Nowadays, the range of functional foods includes products such as baby foods, baked goods and cereals, dairy foods, confectionery, ready meals, snacks, meat products, spreads, and beverages (Ofori and Hsieh, 2013).

Consumers are more and more aware of the role of food and nutrition on the prevention of diseases, as well as on their link with well-being and health; thus, we can imagine an increasing trends towards functional foods in the future, along with the definition of mandatory rules by Governments and National/International Agencies. The first challenge is the building of a common background for the definition of functional foods, able to harmonize traditions from different Countries and gain a real consumer awareness towards the significance and the impact of these products. Moreover, a second threat could be a special focus on the health claims to

define a unique checklist for the definition and the optimization of an effective and international protocol to prove the benefits of functional foods. Finally, these products are still niche foods, due to the relatively high cost, therefore the optimization of simple technologies and/or flow-charts for their production could be a suitable way to reduce the cost and gain a large diffusion all over the world. This PhD thesis proposes an experimental design for the production of a healthy cereal-based beverage, with a special focus on the protocol to avoid the modification of the physical properties of a rice-beverage by probiotic and starter cultures, added with β-glucans.

Many traditional cereal foods contain microorganisms in a living form (biza, tarhana, shalgam, fura, brembali, burukutu, etc.) (Blandino et al., 2003); therefore, the use of probiotic bacteria/functional yeasts for these products or for other traditional cereal foods could be a suitable way for their valorization. Some examples of novel functional beverages are whey-based prickly pear (Baccouche et al., 2013) and grape-based beverages (Di Cagno et al., 2010), cereal-based probiotic drinks (Rathore et al., 2012), fruit-beverages (Gad et al., 2013), and some organic beverages (Awe et al., 2013).

The targets of this PhD project are both yeasts and lactic acid bacteria; namely I focused on 4 different yeasts (*Saccharomyces cerevisiae* var. *boulardii*, *Saccharomyces pastorianus*, *Kluyveromyces lactis* and *Kazachstania exigua*, commonly known as *Saccharomyces exiguus*) and 4 lactic acid bacteria (*Lactobacillus reuteri* DSM 20016, *Lactobacillus plantarum* strain 12, isolated from an Italian sourdough, *Lactobacillus casei* LC01 and *Bifidobacterium animalis* subsp. *lactis* BB12).

A main drawback of probiotics/starter cultures in food could be their active metabolism, e.g. some strains of lactic acid bacteria continue to produce lactic acid and cause the so-called post-acidification (decrease of pH within the storage); therefore many times it is important to control the metabolism of probiotic and starter cultures in foods, without affecting their viability and functional properties (Bandiera et al., 2013; Ferdousi et al., 2013). This goal could be achieved by attenuation.

Some authors studied homogenization as a way to attenuate/modulate starter cultures in dairy products (Lanciotti et al., 2004, 2006, 2007); in this project I have used homogenization and compared it to a new approach for attenuation, i.e. ultrasound.

The PhD project consists of 3 different steps:

Step 1: Screening on yeast metabolism by using a head-space gas analyzing approach and viability and acidification in an organic rice-drink. The final goal of this step was to choose the best yeast.

Step 2: Metabolism of lactic acid bacteria: cell viability and pH change in cereal-based media and rice drink. *L. plantarum* strain 12 and *L. reuteri* were used as targets.

Step 3: Attenuation of the best strains thorugh homogenization and ultrasound and confirmation of the results on two commercially available probiotics (*L. casei* LC01 and *B. animalis* BB12). As an additional goal of this last step, the best strains were also combined with β-glucans, added as healthy compounds; thus, I studied the effects of this combination on the sensory scores.

Chapter 1. LITERATURE BACKGROUND

1.1 Introduction: the state of art of the research

Consumers have become more health conscious due to the wide availability of scientific evidence on various aspects relevant to food quality and safety. During the last decade, some studies opened a new field of research dealing with the health promoting traits of functional foods (Gobbetti et al., 2010). Although yogurt and fermented milks are the most popular functional products, consumer demand for non-dairy beverages is increasing. The main drawbacks for fermented milks are mainly the ongoing trend of vegetarianism, the increasing prevalence of lactose intolerance, and the level of cholesterol (Prado et al., 2008). Traditionally dairy fermented products have been considered as the best carrier for probiotics. Therefore, several raw materials, including cereals and cereal-based beverages, have been extensively investigated to determine if they are suitable substrate to produce novel non-dairy functional foods (Rivera-Espinoza and Gallardo-Navarro, 2010). The concept of functional foods includes food or food ingredients that, in addition to basic nutritional functions, exert a beneficial effect on host health or reduce the risk of chronic disease (Huggett and Schliter, 1996). Fermented foods containing probiotic microorganisms are generally considered as functional foods. Probiotics need to be viable at the time of consumption, although non-viable "probiotics" are not necessarily without health effects. As a general rule, a lower limit of 10^9 cfu *per* dose is often used, although this may be different depending on the strain, health effect, and the raw material (Forssten et al., 2011). In addition to the classical traits

for a probiotic culture, some additional features are required in functional beverages, like the interaction with the starter cultures, as antagonistic interaction between probiotics and starter cultures may result in growth inhibition by acid, peroxide, bacteriocins and other metabolites. As a result, the selection of compatible probiotic and starter cultures could be pivotal to prevent inhibition (Nagpal et al., 2012). Another increasing trend is the focus on synbiotics, a combination of pre- and probiotics in a single product (Bielecka et al., 2002). A prebiotic is a polysaccharide compound; it should not be digested by the human host in the upper gastrointestinal tract but can selectively stimulate the growth of one or more species of colonic bacteria (Gibson and Roberfroid, 1995). Acid and bile tolerances are two fundamental properties that indicate the ability of a probiotic microorganism to survive the passage through the gastrointestinal (GI) tract, resisting the acidic conditions in the stomach and the bile acids at the beginning of the small intestine (Prasad et al., 1998). The selective promotion of beneficial colonic bacteria can be mediated via the use of indigestible carbohydrates. These compounds, which are present in various cereal grains, are known as prebiotics and selectively stimulate the activity or growth of beneficial bacteria in the colon. The success of new probiotic formulations does not rely only the ability to provide enough probiotic cells that may survive the human gastrointestinal tract. The organoleptic properties of these products must also be acceptable for consumers. An appropriate selection of substrate composition and strains is necessary to efficiently control the distribution of the metabolic end-products (De Vuyst, 2000). Several factors have been suggested to influence probiotic survival during processing and storage. In the case of cereal-based fermented products the most likely factors influencing probiotic survival

during refrigerated storage are pH and lactic acid concentration. The former usually ranges between 3 and 4 depending on the strain, the composition and buffering capacity of the growth medium (Angelov et al., 2006).

Some strains of the genus *Lactobacillus*, used as probiotics, have proven to exert a range of health promoting activities such as immunomodulation, enhancement of resistance against pathogens and reduction of blood cholesterol levels. The growth and the metabolism of these microorganisms can be affected by medium composition, presence of oxygen, pH and product concentration. Thus, it is important to optimize the concentration of the nutrients in order to get maximum growth and survival of lactobacilli.

1.2 Cereal fermentation

Recently, the fermentation of cereals has been extensively studied. Cereals are grown over 73% of the total world harvested area and account for 60% of world food production. Cereals have higher content of certain essential vitamins, prebiotic dietary fiber, and minerals than milk, but have lesser quantities of readily fermentable carbohydrates (Charalampopoulos et al., 2002a). The studies using cereal substrates and cultures as delivery vehicles for potentially probiotic lactic acid bacteria (Charalampopoulos et al., 2003; Angelov et al., 2006; Helland et al., 2004). Kedia et al. (2007) have also used mixed cultures for the fermentation of single cereals and cereal fractions. In the past, cereals were regarded as good substrates for the growth of probiotic strains (Charalampopoulos et al., 2002b) and cereal extracts were found to enhance acid and bile tolerance. The results showed that malt, wheat, and barley extracts were able to exhibit a significant protective effect on the viability

of lactobacilli under acidic and bile conditions (Patel et al., 2004). Cereal grains and their fractions contain many functional compounds, such as essential fatty acids, phytosterols, phenolic compounds or resistant starch, and the consumption of whole grains has been associated with lower incidences of certain cancers and cardiovascular diseases (Baublis et al., 2000; Peterson, 2001; Truswell, 2002). Flavour is one of the most important characteristic in the sensory profile of a specific food and plays a decisive role in consumer acceptability. The analysis of volatile flavour components tends to be one of the main parameters to take into account for the development of a novel fermented food (McFeeters, 2004). The use of oat, wheat, barley and malt substrates to support the growth of probiotic strains of *Lactobacillus plantarum* has been previously reported (Kedia et al., 2007, 2008). Furthermore, the interaction between lactic acid bacteria and yeasts may affect the synthesis of volatile compounds.

Nowadays, cereals are used for the production of traditional fermented beverages as well as to design new foods with enhanced healthy properties (Blandino et al., 2003) for their high content of essential vitamins, dietary fibre and minerals (Charalampopoulos et al., 2002a). Unfortunately, the low content in proteins and essential amino acids (lysine), the low starch availability, and anti-nutrients (phytic acid, tannins and polyphenols) represent a drawback compared to milk and dairy products (Blandino et al., 2003). However, fermentation could improve the quality of whole grain and cereal-based products (Gobbetti et al., 2010).

Rice is largely used for the manufacture of traditional fermented foods and beverages (Blandino et al., 2003). Due to the high levels of soluble dietary fibers (e.g., β-glucans) (Johansson et al., 2004), selenium and zinc, and antioxidant activity, oat and

barley were used for making yogurt-like beverages (Gee et al., 2007; Walsh et al., 2010). Based on nutritional and rheological properties, emmer flour was recently used for the manufacture of fermented beverages (Coda et al., 2011).

1.3 Fermented cereal-based drinks

Some innovative fermented functional cereal beverages were designed over the last ten year, as a consequence of the focus on the nutritional properties of cereals (Vander Wal et al., 2007; Charalampopoulos et al., 2002a; 2002b). Non-alcoholic fermented functional cereal beverages can be referred to as a new generation of fermented cereal beverages; the concept of fermented cereal beverages is not a new one as products such as beer fall into this category. Traditional non-alcoholic fermented cereal beverages have been previously defined by Roberfroid (1998) as non-dairy vegetarian fermented drinks suitable for vegetarians or consumers with lactose allergies. These beverages are generally homogeneous suspensions with an appearance from light to dark beige with a sweet to slightly sour taste. In addition, the incorporation of probiotic microorganisms into cereal substrates produces a range of foods and beverages with distinct and enhanced sensory and nutritional properties (Blandino et al., 2003). There are many traditional non-alcoholic fermented cereal beverages on global markets which have been purchased by consumers as healthy supplements to their diets (Charalampopoulos et al., 2002a; Blandino et al., 2003). Nutritional research on the benefits of cereals has found that daily consumption of cereals could help consumers to lower the risk of some diet-related diseases, such as obesity, diabetes and bowel diseases (Charalampopulos et al., 2002b). In addition, fermented functional cereal beverages could be safely and effectively consumed as

meal replacements for weight conscious consumers as fibre and protein of cereal-based products slow digestion and extend satiety (Vander Wal et al., 2007).

The application of probiotic cultures to cereal products represents a great technological challenge for the development of market-oriented functional cereal products (Dean et al., 2007). Although cereal products provide manufacturers with the opportunity to enter a relatively high margin food market, many technology-oriented products often result in low consumer acceptance. Therefore, in order to successfully introduce cereal-based functional beverages to consumers, an understanding of consumers' perceptions of cereal products is necessary at an early stage of the new product development process. Despite several approaches have been proposed for the storage of cereals, fermentation remains today, especially in the Developing Countries, the most used technology for several reasons: the low energy inputs required, the simple production protocols and the possibility of biofortification of the raw material (Hjortmo et al., 2008).

Many cereals are used for the preparation of various fermented beverages, such as beer and whiskey, vodka, American bourbon, Japanese sake (Hammel et al., 2005) and some traditional foods, like ogi (Nigeria), togwa (Tanzania), poto poto (Congo). The fermentation of cereals, defined by Nout (2009) as "rich nutrition for the poorest", involves a composite microflora of lactic acid bacteria (*Lactobacillus*, *Pediococcus, Enterobacter*) yeasts (*Candida*, *Debaryomyces*, *Pichia*, *Saccharomyces*) and molds (*Aspergillus*, *Mucor*, *Rhizopus*). In Western countries the increasing trend towards the green consumerism determined a special focus on cereal-based beverages (Coda et al., 2011, 2012; Mukisa et al., 2012). Cereal fermentation leads to the decrease of the level of carbohydrates as well as of some

non-digestible poly- and oligo-saccharides, while the availability of certain amino acids and B vitamins is improved (Gobbetti et al., 2010). Indeed, the selection of appropriate starter cultures for each variant of cereal beverage is an industrial need to drive, accelerate and standardize the fermentation (Nionelli et al., 2014). Many traditional cereal foods contain yeasts in a living form (biza, tarhana, shalgam, fura, brembali, burukutu, etc.) (Blandino et al., 2003); therefore, the use of probiotic/functional yeasts for these products or for other traditional cereal foods could be a suitable way for their valorization**. Figure 4.1** proposes a classification of cereal-based drinks.

1.4 The design of new cereal-based beverages

In the literature it is possible to find different definitions for cereal-based drinks, depending on the flow-sheet, raw materials and economic value. Some commercial products contain fats, thus the appearance of the beverage looks like a milky emulsion ("vegetable milk" or cereal milk). In 2005 a law was published in Switzerland (Ordinance of the DFI 817.022.111, 2005 on soft drinks); this regulation states that this kind of products should be referred as "cereal-based drinks". Nowadays, this kind of food could be one of the most promising way for the innovation in the food industry, due to the health-focused value of many cereal-based drinks. **Table 4.1** reports the different species of cereals used for the preparation of beverages (Casanova et al., 2013). All the cereals can be suitable for the preparation of beverages; generally, these products contain natural sugars such as maltose or dextrin obtained from carbohydrates, low amounts of fat, protein, minerals and vitamins. Due to the high percentage of water (about 90%), these beverages are

diluted products if compared to the cereal grains. Some producers often add a fat milky emulsion to obtain "vegetable milk" or cereal milk. You can add oil seeds (sunflower), to soften the taste, and possibly a small amount of sea salt (which contains valuable trace elements such as iodine, magnesium and fluoride) to improve the taste. To avoid the formation of solid particles, the cereal drinks are homogenized; finally, the beverages are heat-processed (pasteurization or sterilization). **Figure 4.2** shows a simplified flow chart for the production of cereal-based beverages (Casanova et al., 2013).

The innovation on cereal drinks can take place at different levels. Some strategies are focused on the use of enzyme preparations, such as Betamalt (produced by Sternenzym), extract of plant origin containing β-amylase which hydrolyzes efficiently dextrin and maltose, or Optizym (α-amylase); these enzyme preparations regulate the viscosity, mouthfeel and sweetness of the drink. Another possible innovation concerns a controlled fermentation with the use of some microorganisms: sugars released flavor-active and organic acids; when these compounds are combined together, they create a dry, fruity and slightly acidic. Microorganisms used for the fermentation of cereal-based beverages include *Gluconobacter oxidans, Saccharomyces cerevisiae, Lactobacillus* spp. and other microorganisms such as the Kombucha mushroom (belonging to a symbiotic community of *Acetobacter*, *Brettanomyces bruxellensis*, *Candida stellata*, *Schizosaccharomyces pombe*, *Torulaspora delbrueckii* and *Zygosaccharomyces bailii*).

An important direction in the development of functional foods is the combination of numerous ingredients to achieve a specific set of goals; an increasing trend in cereal

beverages is the combination of probiotics with some prebiotic compounds, like β-glucans.

1.5 Functional beverages: the state of art of the research

Functional drinks constitute an important category of functional foods (**figure 4.3**). What is a functional drink? There is not a commonly accepted definition of functional drinks; however, keeping in mind the different reports available in the literature, functional beverages can be defined as ethanol free drinks that satisfy thirst and contain some non-traditional ingredients, such as herbs, vitamins, minerals, amino acids or additional fruit/vegetable raw ingredients, depending on the purpose for which they are designed for improving immunity and digestion, maintenance of health and well-being, satiety, and energy-boosting. Some examples of "active ingredients" used to design functional drinks are reported in **figure 4.4**. A pre-requisite for these ingredients is their stability through product shelf life and the absence of the interaction with other components of the beverage.

In recent years, cereals have been also investigated as fermentable substrates for the growth of probiotic microorganisms (Nyanzi and Jooste, 2012). Cereals have also a huge potential as vehicles for functional compounds such as antioxidants, dietary fiber, minerals, prebiotics and vitamins (Nionelli et al., 2014). Examples of commercial products are Proviva® (Skane Dairy, Sweden), the first oat-based probiotic food beverage, where the active probiotic component is *L. plantarum* 299v (Prado et al., 2008), and Whole Grain Probiotic Liquid® (Grainfields, Australia), a refreshing, effervescent liquid containing both LAB (*L. acidophilus*, *L. delbrueckii*) and yeasts (*Saccharomyces cerevisiae* var. *boulardii* and *S. cerevisiae*), as well as

vitamins, amino acids, and enzymes (Soccol et al., 2012). **Table 4.2** lists some commercially available probiotic beverages (Corbo et al., 2014b).

The optimization of the fermentation of cereal-based beverages is an increasing trend in the literature (Coda et al., 2011, 2012); some examples of novel functional beverages are whey-based prickly pear (Baccouche et al., 2013) and grape-based beverages (Di Cagno et al., 2010), cereal-based probiotic drink (Rathore et al., 2012), fruit-beverages (Gad et al., 2013), and some organic beverages (Awe et al., 2013). Many researchers report that functionals food represent one of the most interesting area of research and innovation in the food industry (Bigliardi and Galati, 2013), as suggested by the increasing number of scientific papers dealing with this topic since 2007 (**table 4.3)**. The different kind of approaches could be grouped as follows: (1) exploitation of microorganisms functionality, (2) optimization of the production and formulation of novel functional beverages, (3) use of prebiotics and synbiotics, (4) use and processing of natural ingredients, (5) use of by-products of fruit and food industries as functional ingredients. In addition, some papers focused on the application of novel technologies to improve the production of functional beverages without compromising their sensory and functional properties (Corbo et al., 2014b).

Soluble fibers such as FOS, β-glucan, and inulin have successfully been added to functional beverages. The beneficial physiological effect of soluble dietary fibers seems to be closely related to an increase in the viscosity of gastro-intestinal tract's contents that, in turn, reduces the rate of gastric emptying and nutrient absorption by profusely increasing the unstirred layer in the small intestine (Paquet et al., 2014). Therefore, the addition of dietary fiber into a diet through the use of functional beverages is a challenge of great concern in the area of nutritional deficiencies. For

instance, people from North America consume only about half of the recommended daily amount of dietary fiber which should be 38 g/day for men and 25 g/day for women (Chen et al., 2010).

The design of synbiotic products (the combination of probiotics and prebiotics) is the new challenge for functional beverages, as prebiotics could enhance and/or improve the viability of probiotic bacteria and actively stimulate the beneficial microbiota in the gut (Sip and Grajek, 2009). Nazzaro et al. (2008) designed a functional fermented carrot juice beverage with *L. rhamnosus* DSM 20711 and *L. bulgaricus* ATCC 11842 supplemented with inulin and FOS. Renuka et al. (2009) reported that fruit juice beverages could be successfully fortified with FOS with a shelf-life of 4 months and 6 months at ambient temperature and under refrigeration, respectively, without undesirable changes in their physicochemical characteristics. Moreover, Brajdes and Vizireanu (2013) reported that inulin induced better stress resistance in *L. plantarum*, as compared to glucose, whereas the adhesion of probiotics to the surface of enterocytes and of mucosal cells through self-assembly and co-aggregation is about 10 times higher.

The European Union (EU) defined two kinds of health claims: the "function claims" and the "claims connected with the reduction of disease" (EC No. 1924/2006, in the articles 13 and 14); later, the European Commission (Commission Regulation No 432/2012) approved the list of the permitted health claims (health claims for foods, health claims referred to the reduction of disease risk, health claims connected with food designed for children and/or infant formula); for example, there are two different claims for β-glucans, as follows:

1. β-glucans contribute to the maintenance of normal blood cholesterol levels. This claim can be used only for a food containing at least 1 g of β-glucans from oats, oat bran, barley, barley bran, or from mixtures of these sources. Consumers should be informed that the health benefit relies upon a daily intake of 3 g of β- glucans (EFSA, 2011a).
2. The consumption of β-glucans from oats or barley as part of a meal reduce significantly the impact of the post-prandial hyperglycemia. This claim can be used only for a food containing at least 4 g of β-glucans from oats or barley for each 30 g of available carbohydrates. Consumers should be informed that the beneficial effect relies upon the consumption of glucans from oats or barley (EFSA, 2011b).

1.6 Starter attenuation

The supplementation of probiotics to cereal-based matrices requires special technologies because of the active metabolism of probiotics, which could lead to a significant changes in the flavor and in the rheology of the beverages. A possible way to overcome this problem is the attenuation of the probiotic, through a physical method. A starter culture is any active microbial preparation intentionally added during product manufacture to start desirable changes, and a starter could be attenuated to create populations of both lysed and dormant cells with a greater potential to control and/or accelerate process (Yarlagadda et al., 2014). Attenuation can be defined as a technological method to enhance the total pool of intracellular enzyme released into the matrix, positively influencing flavor and quality of the final product. Attenuated starters are generally lactic acid bacteria unable to grow and to

produce significant levels of lactic acid but still delivering active ripening enzymes during cheese ageing. The use of attenuated starters was first proposed by Petterson and Sjöström (1975) to accelerate the ripening of Svecia, a Swedish semi-hard cheese, by thermal treatments (e.g., 69°C for 15 s). At this time, their main purpose was to accelerate proteolysis and shorten ripening time, which has great economic interest. Today, in most developed countries the hygienic quality of milk is so high that the problem is not only to shorten the ripening time but also to improve the flavor. Starter bacteria are mainly responsible for the formation of small peptides, amino acids and for flavour development in cheese. Increasing the cell count of starters might represent a tool to accelerate the ripening of cheese, but in some cases (e.g. Cheddar cheese) high levels of starter cells have been associated with bitterness (Ristagno et al., 2012).

Attenuation of the cells is also necessary to eliminate the overproduction of lactic acid during cheese making (Johnson at al., 1995).

Some chemical an physical techniques intended for attenuation have been extensively reviewed (El-Soda et al., 2000; Geciova et al., 2002; Klein and Lortal, 1999). According to Yarlagadda et al. (2014) these techniques can be generally divided into two types of treatment: (a) chemical treatments using hexadecyltrimethylammonium bromide (CTAB), ethylenediaminetetraacetic acid (EDTA), isopropyl alcohol (IPA), sodium dodecyl sulphate (SDS), or n-butanol (Doolan and Wilkinson, 2009; Exterkate, 2006); (b) physical treatments including heat or freeze shocking, and/or mechanical treatments such as sonication (Exterkate, 2006), bead mill, high-pressure homogenization and microfluidization (Geciova et al., 2002). Each method shows its own advantages and disadvantages. Chemical

treatment using chelating agents, such as EDTA, have strain-specific effects that are influenced by buffers and are most effective towards Gram-negative bacteria. SDS is used mainly for Gram-negative bacteria; however, it can also cause denaturation of proteins. The use of alkanols like n-butanol increased the permeabilisation of lactococcal cells; however, the enzymes are sensitive to irreversible inactivation (Exterkate, 2006). On the other hand, use of mechanical treatments like bead milling is more efficient in yeasts or moulds compared with bacteria but the effectiveness is dependent upon the size of beads.

1.6.1 Focus on ultrasound and homogenization

Many authors proposed the use of homogenization in some dairy-based products (Ciron et al., 2010, 2011); hereby we investigated the possibility of achieve this goal by using another physical treatment, i.e. ultrasound (US). US is one of the emerging technologies that were developed to minimize processing, maximize quality, and ensure the safety of food products and at the same time to maintain a high level of proprieties and sensory qualities. It is applied to impart positive effects in food processing such as improvement in mass transfer, food preservation, assistance of thermal treatments and manipulation of texture and food analysis (Knorr et al., 2011). Sound propagates through food materials as mechanical waves causing alternating compressions and decompressions. The efficiency of ultrasound waves is due to some important parameters, like wavelength, rate, frequency, pressure and period. The interaction of sound waves with matter alters both the velocity and attenuation of the sound waves *via* absorption and/or scattering mechanisms (Di Benedetto et al., 2010). Sonication is a laboratory scale method that not only

increases cell lysis, but also significantly increases the degradation of enzymes due to heat denaturation (Geciova et al., 2002), as the ultrasonic waves have the potential to influence the microorganisms and living cells (Tabatabaie and Mortazavi, 2010). Ultrasound is defined as a pressure wave with a frequency of 20 kHz or more. Generally, it uses frequencies from 20 kHz to 10 MHz, and for industrial purposes, it has two main requirements: a liquid medium, even if the liquid element forms only 5% of the overall medium, and a source of high energy vibrations (the ultrasound) (Patist and Bates, 2008). The effects of destructive ultrasound rely upon the acoustic cavitation (Cárcel et al., 2012; Leighton, 1998; Soria and Villamiel, 2010). The antimicrobial effect depends on amplitude of waves, temperature and duration of the treatment, volume and composition food (Chemat et al., 2011), microorganism shape and dimension (Heinz et al., 2001). Gram-positive bacteria are more resistant than Gram-negative ones (Drakopoulou et al., 2009) and cocci are more resistant than rods (Chemat et al., 2011). During the sonication process, the sonic wave encounters a liquid medium creating longitudinal waves, that generates regions of high pressure alternating with areas of low pressure. These regions with different pressure cause cavitation and gas bubbles formation that increase gradually their volume until they implode, creating regions of high temperature and pressure. Pressure resulting from these implosions cause the main bactericidal effect of ultrasound (D'Amato et al., 2010).

High pressure (HP) treatment dates back to end of 19th Century (Hite, 1899), as a suitable mean for reducing food contamination by pathogens and spoiling microorganisms. It is defined as non-thermal treatment that uses the pressure (300-700 MPa, in some cases up to 1000 MPa) as the main preservation method. Due to

the fact that pressure increase is achieved through a fluid (for example water), this process has been also referred to as high hydrostatic pressure (HHP) as opposite to the high pressure of homogenization (HPH), where the increase of the pressure is obtained forcing the product through a small valve (homogenizing valve) (Bevilacqua et al., 2010a). From 1990, a new generation of homogenizers, referred to as high-pressure homogenizers, has been developed and used by pharmaceutical, cosmetic, chemical and food industries for the preparation or stabilization of emulsions and suspensions or for creating physical changes, such as viscosity changes, in products. Other applications are cell disruption of yeasts or bacteria (in order to release intracellular products such as recombinant proteins) and/or reduction of the microbial cell load of liquid products (Bevilacqua et al., 2008). A homogenizer consists of a positive displacement pump and a homogenizing valve. In the homogenizing valve (often referred to as radial diffuser), the fluid is forced under pressure by pump, through a small orifice between the valve and the valve seat (Diels and Michiels, 2006). HPH was proposed for the non-thermal fluid food microbial decontamination (Bevilacqua et al., 2010a; Lanciotti et al., 2007). Cavitation and viscous shear have been identified as the primary mechanisms of microbial cell disruption during HPH treatment (Kleinig and Middelberg, 1998; Middelberg, 1995). Furthermore, it appears that even this technology is active on food constituents, especially proteins, leading to changes in their functional properties and activities (Kheadr et al., 2002; Vannini et al., 2004). Specifically, HPH treatment of skim and whole milk has been reported to modify the ratio of the nitrogen fractions and the soluble forms of calcium and phosphorous, improve the coagulation of milk as well as increase cheese yields (Lanciotti et al., 2007). HPH treatment of milk was

associated with an acceleration of lipolysis activities observed in Crescenza (a soft cheese), when produced using milk HPH-treated at 100 MPa (Lanciotti et al., 2004). Temperature increase during the process is a key process parameter for enzyme activity modification and microbial inactivation (Hayes et al., 2005). According to Grandi et al. (2005) the temperature increase derives during the pressure drop from the pressure energy transformation into thermal energy and corresponds to about 12°C each 50 MPa. Activation of endogenous and microbial proteolytic enzymes was observed in cheeses obtained from cow and goat milks treated at 100 MPa (Lanciotti et al., 2006), suggesting that the treatment increased the activity of extracellular enzymes and of enzymes located on cell envelope.

Chapter 2. MATERIALS AND METHODS

2.1 Strains and inocula preparation

The following microorganisms were used throughout this study:

Saccharomyces cerevisiae var. *boulardii* ATCC MYA-796 (GenBank: JQ070086.1) was purchased from American Type Culture Collection (Manassas, USA), whilst *Kluyveromyces lactis* x *Saccharomyces cerevisiae* DBVPG 6530 (previously known as *Saccharomyces distaticus* and proposed for brewing; Fontana et al., 1992), *Saccharomyces pastorianus* var. *pastorianus* DBVPG 6033 (type strain of *Saccharomyces carlsbergensis* E.C. Hansen, isolated from brewery), *Kazachstania exigua* DBVPG 4384 (previously known as *S. exiguus*, isolated from sea water) were from the Industrial Yeast Collection, University of Perugia (Perugia, Italy); *Lactobacillus plantarum* strain 12 isolated from an Italian sourdough (Corbo et al., 2014a) and belonging to the Culture Collection of the Laboratory of Predictive Microbiology (Dept. of the Science of Agriculture, Food and Environment-University of Foggia); *L. reuteri* (DSM 20016, Deutsche Sammlung von Mikroorganismem und Zellkulturen's collection, Braunschweig, Germany), originally isolated from human feces (Sriramulu et al., 2008); *Lactobacillus casei* LC01 and *Bifidobacterium animalis* subsp. *lactis* BB12 purchased from Chr. Hansen (Hørsholm, Denmark) (**table 4.4**). Lactic acid bacteria were stored at -20°C in MRS broth (Oxoid, Milan, Italy), containing 33% of sterile glycerol (J. T. Baker, Milan), whilst yeasts were stored at 4°C on YPG slant (bacteriological peptone, 20 g/l; yeast extract, 10 g/l; glucose, 20 g/l; all the ingredients were from Oxoid); before each

assay the strains were grown under the optimal conditions. LAB cultures were centrifuged at 4000 rpm/4°C for 10 min, while yeasts were centrifuged at 6900 rpm/4°C for 15 min. The strains were washed with sterile tap water.

2.2 Rice drink

A commercial organic rice-drink gluten and dairy free and with no added sugar (water; Italian organic rice, 20%; organic sunflower seed oil cold-pressed, 1.2%; sea salt, 0.1%; pH, 6.8-6.9; soluble solids, 14.1°Bx) was purchased from a local market in Foggia.

2.3 Screening on yeasts

2.3.1 Evaluation of CO_2 production by yeasts

The experiments were performed in glass vials (volume 20 ml, Dani Instruments, Cologno Monzese, Italy) (**figure 4.5**) containing 10 ml of media (YPG broth, Malt Extract broth, Malt Extract broth diluted to 15%). After yeast inoculation (ca. 5 log cfu/ml), vials were sealed with a butyl cap and a metal ring and stored at 15 and 25°C (for 48-96 h); the content of CO_2 in the headspace (%, v/v) was evaluated through a headspace gas analyzer Checkmate II (PBI Dansensor, Ringsted, Denmark) (**figure 4.6**). The initial level of yeasts was assessed through spread plating on YPG agar, incubated at 25°C for 72 h.

The analyses were performed over at least four different batches for each time and sample. CO_2 values were fitted through a positive Gompertz equation, reparameterised by Zwietering et al. (1990) and Bevilacqua et al. (2013a) and cast in the following form:

$$CO_2 = (CO_2)_0 + (CO_2)_{max} \cdot \exp\left\{-\exp\left\{\left[\left(k_{max} \cdot 2.71\right) \cdot \frac{\lambda - time}{(CO_2)_{max}}\right] + 1\right\}\right\}$$

where: $(CO_2)_0$ and $(CO_2)_{max}$ (v/v) are respectively the initial and the maximum contents of CO_2 in the head space; k_{max}, the rate of production of CO_2 in the exponential phase (CO_2/h); λ (h), the time before the beginning of the production of CO_2; time is the independent variable, i.e. time of sampling.

2.3.2 Acidification and metabolism in model systems with flour

Malt extract from Oxoid, and soft wheat, rice and Khorasan wheat (Kamut) flours were purchased from a local supplier and singly used for the preparation of cereal extracts. Briefly, 2 g of each substrate was mixed with 98 ml of distilled water and filtered under vacuum (pore size 0.45-µm; Sigma-Aldrich, Milan, Italy). After that, appropriate amounts of filtered extracts were added to 20 ml of distilled water in order to obtain culture media with three different cereal concentrations (5, 10 and 15% w/w). Immediately after yeast inoculation (6-7 log cfu/ml), the samples were stored at 4°C to assess periodically pH and viable count.

Data from pH were modelled as pH decrease referred to the starting of the experiments and used to run a multifactorial ANOVA, using the kind of medium and yeasts as independent factors.

2.3.3 Cell viability and acidification in the rice drink

The rice drink was inoculated with yeasts (ca. 8 log cfu/ml), and the samples (30 ml) were stored at 4°C and 25°C for 40 days to assess periodically pH and viable cell counts. The analyses were done in duplicate over two different batches. Aliquots of rice drink, not inoculated with the microbial targets, were used as negative controls.

2.4 Screening on lactic acid bacteria

2.4.1 Cell viability and pH in cereal-based media

The model systems with flours reported above were inoculated to 7.5-8.5 log cfu/ml with *L. plantarum* or *L. reuteri*, and incubated at 25°C (both the strains), 30°C (*L. plantarum*) or 37°C (*L. reuteri*) for 24 h to determine pH values. In addition, inoculated 15%-media were incubated at 4°C for 61 days to assess periodically viable cell count. All the analyses were performed in duplicate over two different batches. Aliquots of cereal media not inoculated with the microbial targets were used as negative controls.

2.4.2 Cell viability and pH in an organic rice drink

Rice drink was inoculated to ca. 8.8-9.0 log cfu/ml, and the samples were stored at 4°C (both the strains), 30°C (*L. plantarum*) or 37°C (*L. reuteri*) for 40 days to assess periodically pH and viable cell counts. The analyses were done in duplicate over two different batches. Aliquots of rice drink, not inoculated with the microbial targets, were used as negative controls.

2.5 Attenuation

2.5.1 HPH-treatment

An aliquot of rice drink (1 l) inoculated with either *L. plantarum* or *Kl. lactis* was processed in a pressure range of 50-100 MPa for 1 time or for 2-3 consecutive times through a high-pressure homogenizer PANDA 2K (Niro Soavi s.p.a., Parma, Italy) (**figure 4.7**). The circuits were cleaned with sterile distilled water (60-70°C) and cooled with sterile distilled water to obtain an exit temperature of the samples of 40°C. Untreated aliquots of rice drink, inoculated with the targets, were used as controls.

HPH-treated rice drink was used as a mother culture to inoculate fresh samples of rice drink (300 µl of mother culture in 30 ml of fresh rice drink); thereafter the samples were stored at 4°C or 30°C and analyzed periodically to assess the viable count of *L. plantarum* and *Kl. lactis*, pH, lactic acid, and ethanol. The analyses were performed in duplicate over two different batches.

2.5.2 Ultrasound-treatment on *L. plantarum* and *Kl. lactis*

Rice drink inoculated with either *L. plantarum* or *Kl. lactis* was processed for 4 min (pulse set to 2 s) through a VC Vibra Cell Ultrasound (US) equipment, model VC 130 (Sonics and Materials Inc., Newtown, CT, USA) (**figure 4.8**); the power was set to 60-100% (single treatment) or to 80% (2 or 3 consecutive treatments). Before each treatment, the ultrasonic probe was washed with sterile distilled water; immediately after processing, beverage was cooled in ice. US-treated rice drink was used to inoculate fresh samples of rice drink, as reported above; the samples were stored at

4°C and analyzed by assessing cell count, pH, and the content of lactic acid and ethanol. The analyses were done in duplicate.

2.5.3 Confirmation of US-attenuation on *L. casei* and *B. animalis*

Aliquots of rice drink (30 ml) inoculated with either *L. casei* LC01 and *B. animalis* BB12 (ca. 9 log cfu/ml) were US-treated for 2 times (power, 80%; time, 4 min; pulse, 4 s). These aliquots were used as mother cultures to inoculate fresh samples of rice drink, as reported above. The samples were stored at 4°C for 11 days and analyzed to assess cell count, pH, lactic acid, and sensory scores. The analyses were performed twice over two independent samples.

2.5.4 Effect of a thermal abuse on US-attenuation

Aliquots of rice drink inoculated with *L. casei* (9 log cfu/ml) were US-treated for 2 times (power, 80%; time, 4 min; pulse, 4 s) and used to inoculate fresh samples of rice drink, as reported above. Thereafter, the samples were stored as follows:

1. 4°C
2. 4 h at 25°C and then at 4°C
3. 4 h at 37°C and then at 4°C
4. 24 h at 25°C and then at 4°C
5. 24 h at 37°C and then at 4°C.

The samples were periodically analyzed (pH, viable count, lactic acid); the assays were performed over two different batches.

2.5.5 β-glucan added rice drink inoculated with attenuated probiotics

US-attenuated *L. casei* and *L. plantarum* (2 cycles- power, 80%; time, 4 min; pulse 2 s) were inoculated into samples of rice drink added with 1% of β-D-glucans from barley (Sigma-Aldrich, Milan, Italy; product code G6513-5G, CAS 9041-22-9); the following samples were prepared and analyzed:

1. Uninoculated rice-drink (control);
2. Rice drink added with glucans;
3. Rice drink inoculated with either *L. plantarum* or *L. casei*;
4. Rice drink inoculated with either *L. plantarum* and *L. casei*, attenuated by US;
5. Rice drink added with glucans and inoculated with *L. plantarum* or *L. casei*;
6. Rice drink added with glucans and inoculated with either *L. plantarum* and *L. casei*, attenuated by US.

The samples were stored at 4°C and analyzed periodically for the evaluation of viable count, pH, lactic acid, glucans, and sensory scores. The experiments were performed in duplicate.

2.6 Determination of pH and viable count

Viable count assessment was done on MRS agar (37°C for 48-72 h under anaerobic conditions) and on YPG agar (25°C for 48-72 h) for lactic acid bacteria and *Kl. lactis*, respectively. pH measurements were performed through a pH-meter Crison 2001 (Crison Instruments, Barcelona, Spain) (**figure 4.9**), previously calibrated with 2 standard solutions at pH 4 and 7.02. The results were reported as pH decrease

referred to the initial value (ΔpH) and modeled through a modified Gompertz equation as follows:

$$y = \Delta pH_{max} \exp\left\{-\exp\left[\left(\frac{d_{max} e}{\Delta pH_{max}}\right)(\alpha - time)+1\right]\right\}$$

where:

ΔpH_{max} is the maximal extent of acidification (maximal decrease of pH within the running time), **d_{max}** acidification rate (ΔpH/h) and **α** (h) time before the beginning of acidification.

2.7 Lactic acid, ethanol and β-glucans

The concentrations of lactic acid (g/l) and ethanol (% v/v) were evaluated through a Hyperlab automatic multi-parametric analyzer (Steroglass, San Martino in Campo, PG, Italy) (**figure 4.10**) by means of an enzymatic kit. Glucan content was determined through a commercial kit by Megazyme (Bray, Ireland).

2.8 Sensory scores

The sensory test was conducted using the approach proposed by Luckow and Delahunty (2004a, 2004b); 15-20 untrained assessors (students and researchers from University of Foggia; 23-50 years old), analyzed the different samples, immediately after the addition of the target microorganisms and within the storage at 4°C. They were requested to give a score for colour, odour and overall acceptability from 0 (bad) to 5 (very good), being 2 the break point for acceptability. Finally they were requested to answer this question: "Would you buy this product?" Consumers could

choose three possible answers: “definitely would buy”, “maybe buy/maybe not buy”, “definitely would not buy”.

The results of sensory scores were reported as score decrease referred to control (rice drink without microorganisms or glucans); the results were analyzed through the non-parametric test of Friedmann ($P<0.05$).

2.9 Statistical analysis

Data were analyzed thorugh one way or two way ANOVA, using Tukey’s test as the *post-hoc* comparison test ($P<0.05$). Data analysis and fitting were performed through the software STATISTICA for Windows (StatSoft, Inc., Tulsa, OK, USA; software version 10.0.1011.0).

Chapter 3. RESULTS AND DISCUSSION

3.1 CO_2 by yeasts in model systems

The starting point of this research was the article of Bevilacqua et al. (2013a); they proposed a headspace gas analysing approach for the evaluation of the level of *Pseudomonas* spp. in milk. The method is based upon the fact that pseudomonads consume O_2 and produce CO_2, and these changes can be easily evaluated. In this project, the amount of CO_2 produced by four yeast strains was assessed in two different laboratory media (YPG broth containing glucose, and Malt Extract broth, with maltose) or in diluted Malt Extract broth, at 15 and 25°C. The target yeasts were selected on the basis of some beneficial effects on human health reported in the literature such as probiotic activity (*S. cerevisiae* var. *boulardii*), improvement of bioavailability of minerals (*S. pastorianus* and *K. exigua*) and folate biofortification (*Kl. lactis*) (Moslehi-Jenabia et al., 2010).

Data were fitted through a common primary model (Gompertz equation) and three fitting parameters were pointed out: lag phase or time of metabolic adaptation (λ), rate of production of CO_2 in the exponential phase of metabolism (k_{max}) and the maximum concentration of CO_2 in the head space ($(CO_2)_{max}$). Model fitted the experimental data very well, as shown by R^2 values (0.978-0.999) (**table 4.5**); *S. cerevisiae* var. *boulardii*, *S. pastorianus* and *K. exigua* showed the highest values of $(CO_2)_{max}$ (ca. 60%) in YPG broth at 25°C. Otherwise, the parameters k_{max} (2%/h) and λ (7.39 h), as well as $(CO_2)_{max}$ (36%), suggested an attenuated metabolism in *Kl. lactis*. At 15°C the highest value of $(CO_2)_{max}$ was observed for *Kl. lactis* (68.55%),

which also showed the lowest value for k_{max} (1.39%/h). No differences were found for the fitting parameter λ.

S. cerevisiae var. boulardii and *S. pastorianus* showed the highest $(CO_2)_{max}$ (ca. 40%) in Malt Extract broth, although the amount of the gas was lower than in YPG broth. On the other hand, *Kl. lactis* and *K. exigua* experienced lower values of $(CO_2)_{max}$ (ca. 19%); *K. exigua* showed the lowest value of k_{max} (4.24/h), too.

At 15°C *S. cerevisiae* var. *boulardii* produced the highest concentration of CO_2 (23.32%) followed by *S. pastorianus* and *K. exigua* (14.92-13.88%, respectively), and finally by *Kl. lactis* (7.74%).

The fitting parameters $(CO_2)_{max}$ was analysed through two-way ANOVA and the factor "strain" exerted a strong effect (**figure 4.11A**); moreover maltose caused an attenuation of metabolic response (**figure 4.11B**), probably related to microbial inability to fully utilize this sugar (Romano et al., 2006). Some additional interactive effects of temperature x substrate were also observed (**figure 4.11C**).

Figure 4.12 shows the evolution of CO_2 in the headspace of vials containing Malt Extract broth diluted to 15%; this medium was used as a model system to simulate a beverage containing a low amount of sugars. At 25°C the yeasts attained the maximum concentration of CO_2 after ca. 40 h (13% in *S. cerevisiae* var. *boulardii* and *S. pastorianus* and 6% in *Kl. lactis* and *K. exigua*). The lag phase was 6 h for *S. cerevisiae* var. *boulardii* and *K. exigua* and 15 h for *Kl. lactis* and *S. pastorianus*. Yeasts experienced similar trends at 15°C, although the differences for the lag phase (ca. 12-13 h) were not significant ($P>0.05$).

3.2 Acidification and viability of yeasts in model systems with different flours and in an organic rice drink

Cereal fermentation processes depend on specific determinants, which have to be strictly controlled to get standardized and acceptable products; the type of flour is one of the most important determinant. It affects fermentation through the level and type of fermentable carbohydrates, nitrogen sources and growth factors (Pontonio et al., 2014). Therefore, yeasts were preliminarily studied for their ability to grow in four cereal-based substrates (malt extract, soft wheat, rice and kamut flours); a fast acidification was considered as a discriminant technological property. **Table 4.6** shows the statistical effects of two-way ANOVA on pH decrease after 24 h; yeast and kind of substrate were used as input factors. **Figures 4.13A-D** shows the acidification profile.

Both the factors (targets and substrate) were significant, as well their interaction; namely, *K. exigua* and *S. pastorianus* experienced the highest acidification after 24 h (1.22 and 0.75, respectively), since they are respectively a typical sourdough-specific yeast species (De Vuyst et al., 2014) and a lager beer strain (Nuobariene et al., 2011), indeed high adapted to cereal-based environments. Moreover, yeasts attained after 24 h a cell count of 7.0-7.8 log cfu/ml (data not shown).

An important trait for the selection of suitable strains is their persistence and viability (Tripathi and Giri, 2014), particularly for cereal-type beverages that would traditionally be stored at room temperature (Marsh et al., 2014). Thus the viability of target yeasts in a commercial rice drink was assessed for 40 days both at 25 (room temperature) and 4°C (refrigeration) (**figure 4.14**); yeast count was ca. 7 log cfu/ml,

thus the targets fulfilled the basic requirement of a minimal level of useful microorganisms in food (Corbo et al., 2014b).

A topic of great concern in food applications relies upon the cell viability of many probiotics during refrigerated storage (Zhang et al., 2014). The results from this study showed that rice constituents had a positive effect on the survival of target yeasts throughout the cold storage; moreover, the storage temperature was not significant.

This effect was also found on lactic acid bacteria (*Lactobacillus plantarum* and *Lactobacillus reuteri*), inoculated in the same rice beverage, thus suggesting that some undefined compounds could exerted a protective effect against cell aging. Nevertheless, further studies are required to confirm this hypothesis.

Finally, we focused on the acidification, modeled through a modified Gompertz equation as it could be described by a sigmoidal trend, with three different steps: an initial phase when acidification did not occur (α), a tumultuous metabolic step, and a final steady state (ΔpH_{max}).

At 25°C yeasts reduced pH by 2.5-2.7 over 15 days; acidification occurred mainly within 5 days with a reduction of pH by 2-2.1. A further reduction was found for 5-10 days and then the pH attained a steady state (**figure 4.15A**). Under refrigeration, *Saccharomyces* spp. and *Kl. lactis* experienced a similar trend with a ΔpH_{max} of 1.2-1.5 and acidification occurred within 15-20 days. The trend by *K. exigua* was quite different, with a significantly higher acidification rate within 2-3 days (0.87 ΔpH/day *vs* 0.12-0.20 ΔpH/day for the other strains), followed by a steady state after 5 days (**figure 4.16B**).

3.3 Screening on lactic acid bacteria: acidification and viability in model systems with flour and in the organic rice drink

L. plantarum strain 12, previously selected due to its acidification and persistence in model dough (Corbo et al., 2014a) and *L. reuteri* DSM 20016, a promising bacterium for its probiotic traits, including the ability to compete with some pathogens and give some benefits to the host (Sriramulu et al., 2008), were used as microbial targets. Some researchers studied a range of parameters including the ability to perform fast acidification, produce antimicrobial compounds, dominate the indigenous microbiota, and improve the nutritional quality of final products to select promising starter strains for cereal-based foods (Edema and Sanni, 2008). The focus of this paper was quite different, as we propose a simple approach (modeling of kinetic of acidification, evaluation of viable count throughout storage in different model systems and in an organic rice beverage) as a preliminary step to study the suitability of two lactobacilli as possible starter/probiotic cultures for cereal-based beverages.

The decrease of pH after 4 and 24 h (ΔpH) by *L. plantarum* and *L. reuteri* was used an input to run a multifactorial ANOVA, using temperature and kind of medium as the main factors. **Figure 4.16A** shows the acidification experienced by *L. plantarum* after 4 h; both temperature and kind of extract, as well as their interaction, played a significant role. On the other hand the concentration of the extracts in the model system did not exert any significant effect, with some exceptions to this generalized statement. Concerning the effect of temperature, we could pinpoint two different classes of samples: malt extract and rice, with no or slight differences due to temperature, and khorasan and wheat flour, where *L. plantarum* was not able to perform acidification at 25°C and showed an active metabolism only at 30°C (ΔpH

of 1.1-1.2 in khorasan wheat and 1.7 in soft flour). The highest acidification was found in malt extract (ΔpH, 2.0-2.5).

After 24 h the effect of temperature was significant and the decrease of pH was more pronounced at 30°C; in addition, *L. plantarum* experienced the highest ΔpH in malt extract, as reported above, and the differences amongst the other extracts (rice, khorasan, soft flour) were slight or not significant.

L. reuteri showed different trends both after 4 and 24 h; after 4 h (**figure 4.17A**) the bacterium experienced better performances at 25°C rather than at 37°C and this effect was strong for rice extract (ΔpH not significant at 37°C and ca. 2.2 at 25°C). A slight difference was recovered also for malt extract (ΔpH of 2.0 at 37°C and 2.4-2.5 at 25°C).

Acidification was not significant in khorasan, whilst the fall of pH was 0.7-0.9 in soft flour incubated at 37°C. The extent of acidification by *L. reuteri* increased up to 2.5-3.0 after 24 h (**figure 4.17B**), with some differences in wheat flour due to temperature.

Apart from acidification another interesting trait to choose the best strain could be the evaluation of its viability throughout storage; we focused on the systems containing 15% of the different extracts. **Figure 4.18** reports the viable counts of the target bacteria throughout 61 days of storage at 4°C; cell count decreased throughout the storage for both the strains, but the extent of this decrease was strongly strain dependent and relied upon the kind of extract.

In the figure we set a threshold level to 7 log cfu/ml, following the recent trend on the minimum level of probiotic bacteria in food (Rosburg et al., 2010). Some representative sampling points were chosen: 15 days (intermediate storage for many

active drinks), 25 days (shelf life for many probiotic beverage), 61 days (prolonged storage).

L. plantarum fulfilled the requirement of the critical level in all the extracts after 25 days, whilst after 61 days (end of the experiment) the viable count was higher than the critical break point only in the model systems with rice extract. *L. reuteri* showed a more pronounced death kinetic, with an acceptable count after 25 days in rice and malt extract (respectively 8.4 and 7.84 log cfu/ml), whereas at the end of the experiment cell count was below the threshold in all the samples (4.68 log cfu/ml in rice extract, 4.59 log cfu/ml in malt extract, 3.05 log cfu/ml in khorasan flour, and 3.41 log cfu/ml in wheat flour).

Why using *L. plantarum* or *L. reuteri*? *L. reuteri* is an emerging probiotic, proposed for some non-dairy foods and beverages (Perricone et al., 2014). Generally, the use of probiotic bacteria as starter cultures is a compromise between the potential health benefits and the technological robustness of the strains, mainly acidification and persistence in food (Kedia et al., 2007). A possible challenge could be cell viability of many probiotic bacteria during refrigerated storage, as it is often insufficient thus limiting their usefulness in food applications (Zhang et al., 2014).

On the other hand, *L. plantarum* is an ubiquitous species and the strain used in this study was isolated form an Italian sourdough and showed interesting technological traits (Corbo et al., 2014a). Generally *L. reuteri* showed some interesting traits in terms of acidification in some extracts (rice, malt), but the limiting element was its viability. On the other hand, *L. plantarum* fulfilled both the requirement for a robust starter: acidification and prolonged cell viability; this result was probably due to the high acid tolerance of this species (Plumed-Ferrer et al., 2008).

Some interesting results were found for the different extracts; the best systems for cell viability and acidification were malt and rice extracts. This result confirms some literature reports on the ability of rice to support the growth of beneficial bacteria (Subhasree et al., 2013), and on the stimulating effect of malt extract (Patel et al., 2004; Rathore et al., 2012), probably due to the simultaneous presence of considerable amounts of monosaccharides (glucose and fructose) and disaccharides (maltose). As a final step screening both *L. plantarum* and *L. reuteri* were inoculated in an organic rice drink incubated at 4, 30 and 37°C (**figure 4.19**). Cell count of both the targets was always higher than 7 log cfu/ml, thus they fulfilled the basic requirement of a probiotic in a food, as reported above; the storage temperature was not significant. This effect was quite strange and unexpected, as well as the strong tail effect of lactobacilli within the storage.

There are some studies that have focused on the role of specific food components and have indicated several factors that are likely to influence cell viability, i.e. food structure, components increasing the buffering capacity, the levels of fat and dietary fibers and the addition of fruit preparations (Charalampopoulos and Pandiella, 2010). Indeed, it could be suggested that some undefined compounds of organic rice drink could exert a protective effect against cell aging. Another possible idea could be the starvation; rice drink contains a low amount of glucose and we could assume that it was completely consumed within few days. The main other carbon source was starch and both *L. plantarum* and *L. reuteri* possess a weak amylolytic activity; therefore, after consuming the available glucose, they probably entered a kind of physiological stress known as starvation and it is well known that this situation could induce a

prolonged viability (Bevilacqua et al., 2010b). However, further studies are required to confirm this hypothesis.

Figure 4.20 shows the acidification kinetics; pH decrease followed a characteristic sigmoidal trend, quite similar to the classic growth curve modeled by Gompertz equation. This kinetic could be divided into three different steps: an initial phase when acidification did not occur (α), a tumultuous metabolic step, and a final steady state (ΔpH_{max}). Both *L. plantarum* and *L. reuteri* showed a low "α" (few hours) and attained the steady state after 3-4 days, with a ΔpH_{max} of ca. 2.0 at 4°C and 2.7-3.0 at 30-37°C.

3.4 Attenuation by physical methods: effect on metabolism, viability and sensory scores

3.4.1 Screening step: homogenization and US as single passes

Following the results of the screening on yeasts and lactic acid bacteria, *Kl. lactis* and *L. plantarum* were selected as promising strains to be inoculated in a rice-drink; however, they caused post-acidification at 4°C and 30°C. Thus, this last step of my PhD project reports on the optimization of a physical approach to attenuate these microorganisms without affecting their viability throughout storage.

First, homogenization was studied; a single homogenization pass at 50 MPa did not affect post-acidification, whilst a multiple-pass treatment (**figure 4.21**) exerted a significant effect on the parameter ΔpH_{max} (maximal extent of acidification) of *L. plantarum* which was reduced from 1.5 (control) to 0.8 (multiple treatments). The attenuation also influenced the parameter α (time before the beginning of acidification), which was prolonged to 4.50±0.28 days by using 2 or 3 step

treatments; HPH did not affect cell viability within the storage (data not shown). On the other hand, a single HPH-pass at 100 MPa was able to control post-acidification by *Kl. lactis* (data not shown). Homogenization was a promising treatment, however its main drawback relies upon the fact that the conditions to achieve attenuation are too restrictive (high pressure or multiple passes).

Afterwards, we moved on US and studied both single and multiple pass treatments; US at 80% avoided post-acidification by *Kl. lactis* (data not shown), whilst the effects on *L. plantarum* were significant, although the result was not a complete depletion of post-acidification (**figure 4.22**); a single pass at 80 and 90% reduced ΔpH_{max} from 2.7 (control) to 1.5. On the other hand a single pass at the maximum level of power prolonged α to 9.65±2.57 days (in the control it was 1.08±days).

Figure 4.23 confirms that US-attenuation did not negatively affect their viability; moreover, the analysis of lactic acid by *L. plantarum* confirmed the attenuation with a decrease of this compound from 90 mg/l in the control to 60 mg/l after 80% US-treatment, 10 mg/l at 90% and at the undetectable level at 100% (**figure 4.24**).

3.4.2 US-confirmation and multiple pass treatments

The suitability of US as a tool for the attenuation of lactic acid bacteria and yeasts was confirmed through the use of multiple pass treatments and later by using this approach on two commercially available probiotics (*L. casei* LC01 and *B. animalis* subsp. *lactis* BB12). **Figure 4.25** shows the acidification kinetics of rice beverages inoculated with attenuated *L. plantarum* after a single or a multiple pass treatments at 80%. The use of 2 or 3 repeated passes at 80% completely depleted pH decrease; this results was also confirmed by the amount of lactic acid at the end of storage (**figure**

4.26), which was reduced both for *L. plantarum* (from 105 mg/l in the control to 35 mg/l after 3 passes) and *Kl. lactis* (from 60 mg/l at the undetectable level). As aforementioned cell viability of *L. plantarum* was not affected, whilst a 3-pass treatment significantly reduced the initial count of the yeast (from 9.08 log cfu/ml to 7.10 log cfu/ml) (**figure 4.27**).

This approach was confirmed on *L. casei* and *B. animalis* by using a 2 pass-treatment; acidification was reduced for both the targets and **figure 4.28** shows the results for *L. casei*. In the control the microorganism decreased pH by 2.7, while attenuation avoided post-acidification for at least 7 days and the pH at the end of the experiment was decreased by 1. The evaluation of lactic acid (**figure 4.29**) confirmed the inhibition of end-product formation, with an amount of this compound of 16.25 mg/l for *L. casei* and 8.25 mg/l for *B. animalis* (in the controls the amounts were respectively 593.75 mg/l and 26 mg/l).

3.4.3 US-attenuation and sample storage under thermal abuse conditions

3rd part of this research was focused on the storage of inoculated beverage under thermal abuse conditions to mimic a possible scenario throughout food handling: food distribution under not correct conditions. The starting question of this last step was the following one: can US-attenuation control post-acidification by probiotics at room temperature? Thus, the samples were inoculated with US-attenuated *L. casei* and stored in different conditions (refrigeration; 25°C for 4 or 24 h and then refrigeration; 37°C for 4 or 24 h and then refrigeration); 25°C was used as a control temperature (room temperature) whilst a temperature of 37°C mimics summer in Southern Italy. **Figure 4.30** reports actual pH in the samples inoculated with

attenuated *L. casei* and in the control; data were not modeled as pH decrease with the modified Gompertz equation as the experiments were performed only on three sampling points (immediately after the inoculum, and after 2 and 7 days of storage). In the samples with no attenuated cultures, the storage at 25 or 37°C caused a strong decrease of pH before refrigeration up to 4.42 or 3.72; when stored for 4 h, the initial pH was not affected, but acidification throughout storage was more pronounced than in the control (sample always stored at 4°C).

US-attenuation could not avoid post-acidification in the samples stored at 25 or 37°C for 24 h. On the other hand, a short thermal abuse (4 h) did not affect US attenuation; in fact, when the samples were stored at 25 and 37°C for only 4 h, pH did not undergo any significant changes within the storage at 4°C. Cell count was not affected (data not shown).

These samples were also used for a panel test; **figure 4.31** shows the sensory scores modeled as difference referred to uninoculated rice drink. The results were put in a box-whisker plot and analyzed through a non-parametric test, because a preliminary statistic revealed that they did not follow a normal trend. Namely, the worst scores were found for the inoculated samples of the groups D and E, stored at 25 and 37°C for a prolonged time; for the samples of the group A (storage at 4°C), B (storage for 4 h at 25°C and then at 4°C) or C (storage for 4 h at 37°C and then at 4°C) the differences were not significant, thus confirming that both *L. casei* and attenuation did not exert a negative effect on the sensory scores.

3.4.4 US attenuation and samples with glucans

The focus of the last step of this research was the combination of lactic acid bacteria and glucans; the microorganisms were attenuated as reported above. As expected, US-attenuation controlled the acidification of the beverage for at least 7 days and the final pH was 6.0-6.5 for both the rice drink and the rice drink with glucans (data not shown). Neither US attenuation nor glucans exerted a negative effect on cell viability or caused a strong viability loss; in fact, at the end of the running time cell count of both *L. plantarum* and *L. casei* was higher than the break-point for a probiotic in a food (7 log cfu/g or ml) (Rosburg et al., 2010) (data not shown). The data on lactic acid confirmed what previously reported, i.e. the amount of the acid was significantly lower (10-20 mg/l) or below the detection limit in rice drink with US-microorganisms (data not shown).

Figure 4.32 shows the amount of glucans in rice-drink; *L. casei* determined in the control sample a decrease of glucans from 1000 mg/l to 800 mg/l after 11 days, whilst the effect was not significant in US-attenuated culture. On the other hand, we could assume that the concentration of glucans was constant in the samples with *L. plantarum*, as the differences were not significant for the entire running time.

Finally, rice drink with lactic acid bacteria and glucans were evaluated for their sensory scores; **figure 4.33** shows the results after 11 days of storage. The non-parametric test of Friedmann found a significant difference in the values of score decrease with a strong fall in the samples inoculated with non-attenuated bacteria with or without glucans, whilst attenuation prevented the decrease of sensory scores and the median of score fall was 0.57-0.75. The same result was found in the negative control (inoculated rice drink).

3.4.5 Impact and significance of attenuation: possible effects on cells

Attenuation was proposed by Klein and Lortal (1999) as an alternative tool to achieve lactic acid bacteria unable to produce lactic acid, but still delivering active enzymes in cheese. They reviewed different approaches for attenuation (heat-treatment, freeze-thawing, spray and freeze-drying, lysozyme and other chemicals); however, some methods show a possible drawback, i.e. a significant effect on cell viability. Many other authors have used high pressure (Tabanelli et al., 2012, 2013a, 2013b; Upadhyay et al., 2007) or sonication (Doolan and Wilkinson, 2009) as alternative ways; the effects of these treatments were different and relied upon the kind of strain, and the conditions (pressure, time, pulsed treatment, power level).

Some papers focused on lactic acid bacteria intended for dairy products, proposed either as starter cultures or probiotic microorganisms, but to the best of our knowledge there are not references on the attenuation of starter cultures/probiotics for cereal-based drinks. In addition, little is known on the effects of yeast attenuation; we only found the paper by Patrignani et al. (2013), who studied the effect of homogenization on yeasts for sparkling wine.

The microbial targets of this research were selected in a preliminary phase for their viability throughout drink storage at 4°C (*L. plantarum*, strain 12) (Bevilacqua, data not published) or for the weak effect on sugar amount (*Kl. lactis*) (Casanova et al., 2014); unfortunately, they still retained an active metabolism in rice-drink and significantly decreased pH by producing lactic acid. First we studied homogenization; this kind of treatment can be applied at different pressure levels, either as a single or as a multiple pass-process (Bevilacqua et al., 2009); lactic acid bacteria are generally more resistant than yeasts, as they could be significantly

reduced by using 3 passes at 150 MPa (Bevilacqua et al., 2009, 2013b). Keeping in mind the basic principle of attenuation (exerting a significant effect on metabolism, without affecting cell viability), pressure was set to 100 MPa; as expected *Kl. lactis* was strongly affected by homogenization, as it retained viability but a single step inhibited glucose metabolism. On the other hand, this kind of treatment was not able to avoid post-acidification by *L. plantarum*, whereas a multiple step process showed a reversible effect with a reduction of ΔpH_{max} and a prolongation of the parameter α (time before the beginning of acidification). The effect of homogenization on glucose and acidification was also reported by Wouters et al. (1998) who suggested that homogenization could affect the enzymes active on phosphorilation or transportation. Casal and Gomez (1999) found an impaired acid production and viability of lactococci and lactobacilli treated at 400 MPa for at least 20 min. Other authors found an enhanced cell lysis, but this effect was strongly-strain dependent, as some targets did not show any change in the permeability of membrane (Malone et al., 2002).

Homogenization was a promising way, but multiple pass-treatments could exert a strong stress on cells, thus sonication was used in 2nd step of this research; the effect was studied towards *L. plantarum* and *Kl. lactis* and later confirmed on some commercial probiotics (*L. casei* LC01 and *B. animalis* subsp. *lactis* BB12). The effect of sonication on fungi and bacteria relies upon the acoustic cavitation and the diffusion of waves throughout a liquid medium (Sango et al., 2014). Moreover, there is a strong debate on a possible hit of susceptibility, as some authors reported that Gram positive bacteria are more resistant than Gram negative ones and bacilli are more resistant than cocci (Sango et al., 2014).

It is well known that sonication could act on cell membrane and enhance its permeability; this effect relies upon the power and the duration of the treatment. Zhou et al. (2014) used ultrasound as a tool to increase the yield in biomass towards photosynthetic bacteria of wastewater and found the optimal result after 2 min; a prolonged treatment time (10 min or more) caused a significant injury on the cell and determined a strong reduction in cell count. A positive effect of sonication on beneficial bacteria was also reported by Suckova et al. (2014); they used this approach to increase the yield of vitamin B12 produced by *Propionibacterium shermanii*. This effect was attributed to an increase in the permeability of membrane with an enhanced lactose metabolism.

On the other hand, Nguyen et al. (2009) recovered a stimulating effect on bifidobacteria, probably associated with an acceleration of lactose hydrolysis and transgalactosylation in milk (Nguyen et al., 2012). This effect on sugar metabolism was also evidenced by a significant change in acid profile, with a decrease of acetic and propionic acids (Nguyen et al., 2012).

To the best of our knowledge, this is 1st report on the use of sonication as a tool to reduce acidification (i.e. sugar metabolism) without affecting cell viability. Many authors reported that the primary targets of sonication are cell wall and membrane due to the external cavitation; moreover, US could cause an internal cavitation, responsible of enzyme activation or deactivation (Mawson et al., 2011). Molecular size and structure of enzymes could play a significant role in sensitivity/resistance to sonication, being large and less globular molecules the most sensitive (Vercet et al., 2001).

The results of attenuation suggest that US probably acted less on cell wall and membrane, as both lactic acid bacteria and *Kl. lactis* retained their ability to grow on plate, thus suggesting a slight or a not significant injury; on the other hand, the depletion of acidification for lactic acid bacteria and the low amount of ethanol for the yeast could suggest a significant effect of US on the enzymes involved in sugar metabolism. Further investigations are required to elucidate the molecular mechanisms and the target enzymes.

Apart from these theoretical ideas, from a practical point of view the results of this research suggest some key-points. Yeasts are more sensitive than lactic acid bacteria, as the attenuation of *Kl. lactis* could be achieved by a single step-treatment, whilst for lactic acid bacteria a multiple-step treatment is required. This result is directly connected to the susceptibility hierarchy to both homogenization and sonication: in fact, it is well known that larger cells show an increased sensitivity to these treatments (Diels and Michiels, 2006; Chandrapala et al., 2012).

Another key-element is the role of the supplied energy and this phenomenon is strong for US-attenuation; energy supplied by sonication is a function of power and kind of treatment (single or multi-step), pulse and duration. In this research, pulse and duration were set to constant values, whilst power varied from 60 to 100%; moreover, the target microorganisms were also treated using a single step or repeated passes. An increase of the supplied energy could lead to an increased extent of both external and internal cavitation, leading to a more pronounced effect on enzymes. In addition, short-term effects (i.e. depletion of acidification immediately after the treatment) were confirmed on three bacterial strains (*L. plantarum*, *L. casei* and *B. animalis*), although the long-term effects were quite different. For example, US-

attenuation through a 2-pass treatment was reversible for *L. casei* as it acquired an active metabolism after 7 days and showed an active acidification after 11 days (ΔpH=1). Finally, US-attenuation did no exert a negative role on the sensorial trait, as well as on the amounts of glucans added as healthy compounds in rice drink.

3.5 Conclusions

Cereal-based beverages represent a good substarte for probiotic and/or starter lactic acid bacteria and yeasts; the screening in the model systems with different flour, as well as the viability in the organic rice beverage showed that at least two strains (*L. plantarum* and *Kl. lactis*) retained their viability throughout storage.

A challenge is their active metabolism and the so-called "post-acidification", i.e. the decrease of pH within storage. Thus, a new way was used, i.e. attenuation.

L. plantarum and *Kl. lactis* were preliminary processed through homogenization (single or multiple passes) or sonication (US) and then inoculated in the rice drink; the samples were stored at 4°C and analysed to assess pH, production of lactic acid and ethanol, viable count and sensory scores. A single US-pass (power 80-100%) avoided post-acidification by *Kl. lactis*, whilst acidification by *L. plantarum* could be controlled by a 2-step process (power, 80%); viability and sensory traits were never affected by sonication. This result was confirmed on two commercial probiotics (*Lactobacillus casei* LC01 and *Bifidobacterium animalis* subsp. *lactis* BB12).

In 2nd step samples inoculated with attenuated strains were also stored under thermal abuse conditions (25 or 37°C for 4 or 24 h, then at 4°C) and the results showed that US could control acidification for a short thermal abuse. Finally, US-attenuated

starter were inoculated in the rice drink containing β-glucans as healthy compounds; the targets did not cause any significant change of prebiotic.

Chapter 4. TABLES AND FIGURES

Table 4.1: Cereals used for the preparation of cereal-based beverages (Casanova et al., 2013).

Cereal	Beverages	Characteristics	Flavor
Wheat	Wheat beer; spirits distilled from grain	Alcoholic; contains gluten	Color/specific odor of the drink
Spelled	Spelled coffee; drink of spelled (with calcium)	It contains iron, calcium, B vitamins, good percentage of fat; gluten digestible; seed flour of carob as a thickener	Milky color, rustic odor
Khorasan Kamut®	Beverage of kamut	Plant proteins; contains gluten	Milky color, pleasant odor and taste
Rice	Rice drink (with calcium, vanilla or cocoa)	B group vitamins, digestibility	Milky appearance, pleasant odor, delicate flavor
Millet	Millet beer; drink millet	Iron and silicic acid, minerals, vitamin A and group B, fat; carob seed flour as a thickening agent	Milk color, pleasant aroma, sweet and delicate aroma of cereal origin
Rye	Coffee rye, kvass, kanne brottrunk, based drink rye, beer, spirits distilled from rye	Potassium and folic acid	Sweet flavor
Oats	Oat drink	Soluble dietary fiber, energetic with lipids and protein, B vitamins and minerals (phosphorus, sulfur and magnesium)	Delicate sweet taste, milky color
Barley	Beer, barley water, barley coffee, coffee malt and barley drink	Soluble dietary fiber (β-glucans)	Sweet taste, milky color
Corn	Corn beer, drink corn (from white corn)	Vitamin A	Traditional taste
Buckwheat, Amaranth, Quinoa	Drinks of pseudocereals (with agave juice)	Digestibility, fiber, B vitamins, minerals (calcium, iron and phosphorus) and unsaturated fats	Milky appearance, toasted flavor, sweet taste

Table 4.2: Some examples of commercially available dairy beverages (Holzapfel, 2006; Gürakan et al., 2010, Özer and Kirmaci, 2010; Soccol et al., 2012; and World Wide Web).

BRAND	PRODUCER	ACTIVE COMPOUNDS
	Probiotics	
Verum®	Essum AB, Sweden	*Lactococcus lactis* L1A, *Lactobacillus rhamnosus* LB21
Gaio®	MD Foods, Denmark	*Enterococus faecium*, *Streptococcus thermophilus*
Actimel®	Danone, France	*L. casei* Immunitas™
Vifit Drink®	Mona, The Netherlands	*L. casei* GG, *L. acidophilus*, *Bifidobacterium bifidum*
CHAMYTO®	Nestle, France	*L. johnsonii*, *L. helveticus*
Yakult®	Yakult Honsha Co, Japan	*L. casei* Shirota
Yakult Miru-Miru®	Yakult Honsha Co, Japan	*L. casei*, *B. bifidum* or *B. breve*, *L. acidophilus*
Cultura®	Arla Foods, Sweden	*L. acidophilus*, *B. bifidum*
Vitagen®	Malaysia Milk SDN. BHD, Malaysia	*L. acidophilus*, *L. casei*
ProCult Drink®	Müller, Germany	*B. longum* BB536, *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*
	Enriched beverages	
Heart Plus®	PB Food, Australia	Omega-3
Natrel Omega-3®	Natrel, Canada	Omega-3
Night-Time Milk®	Cricketer Farm, UK	Melatonin
Meiji Love®	Meiji Milk, Japan	Calcium and iron
Dairyland Milk-2-	Saputo, Canada	Calcium, omega-3, vitamins
Natural Linea®	Corporacion Alimentaria Penanata S.A., Spain	Conjugated linoleic acid
Benecol®	Mc Neil Nutritionals, UK	Phytosterol
Danacol®	Danone, France	Phytosterol
Zen®	Danone, Belgium	Magnesium
Evolus®	Valio Ltd., Finland	Bioactive peptides

Table 4.3: Functional beverages: the state of the art research.

PRODUCTS	ACTIVE COMPOUNDS	REFERENCES
Fortified-strawberry beverage	Polyphenols (rose petals, *Rosa damascena* Mill.)	Mollov et al. (2007)
Fermented carrot juice beverage	Prebiotics: inulin and fructooligosaccharides; Probiotics: *L. rhamnosus* DSM20711, *L. bulgaricus* ATCC 11842	Nazzaro et al. (2008)
Fortified fruit juice beverage	Prebiotics: fructooligosaccharides	Renuka et al. (2009)
Fiber-fortified dairy beverage	Fibers (soybean)	Chen et al. (2010)
Grape-based beverage with potential anti-hypertensive effect	Polyphenols (grape must); γ-amino butyric acid (*L. plantarum* DSM19463)	Di Cagno et al. (2010)
Fermented cereal drink	Fibers (oat, *Avena sativa*) Probiotics: *L. plantarum* ATCC 8014	Gupta et al. (2010)
Olive leaf extract-enriched fruit beverage	Polyphenols (olive leaf extract)	Kranz et al. (2010)
Fermented whey beverage	Proteins (whey); Probiotics: *L. acidophilus* CRL 636, *L. delbrueckii* subsp. *bulgaricus* CRL 656, *S. thermophilus* CRL 804	Pescuma et al. (2010)
Antioxidant red-colored beverage	Polyphenols (red-fleshed apples)	Rupasinghe et al. (2010)
Cereal-based alcoholic beverage	Exopolysaccharides (*W. cibaria* WC4); Proteins, fibers, vitamins and minerals (emmer grains, *Triticum dicoccum*); Probiotics: *L. rhamnosus* SP1; LAB: *L. plantarum* 6E	Coda et al. (2011)
Vegetable health-promoting beverage	Nitrogen-sulphur compounds, phenolics, minerals and vitamins (broccoli, *Brassica oleracea* L. var. *italica*); Favonols and flavanols (green tea extract)	Dominguez-Perles et al. (2011)
Peach-flavored yogurt drinks	Prebiotics: fructooligosaccharide; Probiotics: *L. acidophilus* Lafti-L10	Gonzalez et al. (2011)
Beverage with anti-inflammatory properties	Phenolic compounds, parthenolide (feverfew, *Tanacetum parthenium*)	Marete et al. (2011)
Milk beverage	Polyphenols (olive vegetable water); γ-amino butyric acid (*L. plantarum* C48); LAB: *L. paracasei* 15N	Servili et al. (2011)
Fruit and milk-based beverages	Plant sterols (tall oil, and soybean, rapeseed, sunflower and corn oils)	Alemany-Costa et al. (2012)

Antioxidant dairy-based beverage	Antioxidants (extract of oregano, *Origanum vulgare*; essential oil of oregano, *Origanum minutiflorum*)	Boroski et al. (2012)
Vegetable fermented beverage with hypocholesterolemic and hepatoprotective effect	Xanthines, polyphenols and other antioxidants (Herbal mate leaves, *Ilex paraguariensis* A.St.-Hil.); Probiotics: *L. acidophilus* ATCC 4356	Lima et al. (2012)
Vegetable drink	Favonoids (Maqui berry, *Aristotelia chilensis*) and vitamin C (lemon juice)	Gironés-Vilaplana et al. (2012)
Antioxidant beverage	Polyphenols (red grape, *Vitis vinifera* L.; elderberry, *Sambucus nigra* L.)	González-Molina et al. (2012)
Blended drink	Fibers, vitamins and minerals (cucumber, *Cucumis sativus* and muskmelon, *Cucumis melo*)	Kausar et al. (2012)
Fermented cereal-based probiotic drink	Proteins, fibers, vitamins and minerals (malt, barley); Probiotics: *L. plantarum* NCIMB 8826, *L. acidophilus* NCIMB 8821	Rathore et al. (2012)
Fortified blackcurrant juice	Polyphenols (crowberry, *Empetrum nigrum*)	Törrönen et al. (2012)
Antioxidant blended-beverage	Antioxidants (cocoa, i.e. *Theobroma cacao*; *Hibiscus*-flower-extract; ginger, *Zingiber officinale*)	Awe et al. (2013)
Whey-based prickly pear beverage	Minerals, proteins, free amino acids (prickly pear fruit of *Opuntia* spp.)	Baccouche et al. (2013)
Fermented sprouts buckwheat beverage	Antioxidants (buckwheat, *Fagopyrum esculentum* Moench) Probiotics: *L. plantarum* Prebiotics: inulin	Brajdes and Vizireanu (2013)
Fortified vegetable-beverage	Vitamins, minerals, polyphenols omega-3 fatty acids, proteins, digestible carbohydrates (whey, mango fruit)	Gad et al. (2013)
Cardio-protective fruit-based beverage	Ginger, amino acids, vitamins and minerals (available commercial products)	Gunathilake et al. (2013a)
Fruit-based beverage with hypolipidaemic effects	Ginger, amino acids, vitamins and minerals (available commercial products)	Gunathilake et al. (2013b)
Fortified fruit juice	Antioxidants (brewers' spent grain)	McCarthy et al. (2013)
Enriched fruit juice	L-citrulline (watermelon, *Citrullus lanatus* cv. *Motril*)	Tarazona-Díaz et al. (2013)
Natural, minimally processed plant-derived beverage	Antioxidants, phenolic compounds (sugar and red maple, *Acer saccharum* and *Acer rubrum*)	Yuan et al. (2013)
Alcohol-free beverage	Oligosaccharides and exopolysaccharides (*W. cibaria* MG1)	Zannini et al. (2013)

Beverage for reducing body fat accumulation	Antioxidants (coffee silverskin extract)	Martinez-Saez et al. (2014)
Beverage for cardiovascular protection	Phenolic compounds (Jabuticaba berry, *Myrciaria jaboticaba*)	Martins de Sá et al. (2014)
Fermented yougurt-like beverage	β-Glucans (oat flakes); Lactic acid bacteria: *L. plantarum* LP09	Nionelli et al. (2014)
Pomegranate fermented juice	Phenolic compounds (pomegranate, *Punica granatum* L.); *S. cerevisiae* Fermicru VR5 and acetic acid bacteria	Ordoudi et al. (2014)
Fruit juice-based beverage to attenuate blood glucose and insulin responses	Xanthan gum, barley β-glucan, guar gum, and konjac-mannan (available commercial products)	Paquet et al. (2014)
Vegetable juice for cardiovascular diseases, type II diabetes, and obesity	Antioxidants (vegetables, seeds and sprouts of germinated lentils and cowpeas); Lactic acid bacteria: *L. plantarum* VISBYVAC	Simsek et al. (2014)
Apple-based beverage with anti-diabetic properties	Secoiridoid glycosides (*Fraxinus excelsior* seed extract)	Varela et al. (2014)
Carbonated symbiotic milk-based beverage	Probiotics: *L. acidophilus* and *Bifidobacterium* spp. Prebiotics: inulin	Walsh et al. (2014)
Fermented soymilk-tea beverage	Polyphenols (tea), isoflavones (soy); Lactic acid bacteria and *Bifidobacterium* spp.: *S. thermophilus* ASCC 1275, *L. delbrueckii* ssp. *bulgaricus* ASCC 859 and *B. longum* CSCC 5089	Zhao and Shah (2014)
Peanut soy milk	Proteins (Peanut, soy); Probiotics: *L. rhamnosus* LR 32, *L. acidophilus* LACA 4; Lactic acid bacteria: *P. acidilactici* UFLA BFFCX 27.1, *L. delbrueckii* subsp. *bulgaricus* LB 340, *L. lactis* CCT 0360; *S. cerevisiae* UFLA YFFBM 18.03	Santos et al. (2014)
Fermented Pepper Leaves-based beverage	Antioxidants, phenolic compounds (pepper, *Capsicum annuum* L.); Lactic acid bacteria: *L. homohiochii* JBCC 25 and JBCC 46	Song et al. (2014)

Table 4.4: Microorganisms used in this research.

Strains	Growth conditions	Evaluation of viable count
Yeasts and LAB		
S. cerevisiae var. *boulardii* MYA-796 *Kl. lactis* x *S. cerevisiae* 6530 *S. pastorianus* 6033 *K. exigua* 4384	YPG broth, 25°C for 48 h	YPG agar, 25°C for 48-72 h
L. plantarum strain 12 *L. reuteri* 20016 *L. casei* LC01 *B. animalis* subsp. *lactis* BB12	MRS broth, 30/37°C for 48 h	MRS agar, 30/37°C for 48-72 h under anaerobic conditions

Table 4.5: Production of CO_2 in the headspace of sealed vials, containing YPG broth or Malt Extract broth at 25°C and 15°C (initial inoculum, 5 log cfu/ml). Fitting parameters of Gompertz equation ± standard error. $(CO_2)_{max}$, maximum concentration of CO_2 (%, v/v); k_{max}, maximum rate of CO_2 production (%/h); λ, time before the beginning of the exponential phase in CO_2 trend (h). For each parameter, letters indicate significant differences among yeasts (one-way ANOVA and Tukey's test, $P<0.05$) (A, *S. cerevisiae* var. *boulardii*; B, *Kl. lactis*; C, *S. pastorianus*; D, *K. exigua*).

25°C	**$(CO_2)_{max}$**	**k_{max}**	**λ**	**R^2**
YPG broth				
A	62.19±2.06b	5.08±0.28b	6.96±0.48a,b	0.996
B	36.11±1.64a	2.00±0.07a	7.39±0.46b	0.998
C	62.02±0.99b	5.21±0.15b	6.47±0.22a,b	0.999
D	64.21±1.65b	5.16±0.23b	6.36±0.34a	0.997
Malt Extract broth				
A	41.78±1.02b	3.18±0.12b,c	7.47±0.33b	0.999
B	19.08±1.55a	1.12±0.08a	8.41±0.77b	0.996
C	45.04±1.34b	2.91±0.09c	7.10±0.32b	0.998
D	18.44±0.80a	1.47±0.13b	4.24±0.51a	0.996
15°C				
YPG				
A	62.19±1.43a	2.91±0.34b,c	12.01±1.71a	0.998
B	68.55±4.05b	1.39±0.22a	13.11±3.52a	0.993
C	66.08±0.53a,b	2.34±0.09b	12.41±0.64a	0.999
D	66.79±1.10a,b	3.19±0.23c	16.53±0.75a	0.999
Malt Extract broth				
A	23.32±0.34c	/*	21.24±0.37a	0.999
B	7.74±0.57a	0.86±0.24a	20.54±1.24a	0.978
C	14.92±1.32b	0.83±0.99a	/	0.993
D	13.88±1.04b	0.82±0.17a	/	0.994

* Not significant.

Table 4.6: Two way ANOVA for the effects of yeasts and kind of medium on the decrease of pH after 24 h. SS, sum of squares; MS, mean sum of squares (SS/degree of freedom).

Effect	SS	Degree of freedom	MS	F	P
Intercept	5.51	1	5.51	2215.56	<0.01
Yeast	0.37	3	0.12	49.86	<0.01
Medium	1.83	3	0.61	245.56	<0.01
Yeast * medium	0.63	9	0.07	28.27	<0.01
Error	0.04	16	0.002		

Figure 4.1: Classification of cereal-based drinks (Casanova et al., 2013).

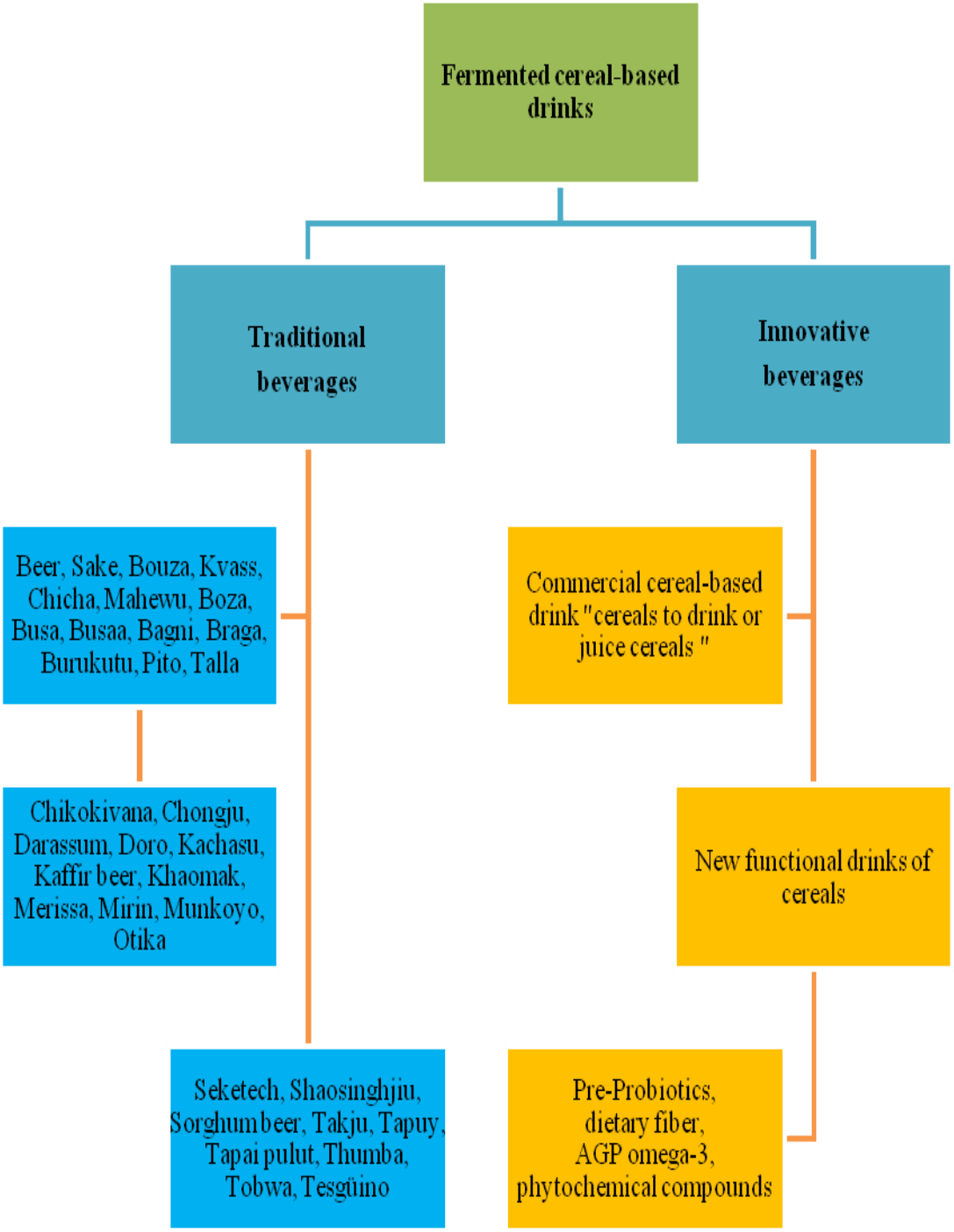


Figure 4.2: Simplified flow-chart for the production of cereal-based beverages (Casanova et al., 2013).

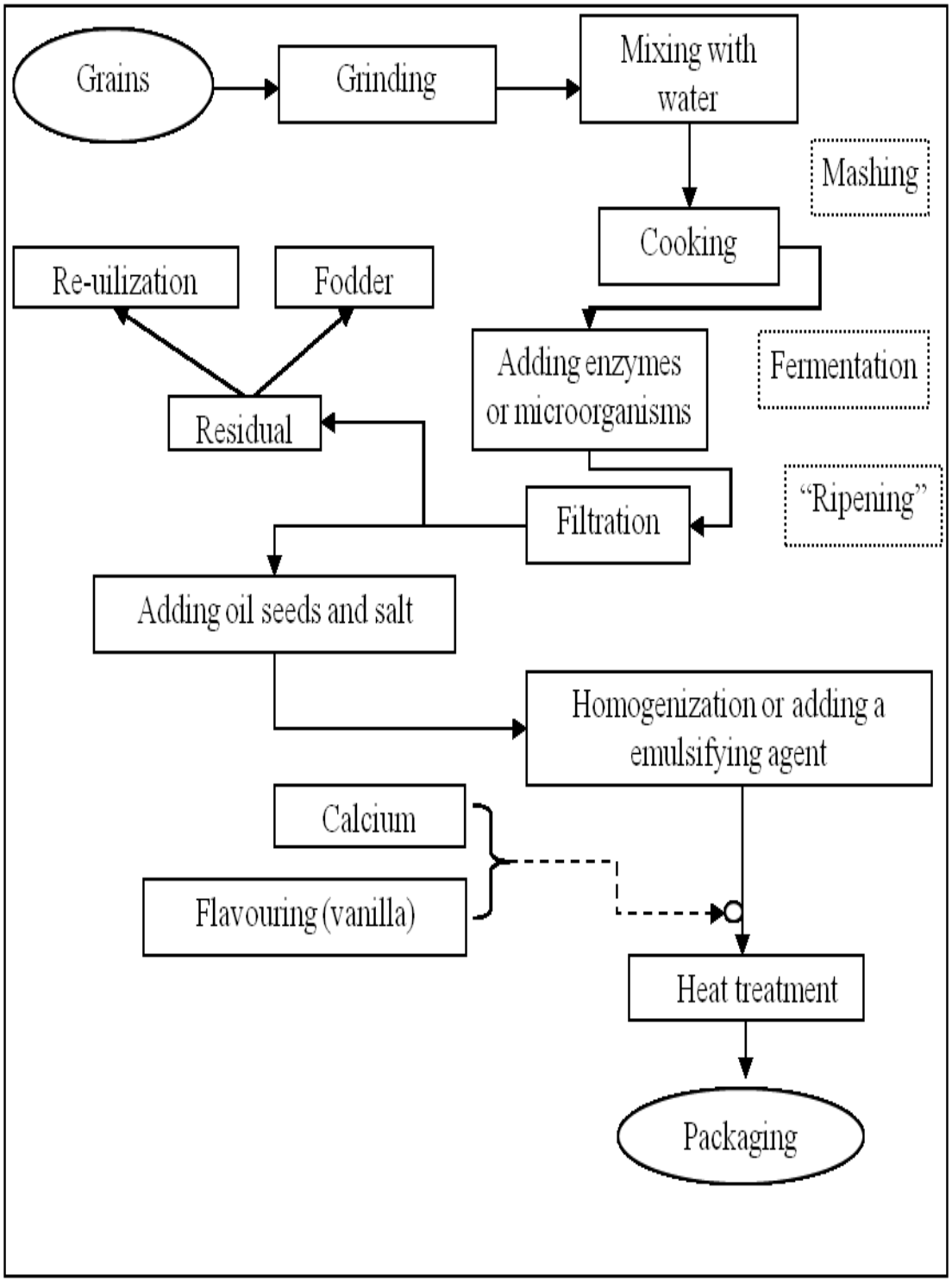


Figure 4.3: Classification of functional beverages.

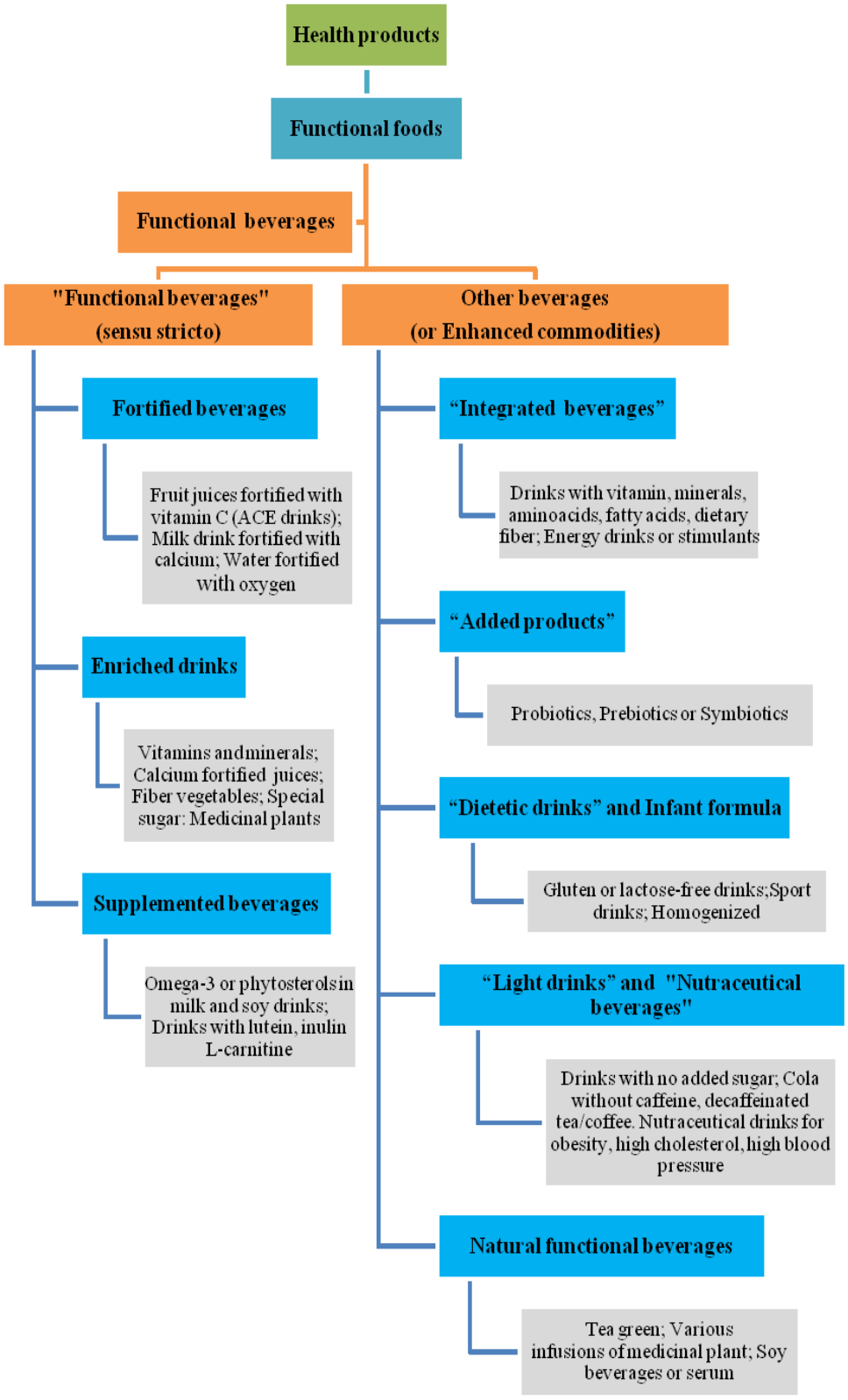


Figure 4.4: Active ingredients for functional drinks.

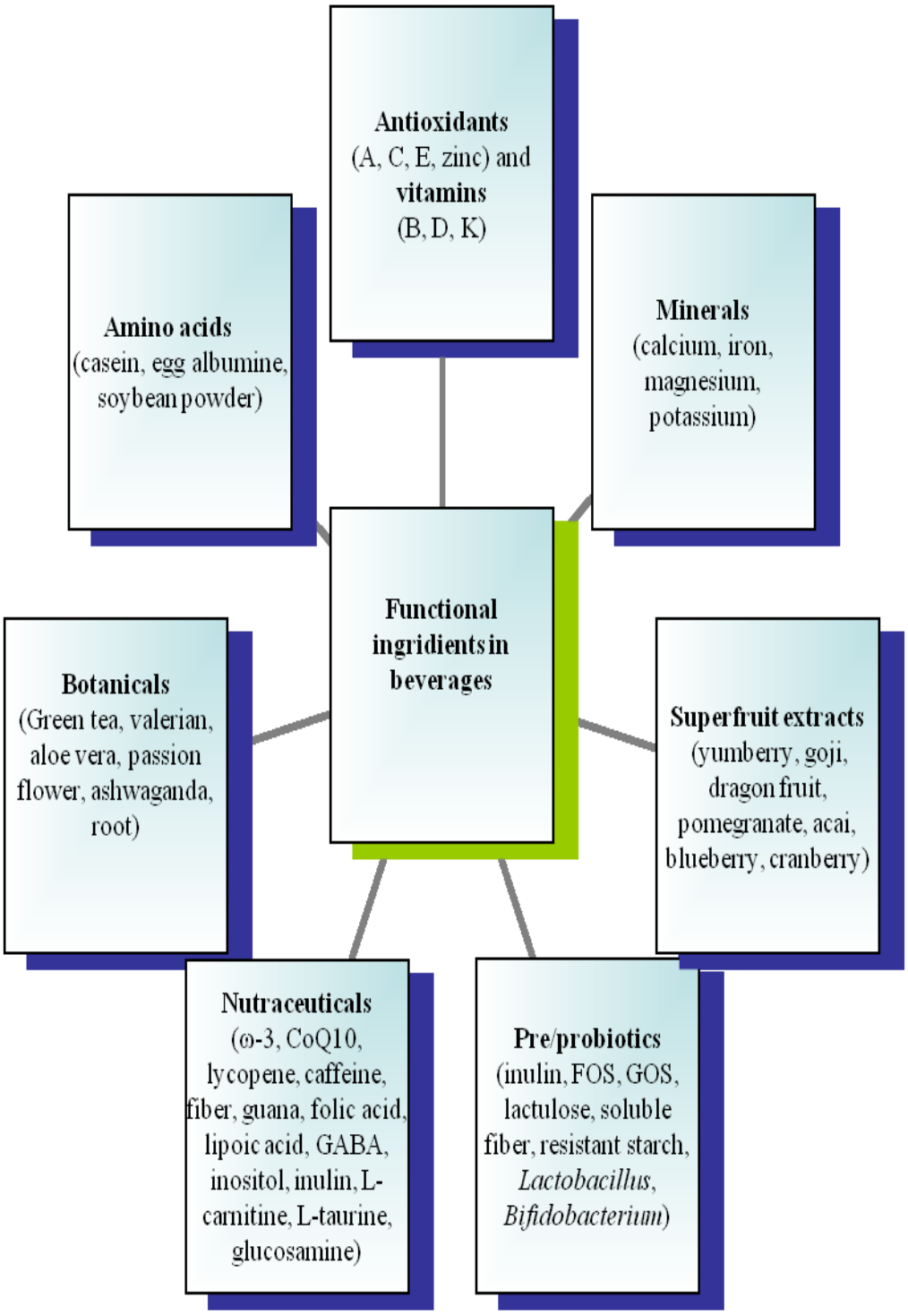


Figure 4.5: Glass vial used for the evaluation of CO_2 produced by yeasts.

Figura 4.6: Checkmate II PBI Dansensor. Gas-analyzer for the evaluation of CO_2 in the head space of sealed vials.

Figura 4.7: High-pressure homogenizer PANDA 2K.

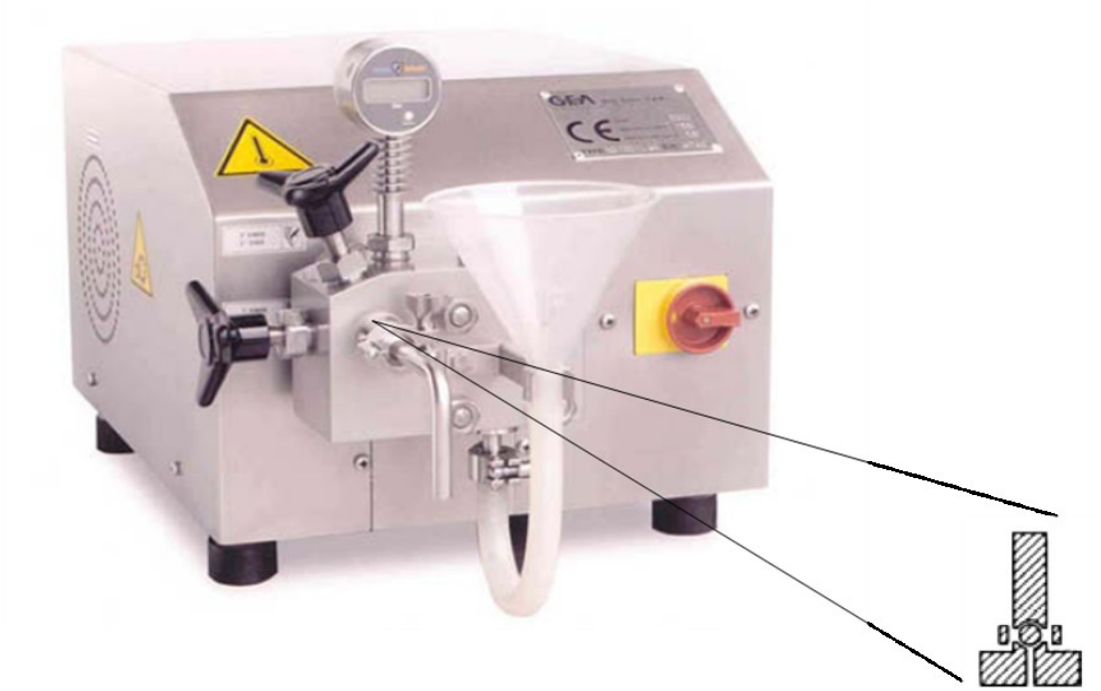
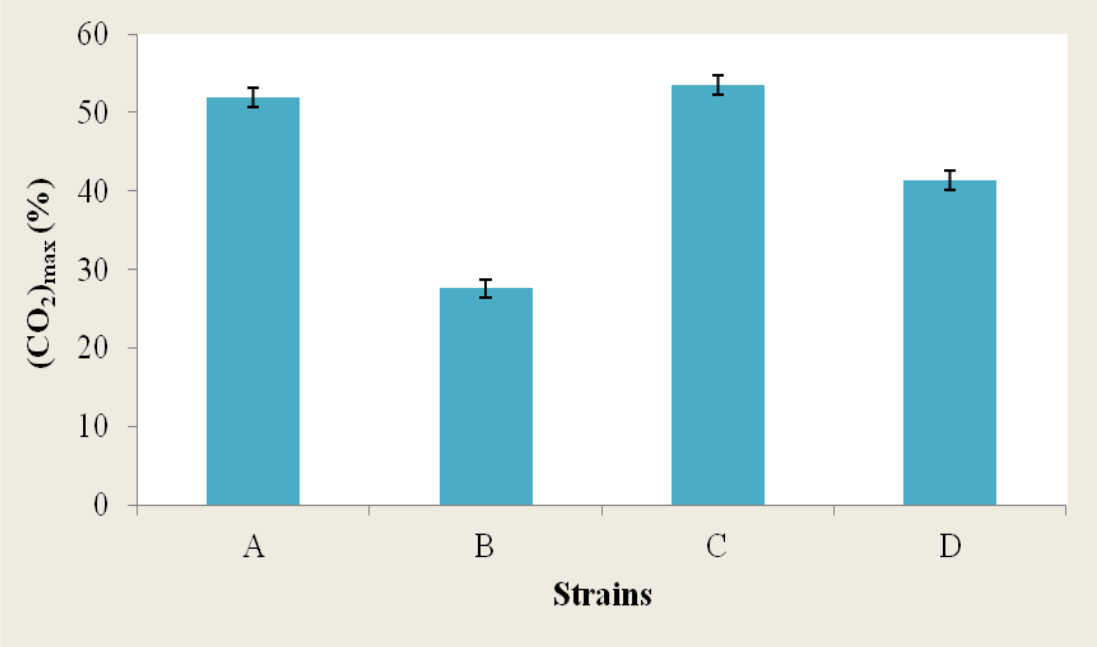


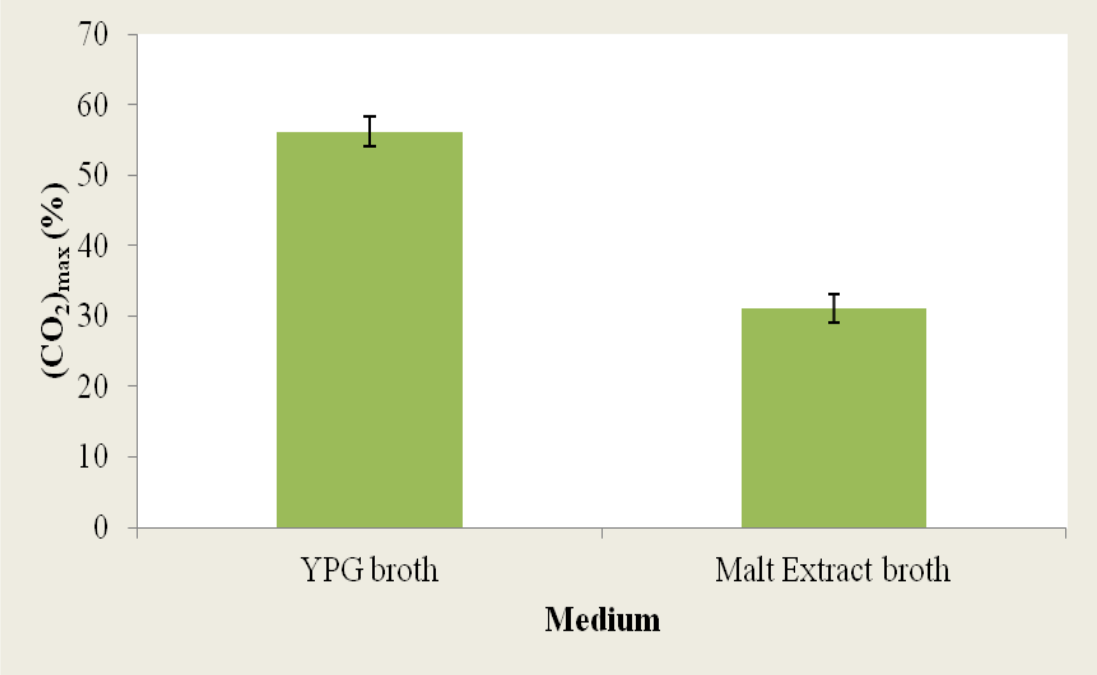
Figure 4.8: VC Vibra Cell Ultrasound (US) equipment, model VC 130.

Figure 4.9: pH-meter Crison 2001.

Figure 4.10: Hyperlab automatic multi-parametric analyzer.

Figure 4.11: Two-way ANOVA for the effects of strain (A), medium (B), and strain *vs* medium (C), on $(CO_2)_{max}$: graphs for the decomposition of the effects of the factors. Vertical bars denote 95%-confidence intervals. (A, *S. cerevisiae* var. *boulardii*; B, *Kl. lactis*; C, *S. pastorianus*; D, *K. exigua*).





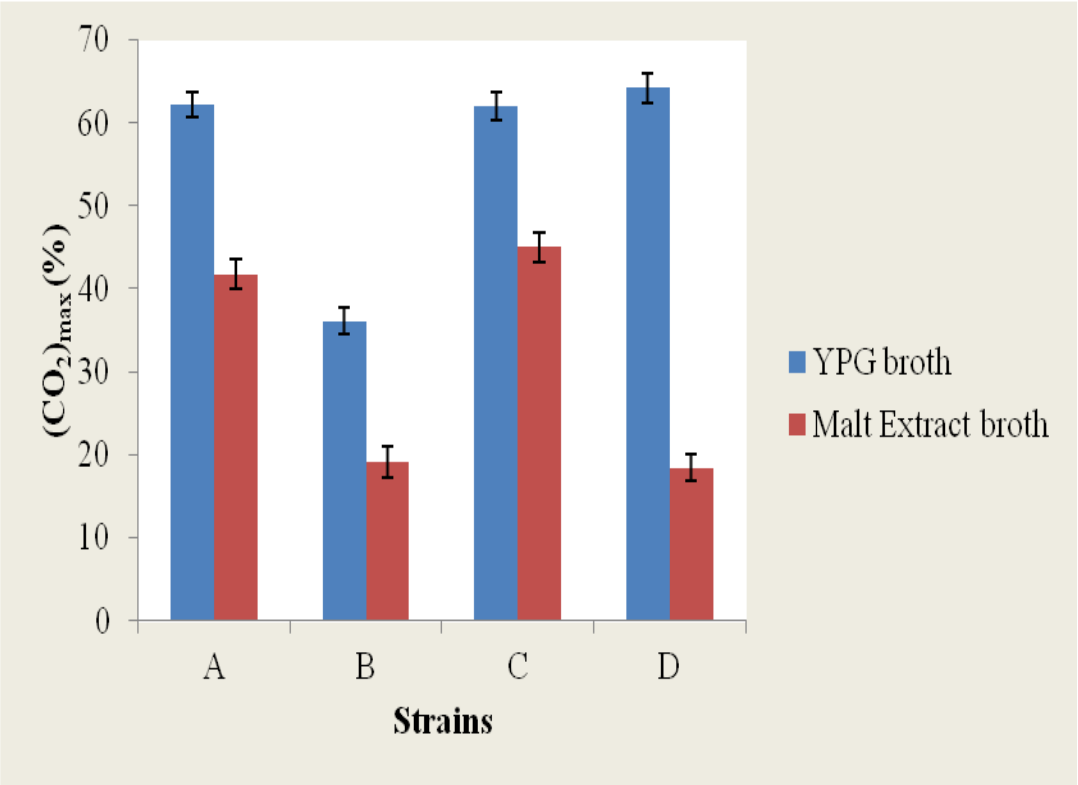

70
60
50
40
30
20
10
0
$(CO_2)_{max}$ (%)
A
B
C
D
Strains
YPG broth
Malt Extract broth

Figure 4.12: CO_2 production in 15% Malt Extract broth at 25°C (A) and 15°C (B). (A, *S. cerevisiae* var. *boulardii*; B, *Kl. lactis*; C, *S. pastorianus*; D, *K. exigua*). For each sampling points, both the replicates are shown.

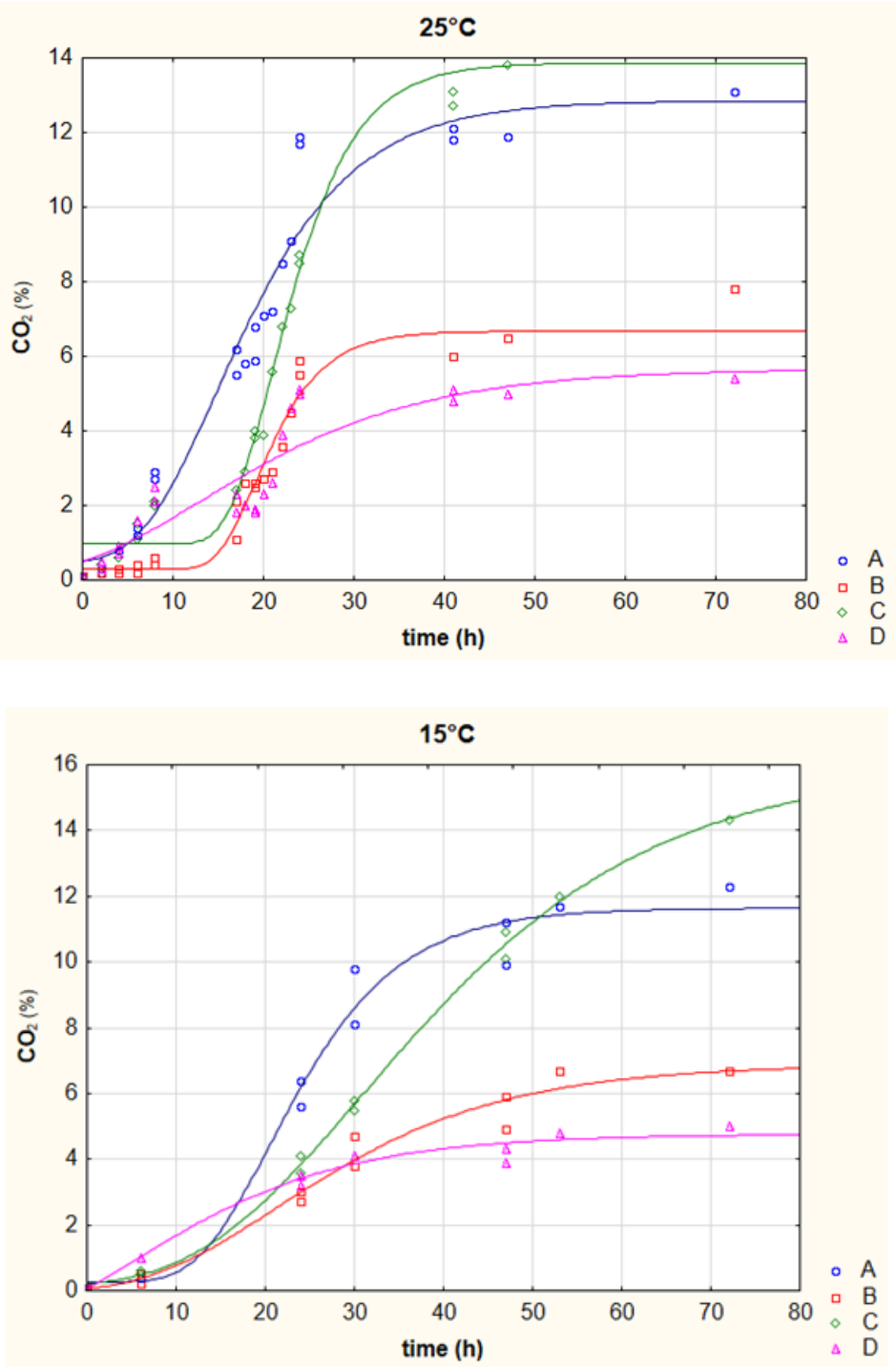
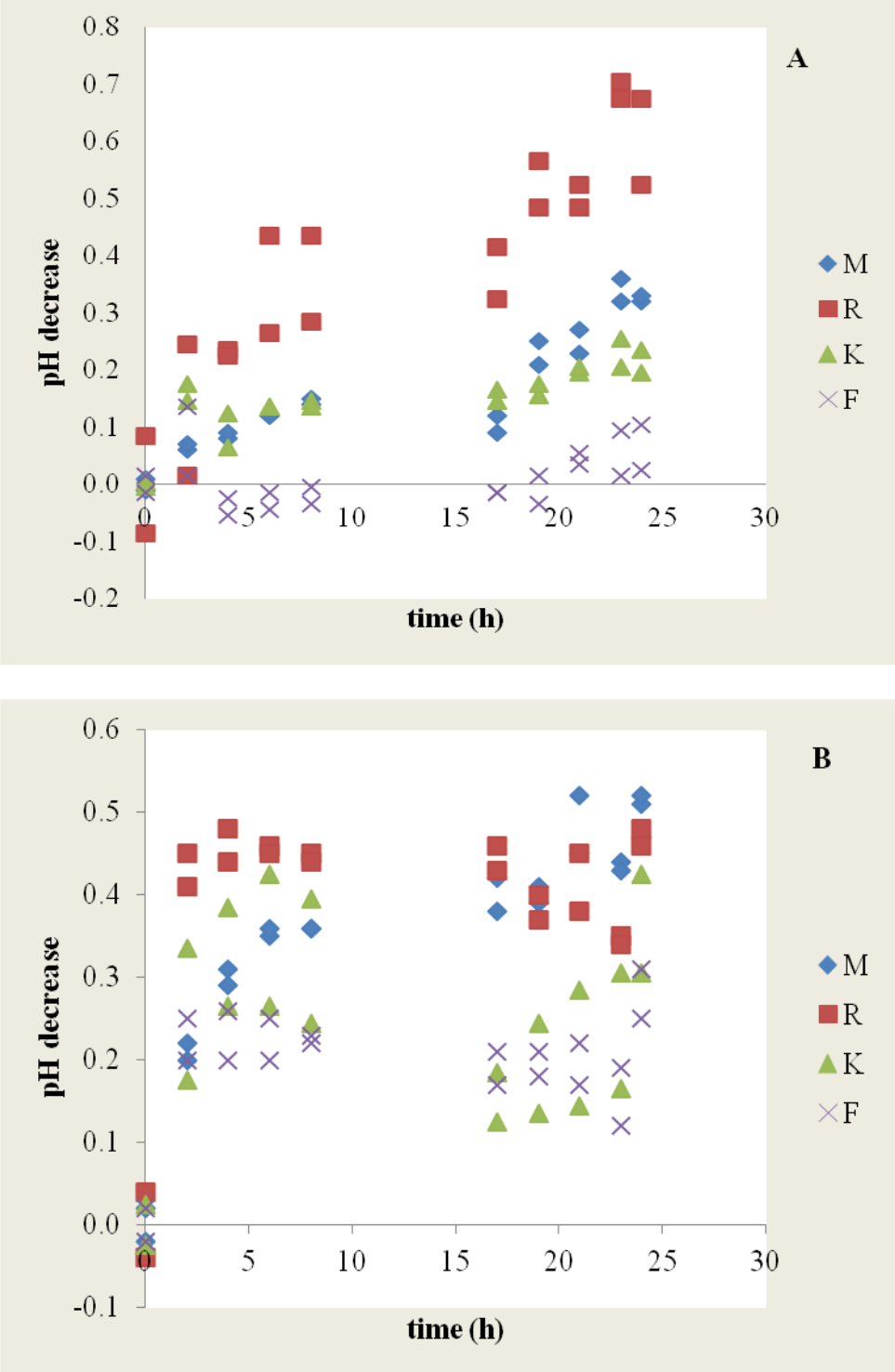
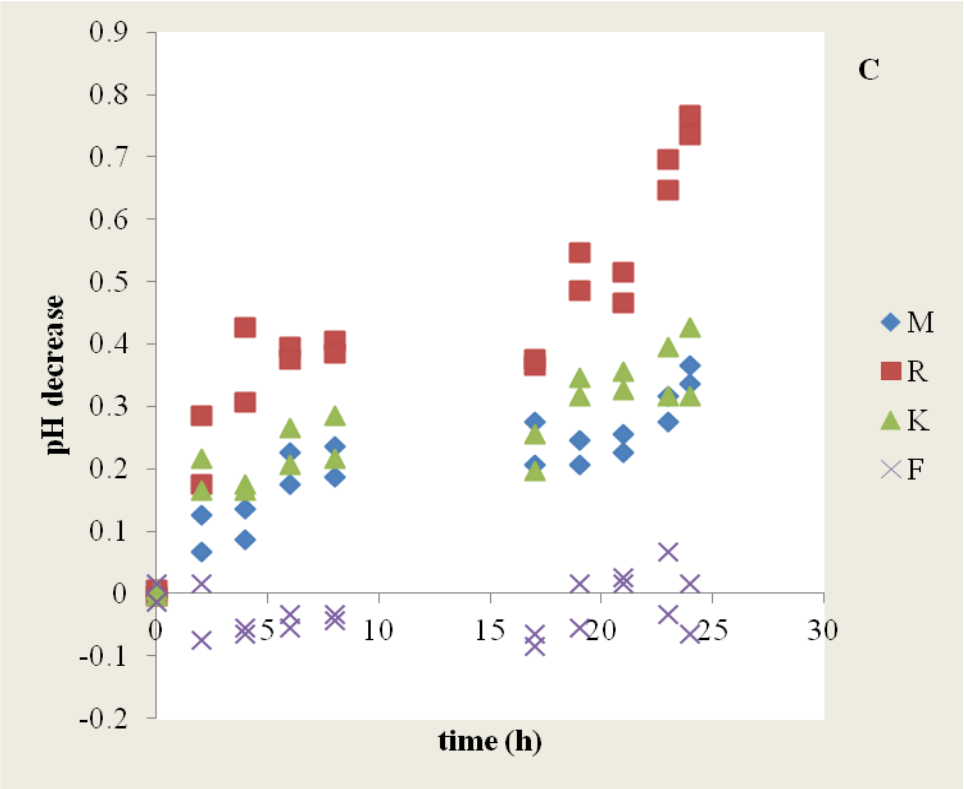


Figure 4.13: pH decrease in the lab media containing the different extracts. M, Malt extract; R, rice; K, khorasan; F, wheat flour. For each sampling points, both the replicates were reported. A, *S. cerevisiae* var. *boulardii*; B, *Kl. lactis*; C, *S. pastorianus*; D, *K. exigua*.




C
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
pH decrease
0
5
10
15
20
25
30
time (h)
M
R
K
F

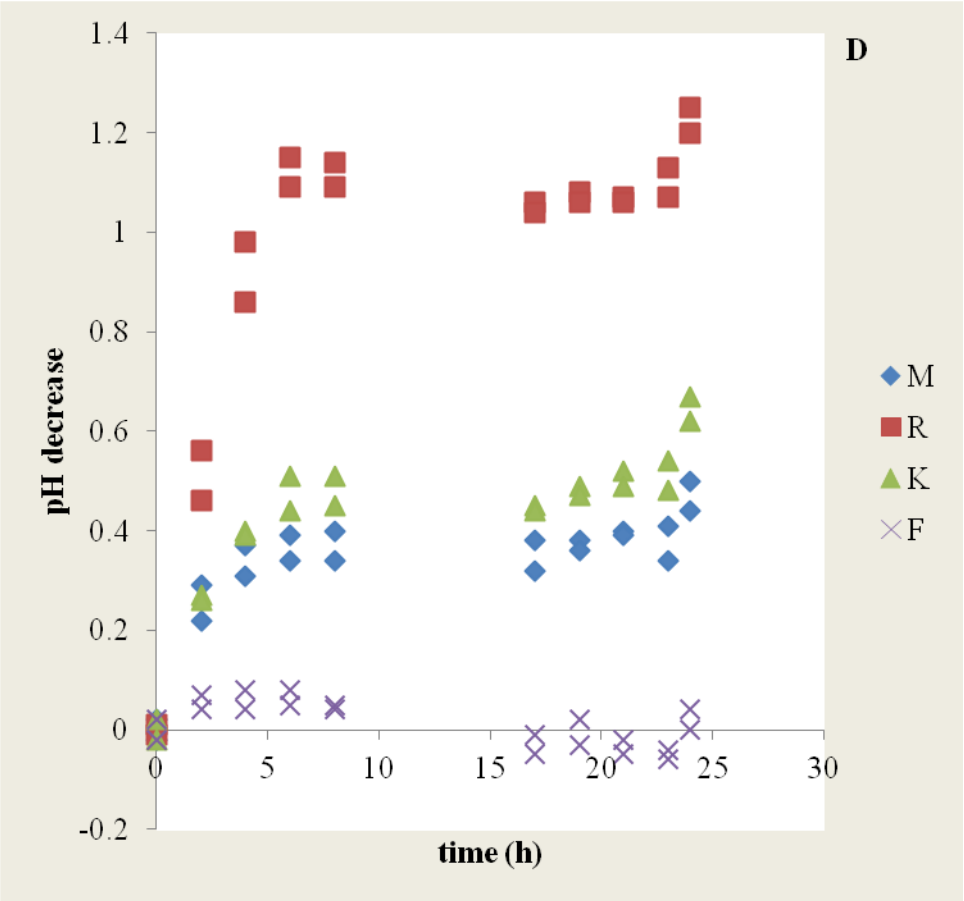
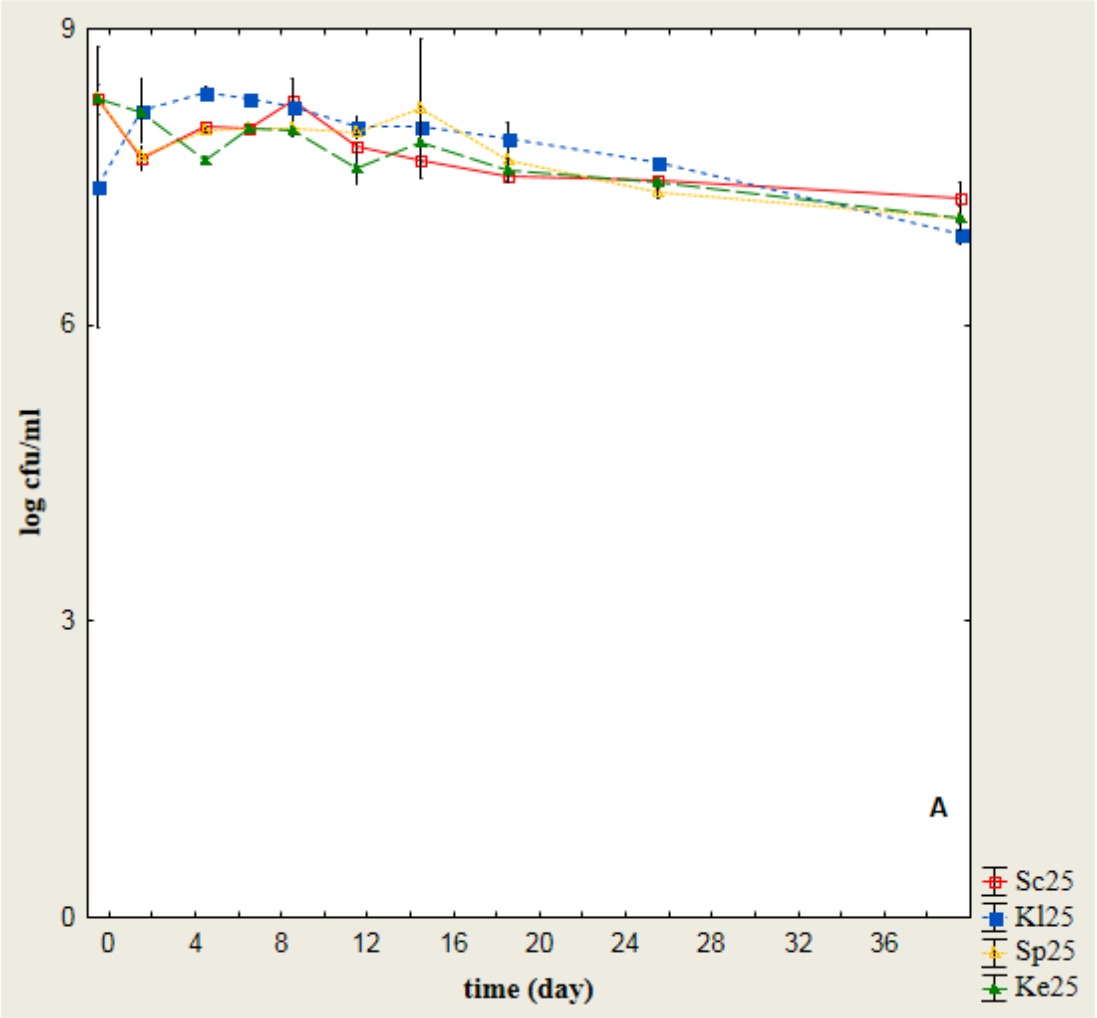

D
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
pH decrease
0
5
10
15
20
25
30
time (h)
M
R
K
F

Figure 4.14: Cell count of target yeasts in the commercial rice drink stored at 25°C (A) and 4°C (B) for 40 days. Mean values ± standard deviation. Sc, *S. cerevisiae* var. *boulardii*; Kl, *Kl. lactis*; Sp, *S. pastorianus*; Ke, *K. exigua*.



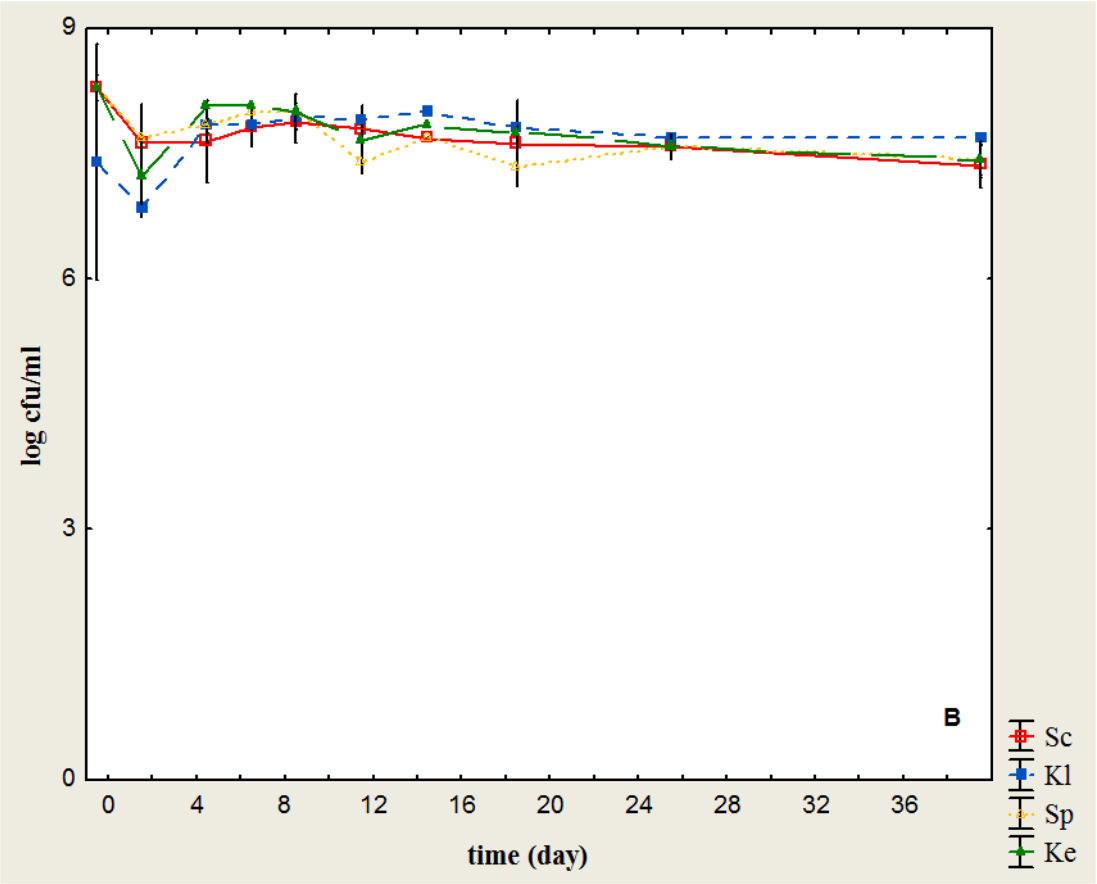
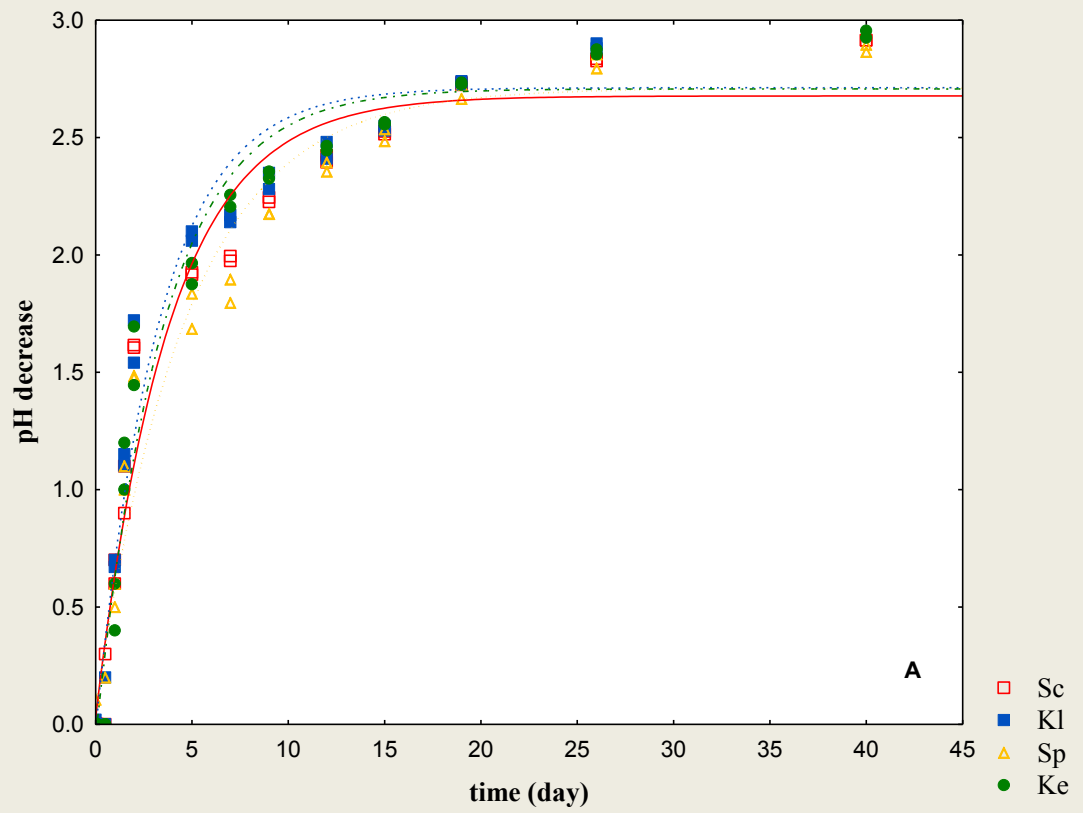

9
6
3
0
log cfu/ml
0
4
8
12
16
20
24
28
32
36
time (day)
B
Sc
Kl
Sp
Ke

Figure 4.15: Acidification profiles of target yeasts in the rice beverage stored at 25°C (A) or 4°C (B) for 40 days. For each sampling points, both the replicates were reported; lines represent the best fit through the Gompertz equation. Sc, *S. cerevisiae* var. *boulardii*; Kl, *Kl. lactis*; Sp, *S. pastorianus*; Ke, *K. exigua*.



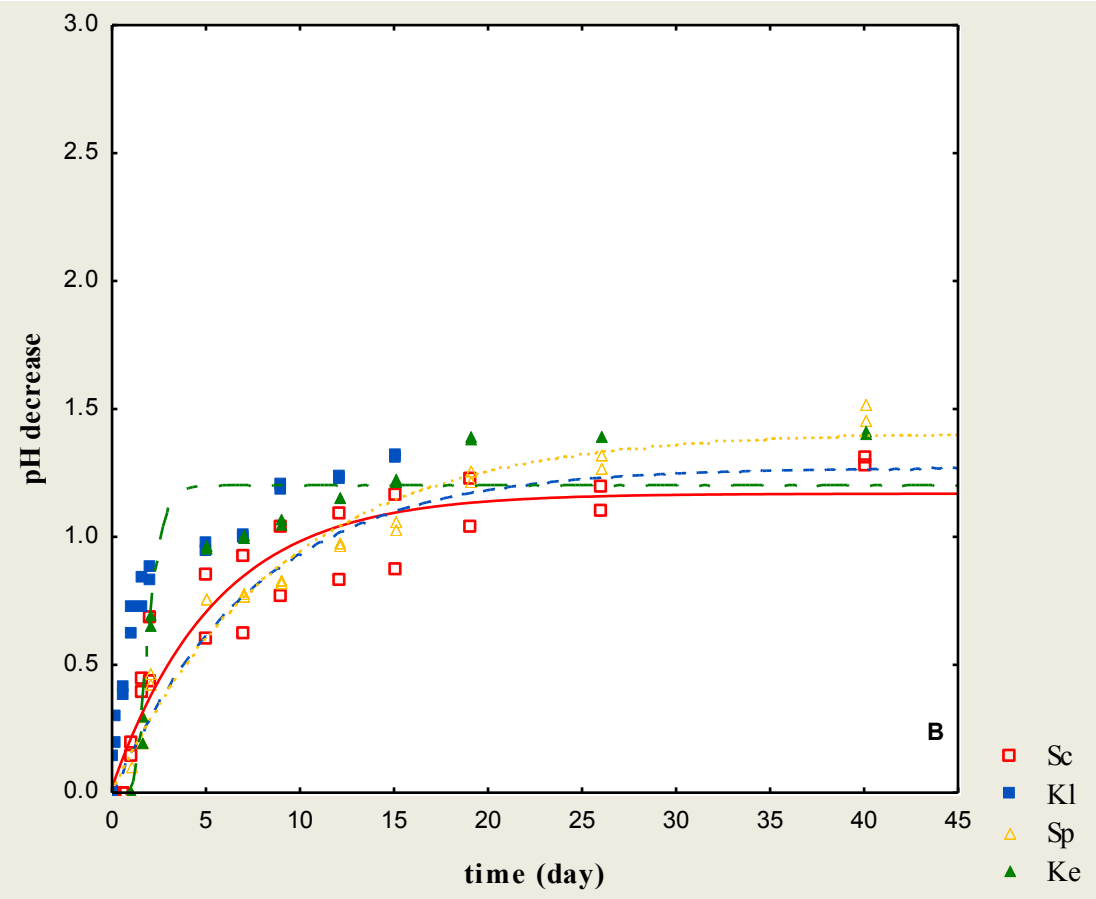
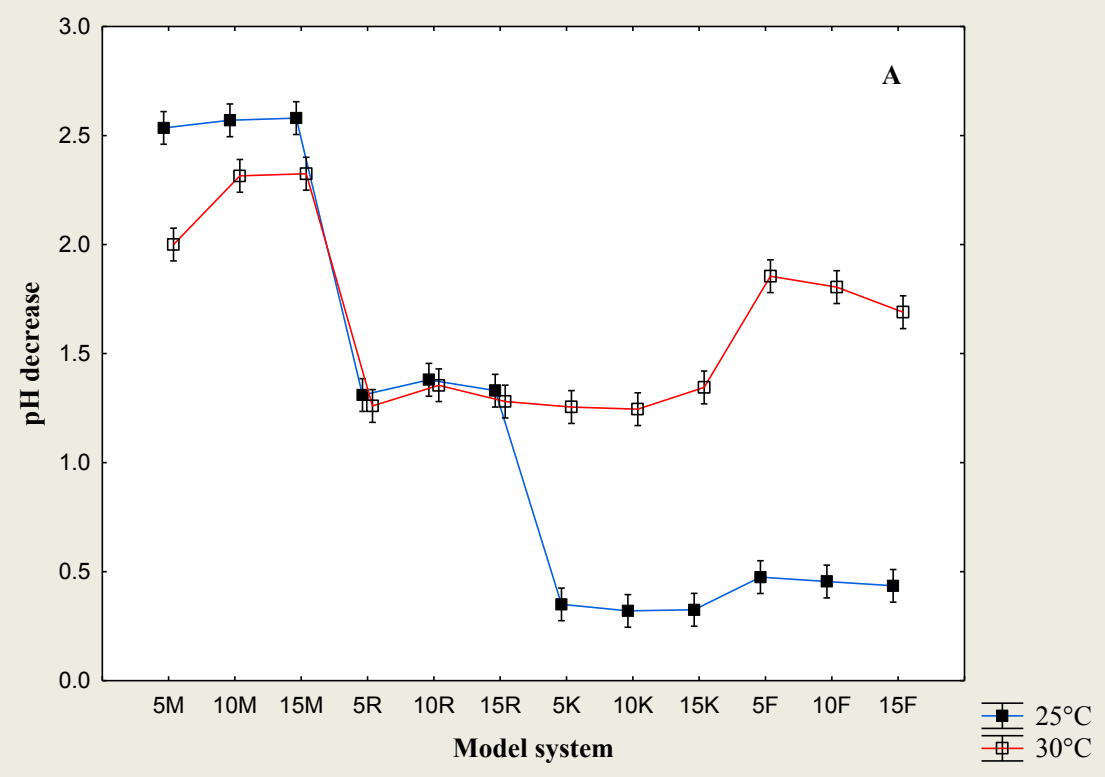

3.0
2.5
2.0
1.5
1.0
0.5
0.0
pH decrease
0
5
10
15
20
25
30
35
40
45
time (day)
B
Sc
Kl
Sp
Ke

Figure 4.16: Hypothesis decomposition for two-ANOVA on the acidification after 4 (A) and 24 h (B) by *L. plantarum* strain 12; the samples were stored at 25 and 30°C. The bars denote 95% confidence intervals. M, Malt Extract; R, Rice extract; K, Kamut; F, wheat flour. The number indicates the amount of the extract in the model system (5, 10, or 15%).



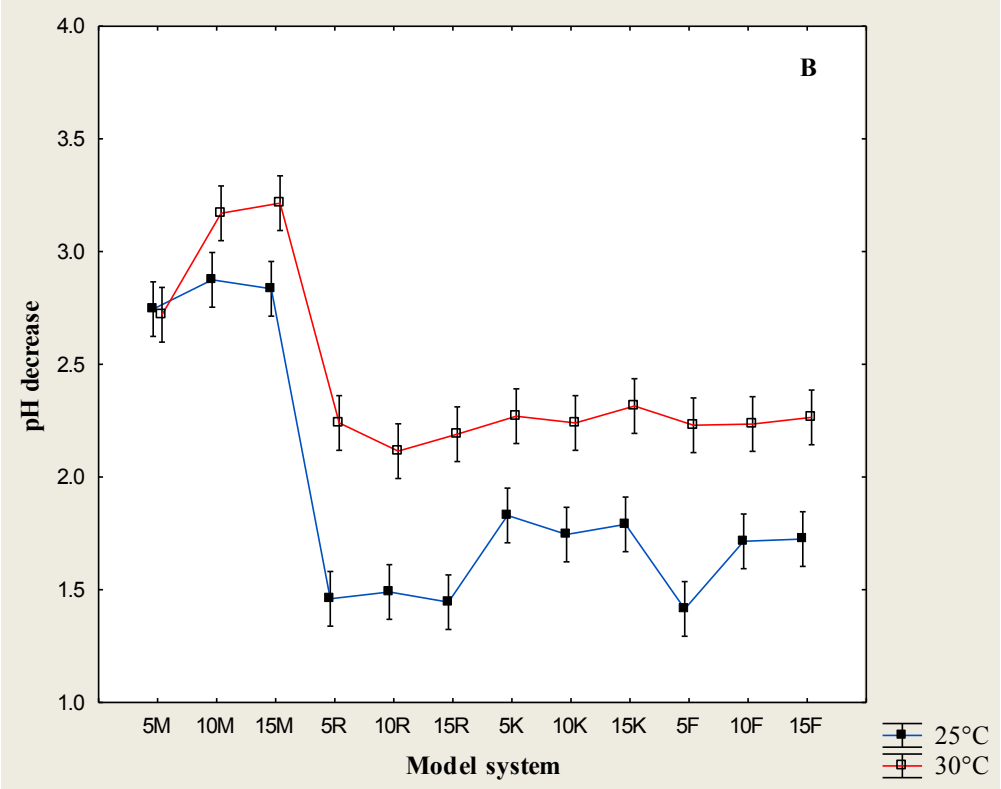
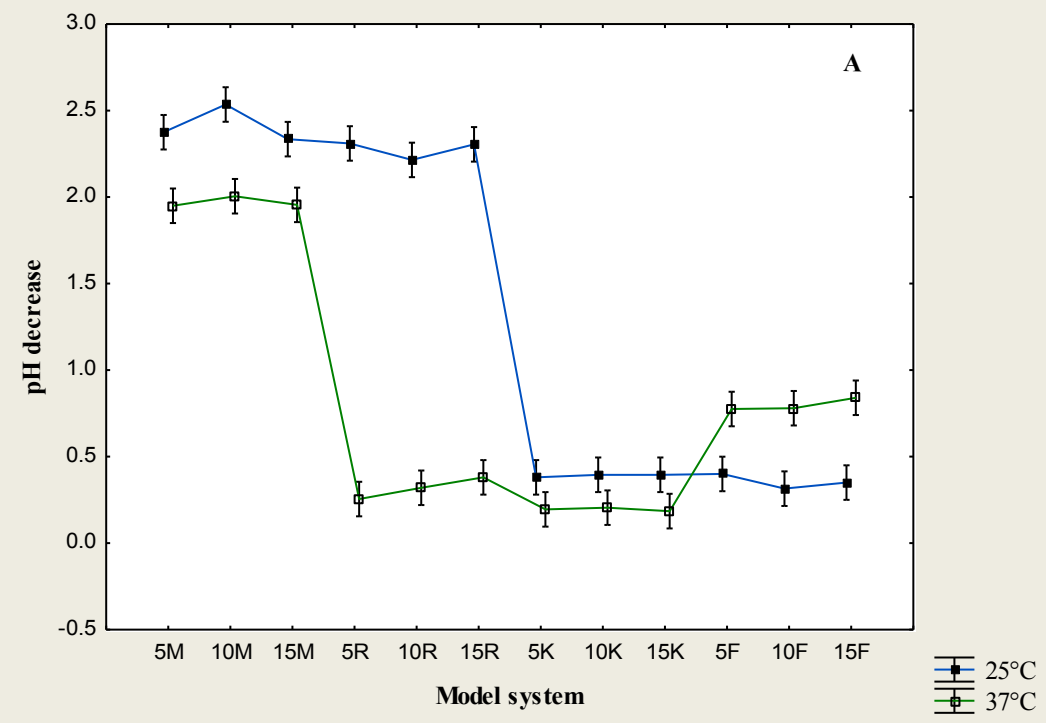

B
4.0
3.5
3.0
2.5
2.0
1.5
1.0
pH decrease
5M
10M
15M
5R
10R
15R
5K
10K
15K
5F
10F
15F
Model system
25°C
30°C

Figure 4.17: Hypothesis decomposition for two-ANOVA on the acidification after 4 (A) and 24 h (B) by *L. reuteri* DSM 20016; the samples were stored at 25 and 37°C. The bars denote 95% confidence intervals. M, Malt Extract; R, Rice extract; K, Kamut; F, wheat flour. The number indicates the amount of the extract in the model system (5, 10, or 15%).



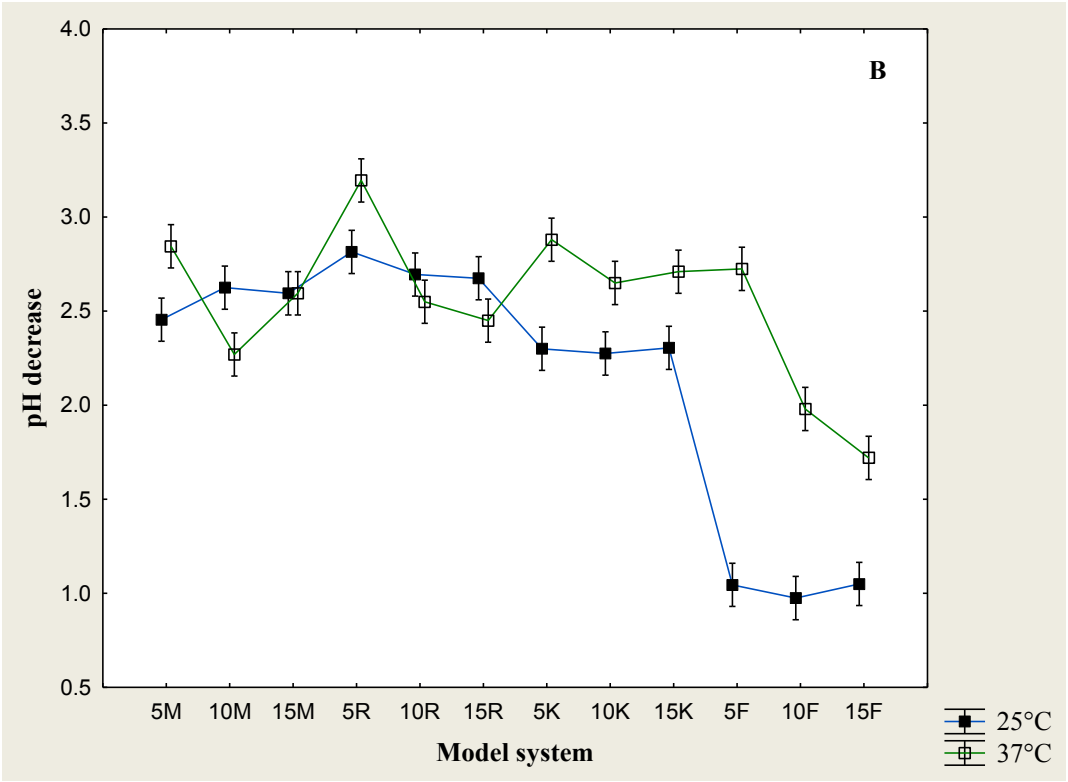
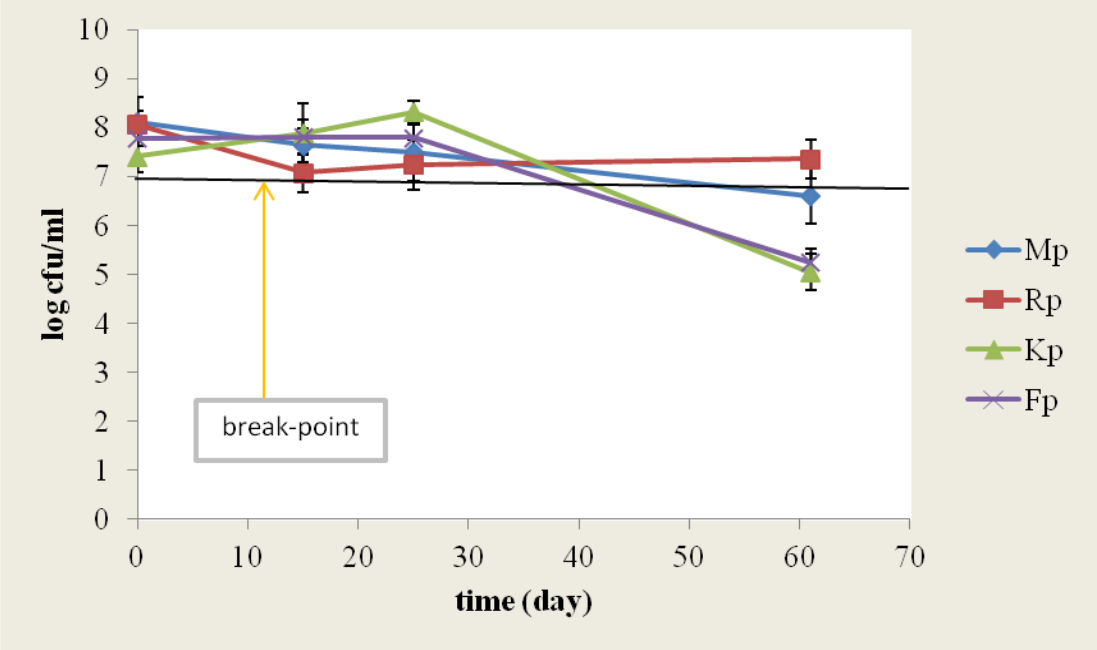

B
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
pH decrease
5M
10M
15M
5R
10R
15R
5K
10K
15K
5F
10F
15F
Model system
25°C
37°C

Figure 4.18: Viability of *L. plantarum* strain 12 (p) and *L. reuteri* DSM 20016 (r) in the samples containing 15% of the different extract (M, Malt Extarct; R, Rice extract; K, Kamut; F, Wheat flour) throughout sample storage at 4°C. Mean values ± standard deviation. The line indicates the break-point of acceptability of probiotics in food (7 log cfu/ml).



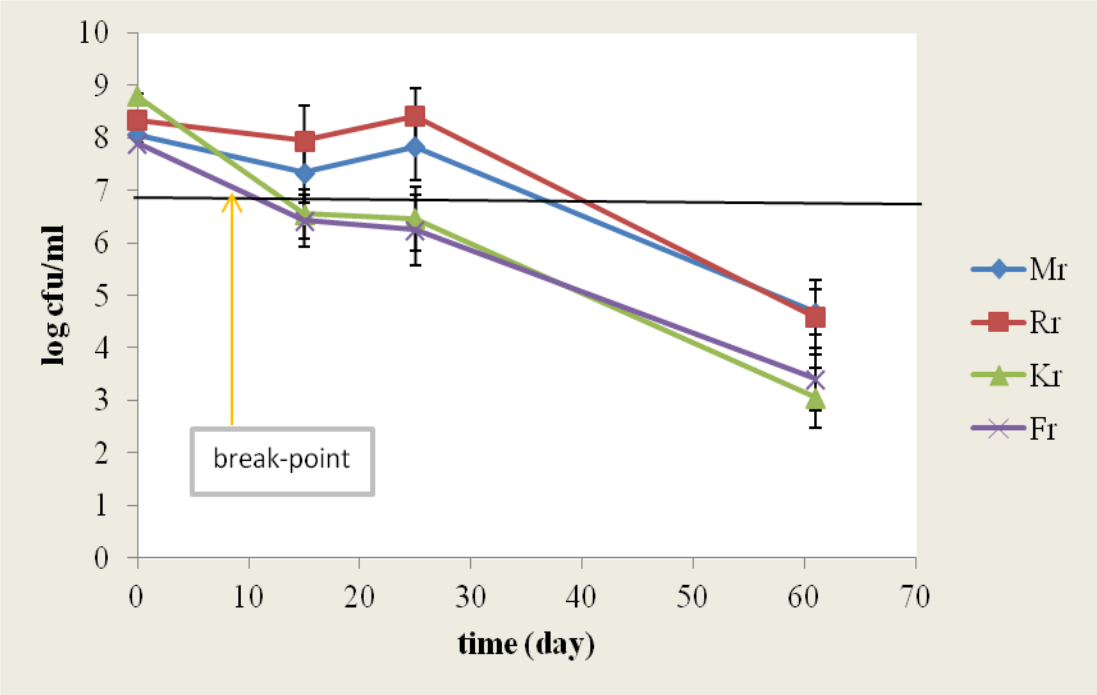


Figure 4.19: Viability of *L. plantarum* strain 12 (Lp) and *L. reuteri* DSM 20016 (Lr) in the organic rice drink stored at 4, 30 or 37°C. Mean values ± standard deviation.

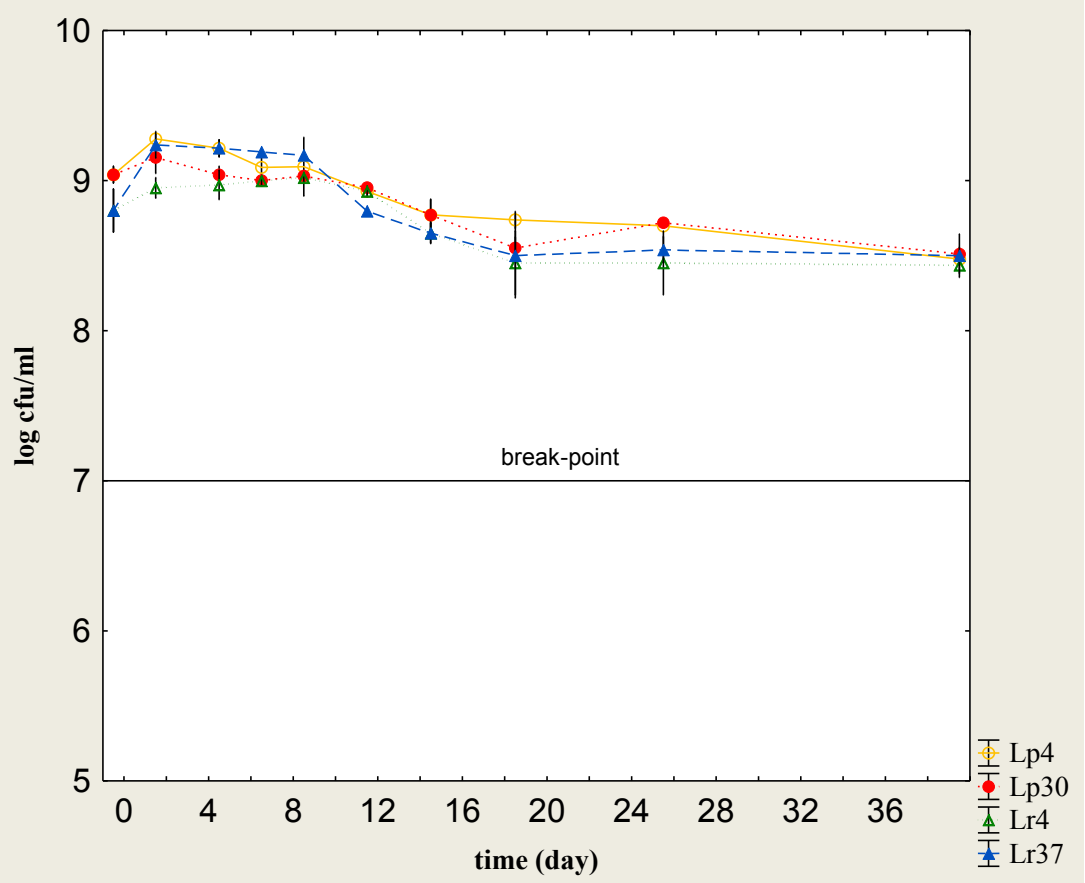


Figure 4.20: Acidification kinetics of *L. plantarum* strain 12 (Lp) and *L. reuteri* DSM 20016 (Lr) in the organic rice drink, stored at 4, 30 or 37°C. Both the replicates were reported for each sampling points; lines represent the best fit through the modified Gompertz equation.

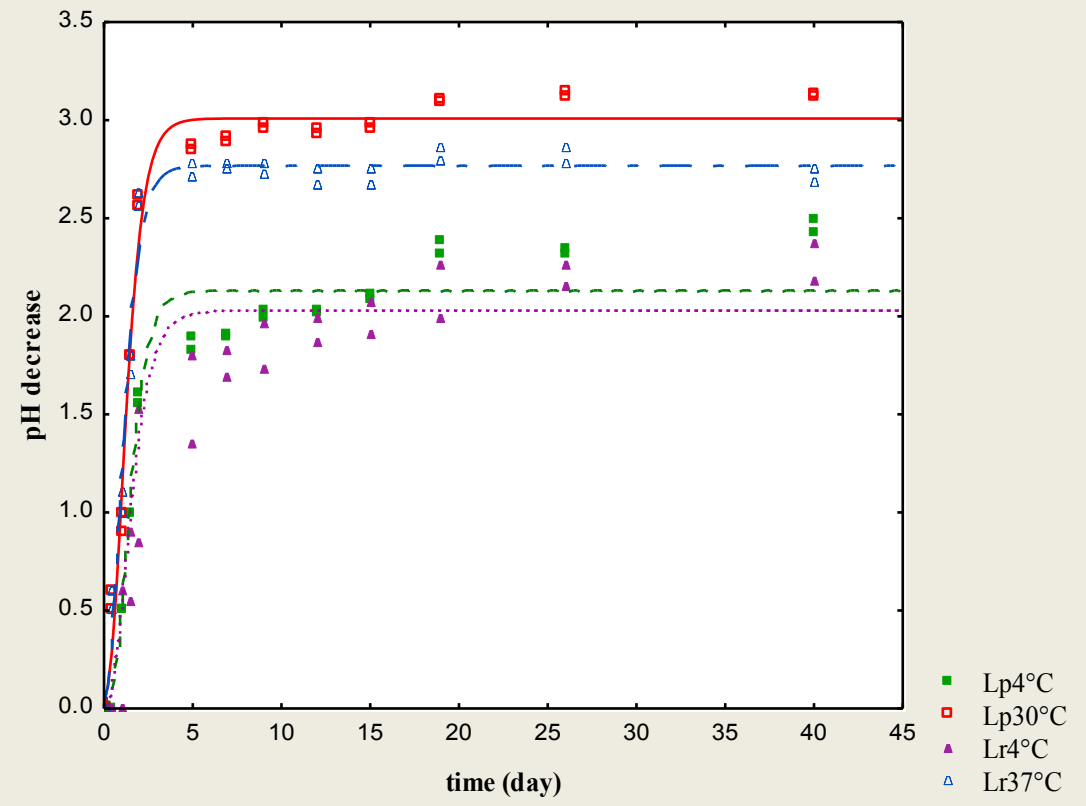


Figure 4.21: Acidification by *L. plantarum* in the organic rice drink stored at 4°C; the bacterium was attenuated through homogenization (multiple passes at 100 MPa). For each sampling points, both the replicates were reported. Lines represent the best fit through Gompertz equation.

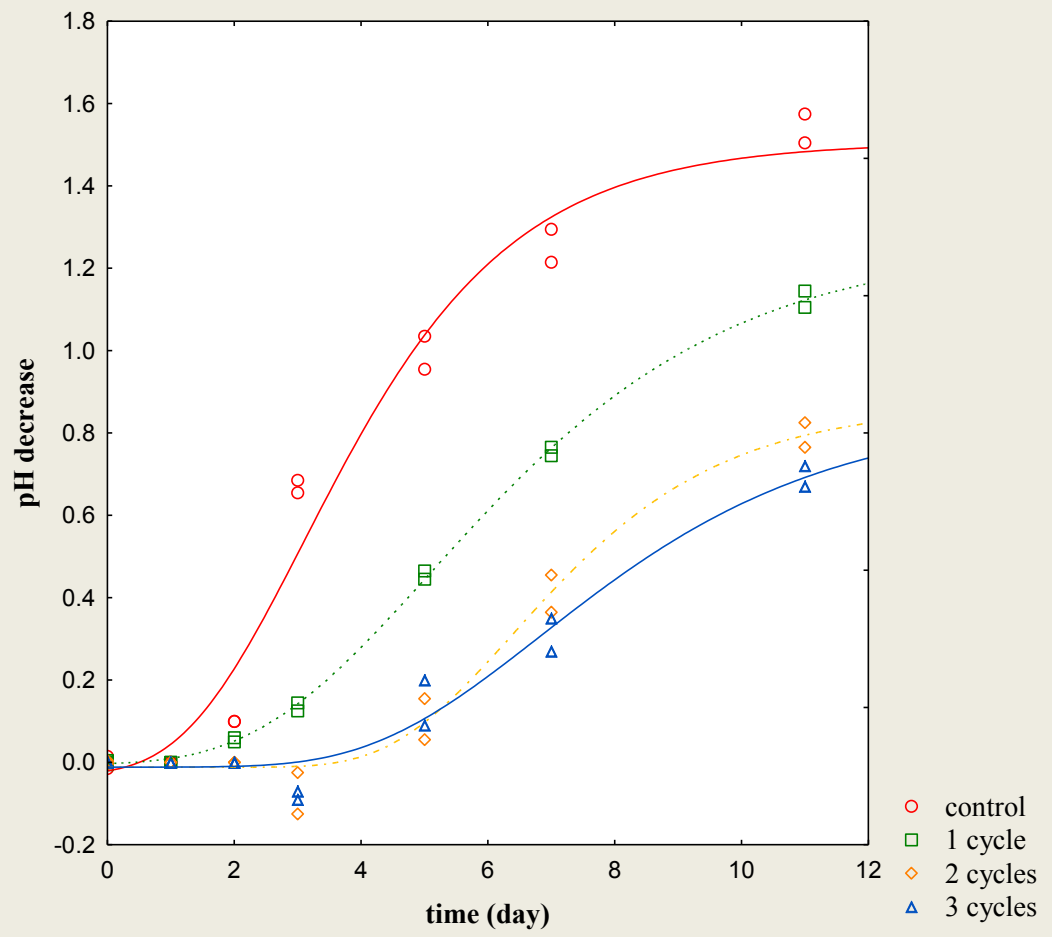


Figure 4.22: Acidification by *L. plantarum* in the organic rice drink stored at 4°C; the bacterium was attenuated through ultrasound (single pass, 80-100%); for each sampling points, both the replicates were reported. Lines represent the best fit through Gompertz equation.

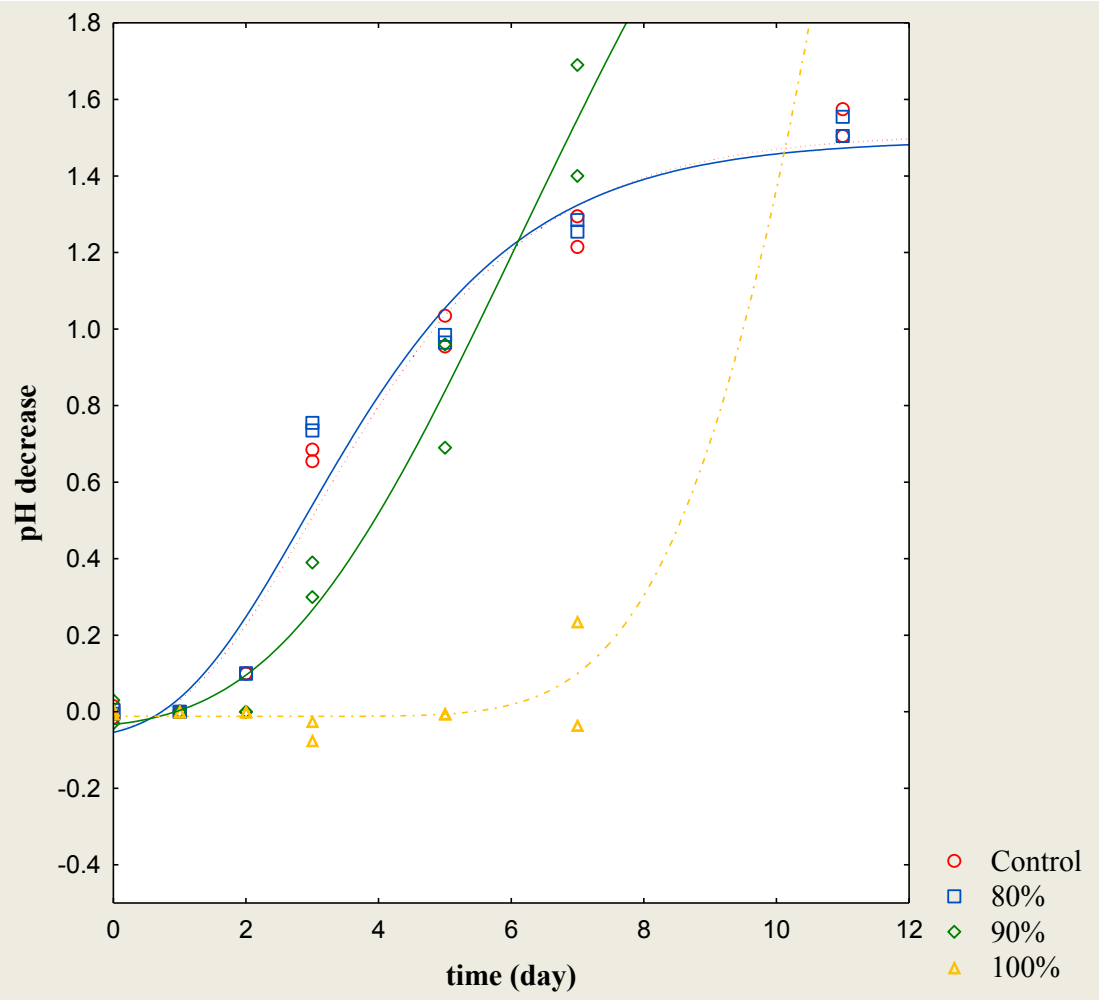


Figure 4.23: Viability of *L. plantarum* attenuated by US (single pass, 80-100%) and inoculated in the organic rice drink, stored at 4°C.* Mean values ± standard deviation.

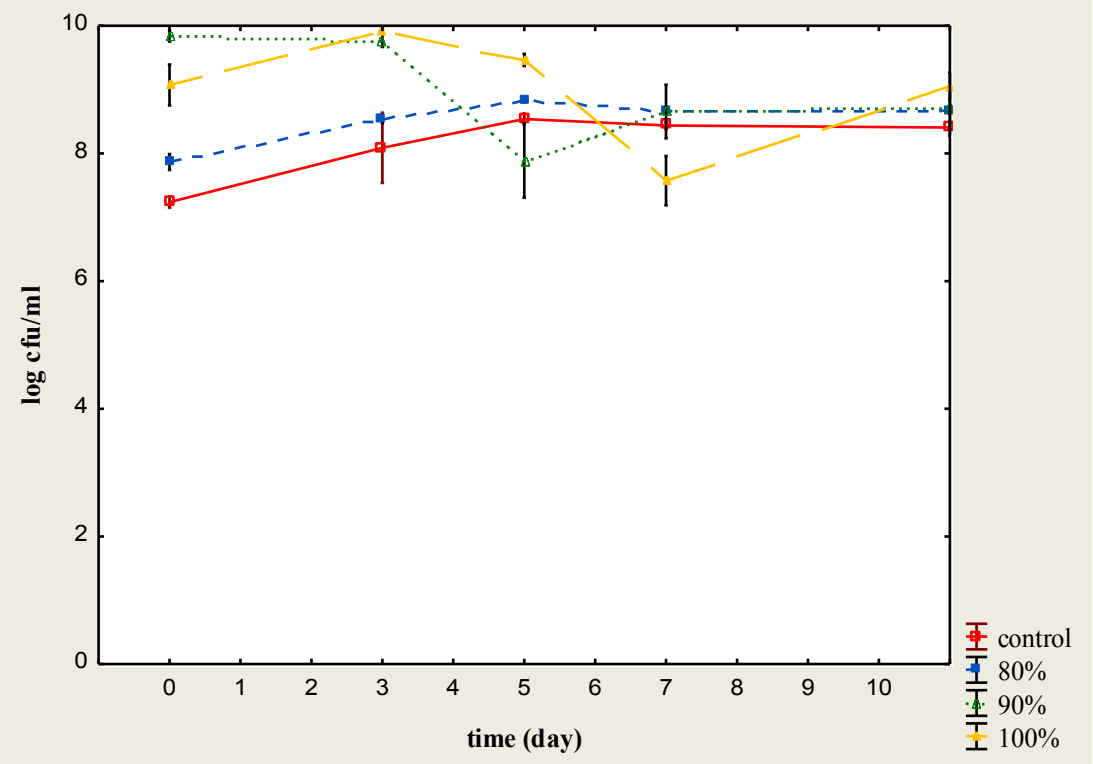


Figure 4.24: Lactic acid produced by *L. plantarum* in the organic rice drink stored at 4°C for 11 days; the bacterium was attenuated by US (single pass, 80-100%). Mean values ± standard deviation.

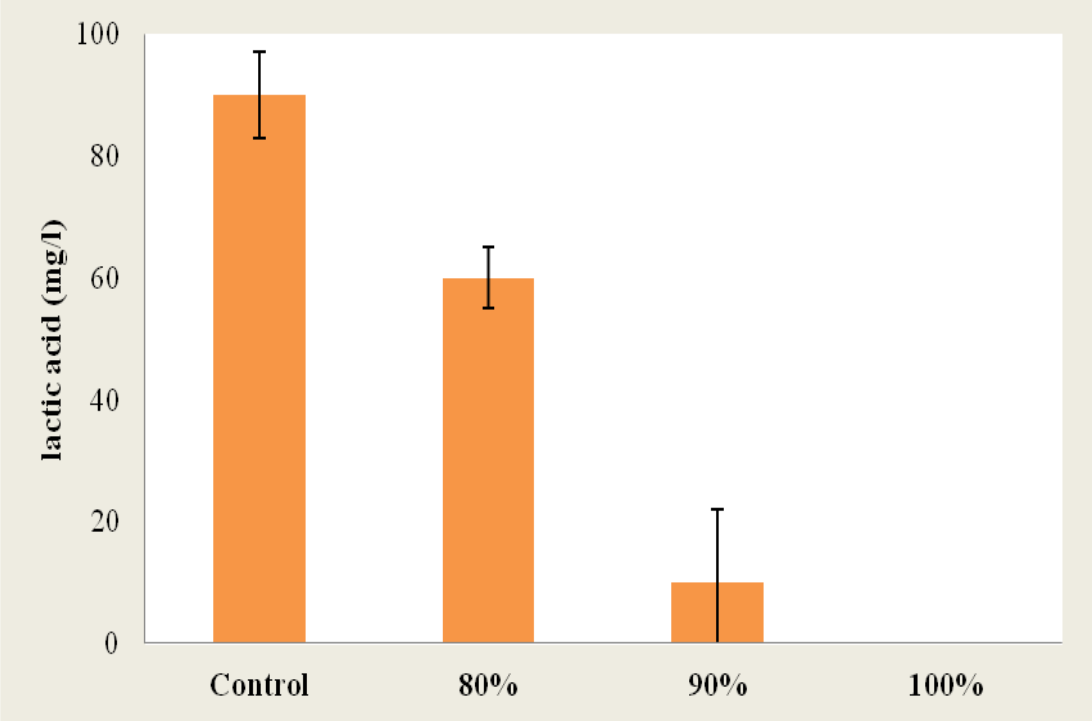


Figure 4.25: Acidification by *L. plantarum* in the organic rice drink stored at 4°C; the bacterium was attenuated through ultrasound (multiple cycles, 80%); for each sampling points, both the replicates were reported. Lines represent the best fit through Gompertz equation.

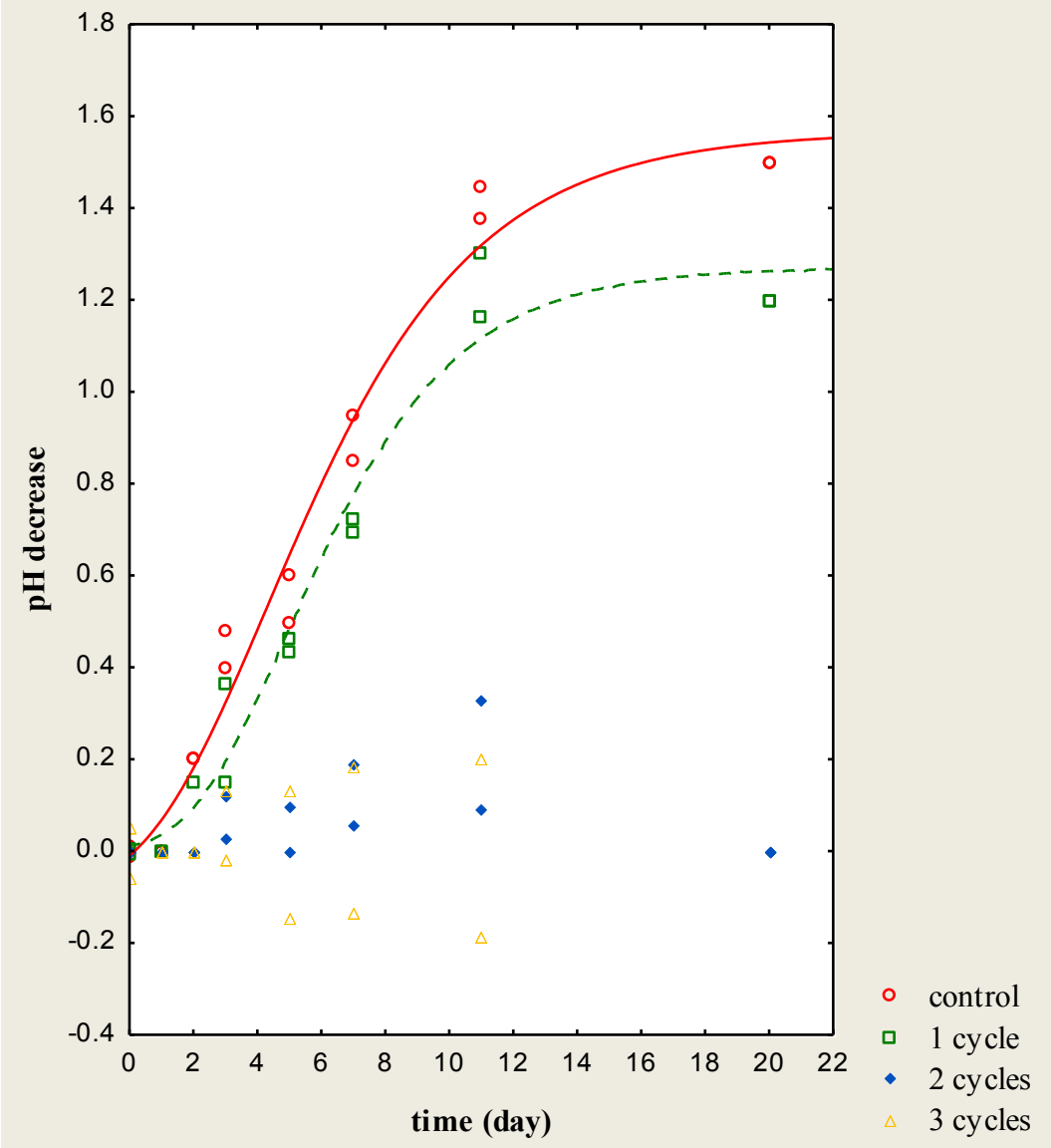


Figure 4.26: Lactic acid produced by *L. plantarum* in the organic rice drink stored at 4°C for 11 days; the bacterium was attenuated by US (multiple cycles, 80%). Mean values ± standard deviation.

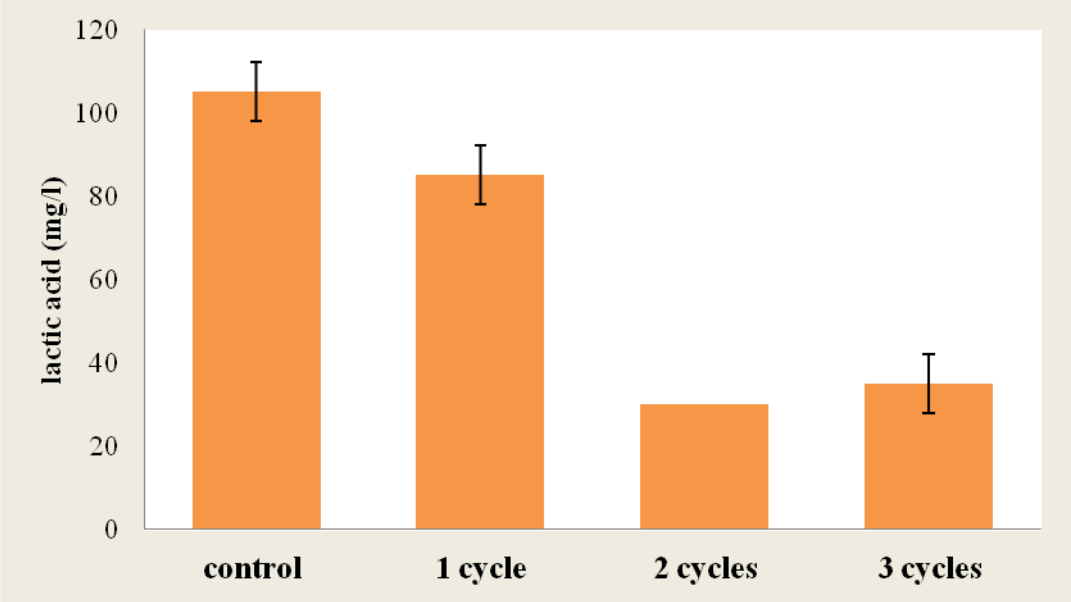


Figure 4.27: Viability of *Kl. lactis* attenuated by US (multiple passes, 80%) and inoculated in the organic rice drink, stored at 4°C. Mean values ± standard deviation.

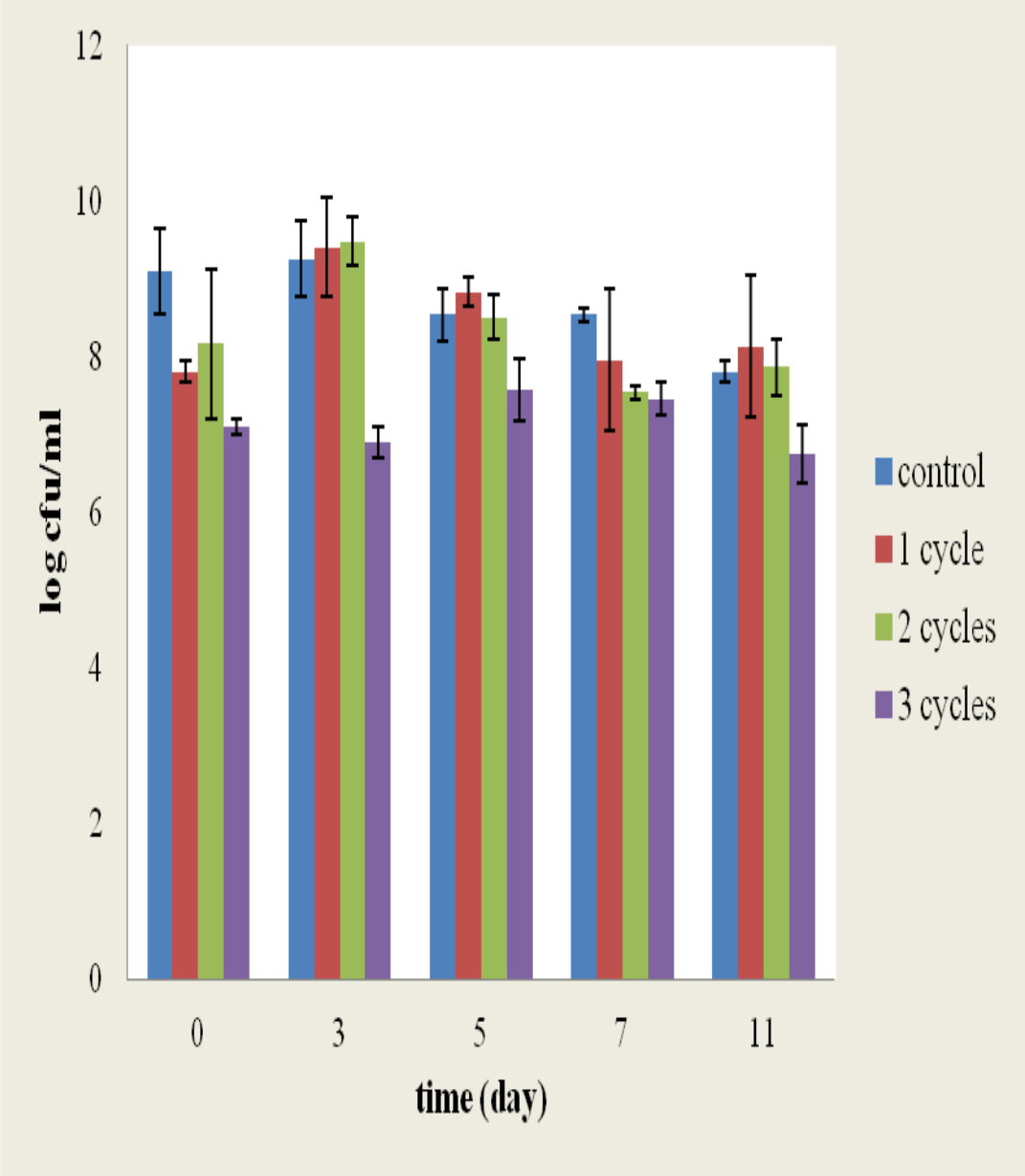


Figure 4.28: Acidification by *L. casei* LC01 in the organic rice drink stored at 4°C; the bacterium was attenuated through ultrasound (2 cycles, 80%); for each sampling points, both the replicates were reported. Lines represent the best fit through Gompertz equation.

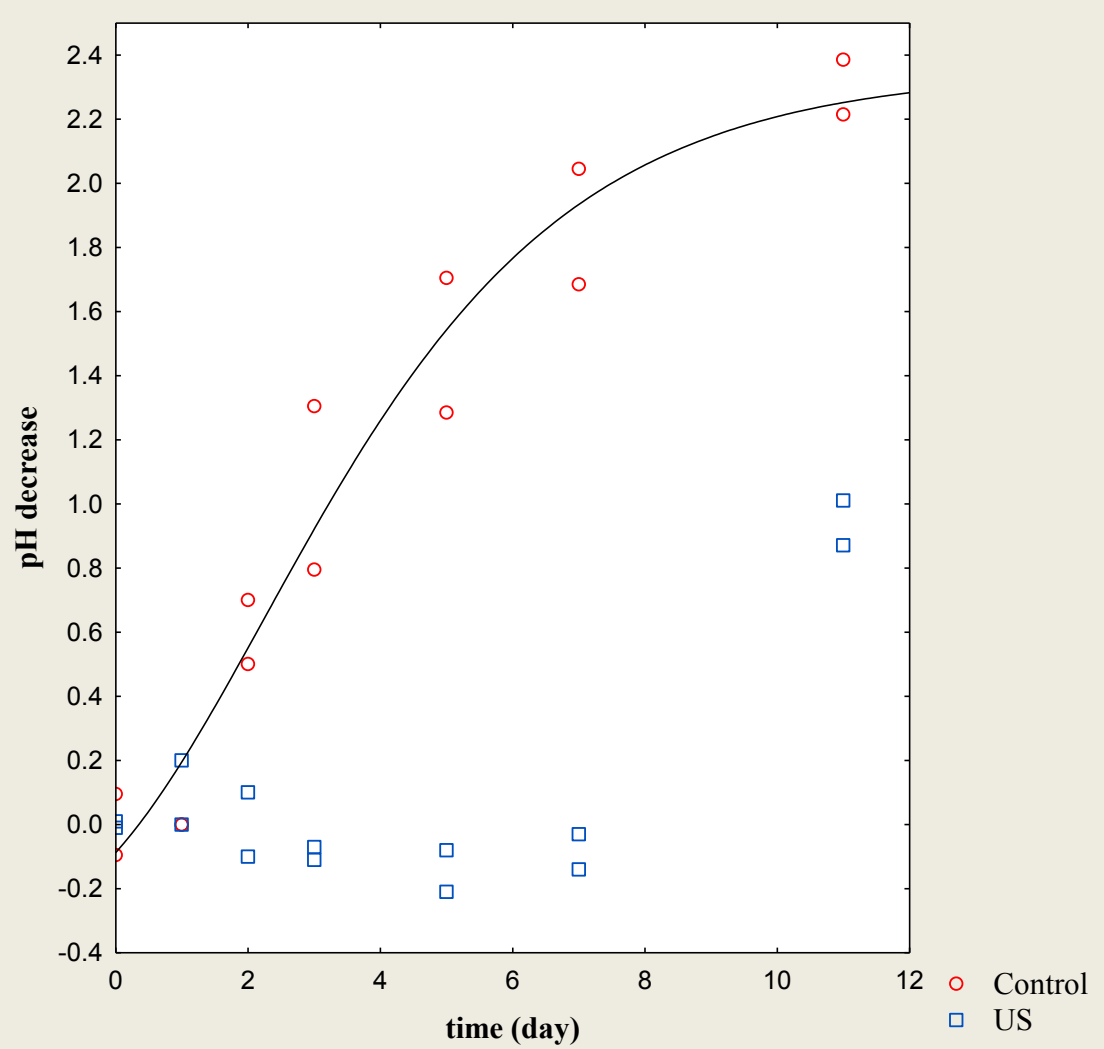


Figure 4.29: Lactic acid produced by *L. casei* LC01 and *B. animalis* subsp. *lactis* BB12 in the organic rice drink stored at 4°C for 11 days; the microorganisms were attenuated by US (two cycles, 80%). Mean values ± standard deviation.

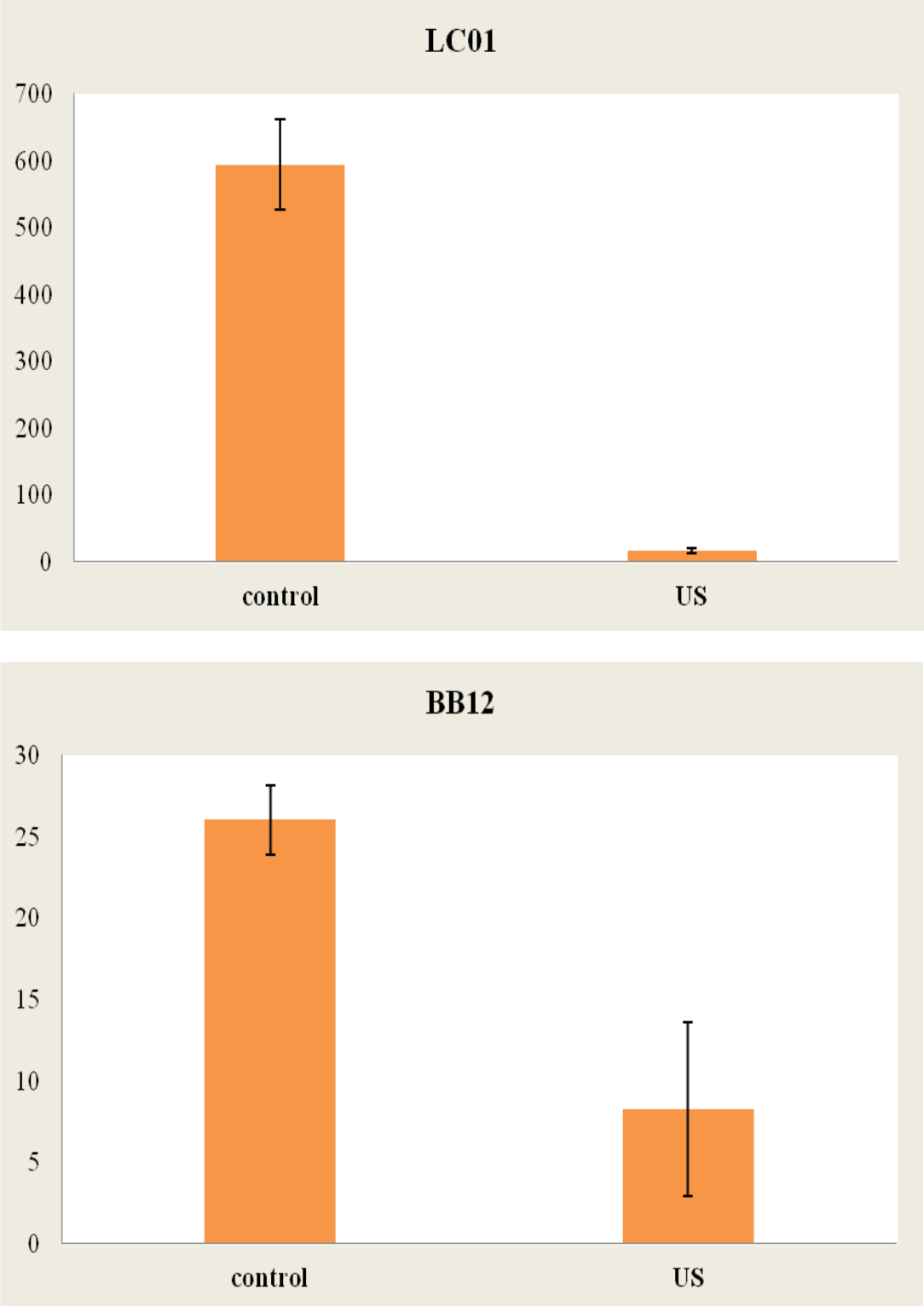
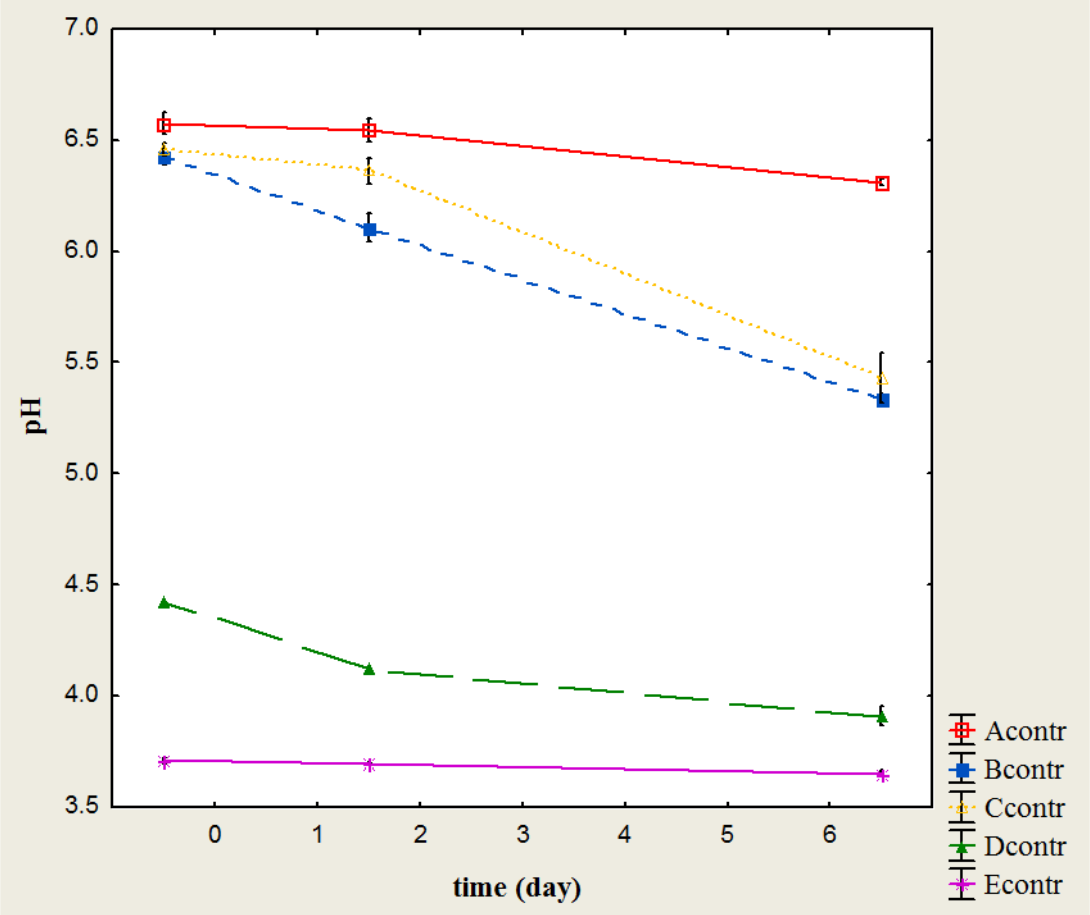


Figure 4.30: pH of organic rice drink inoculated with US-attenuated *L. casei* LC01 (two cycles, 80%). A, storage at 4°C; B, 4 h at 25°C and then at 4°C; C, 4 h at 37°C and then at 4°C; D, 24 h at 25°C and then at 4°C; E, 24 h at 37°C and the at 4°C. Contr, samples inoculated with not-attenuated *L. casei*. Mean values ± standard deviation.



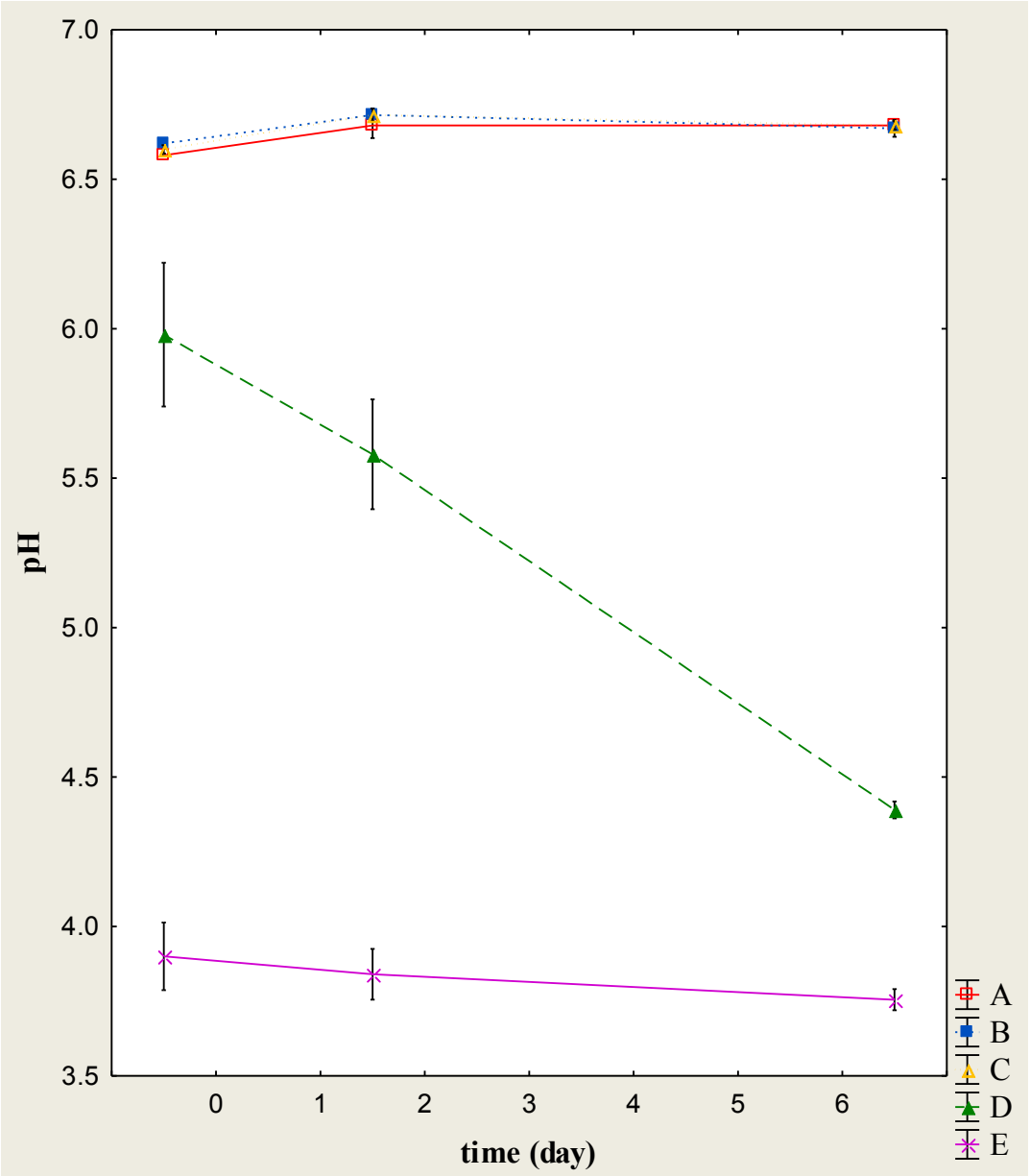

7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
pH
0
1
2
3
4
5
6
time (day)
A
B
C
D
E

Figure 4.31: Sensory scores of organic drink inoculated with *L. casei* LC01 after 7 days of storage; data were modeled as decrease of score referred to uninoculated beverage, stored at 4°C. A, storage at 4°C; B, 4 h at 25°C and then at 4°C; C, 4 h at 37°C and then at 4°C; D, 24 h at 25°C and then at 4°C; E, 24 h at 37°C and the at 4°C.

US, rice drink inoculated with US-attenuated *L. casei*; contr, rice-drink inoculated with not-attenuated *L. casei*; NI, uninoculated rice-drink.

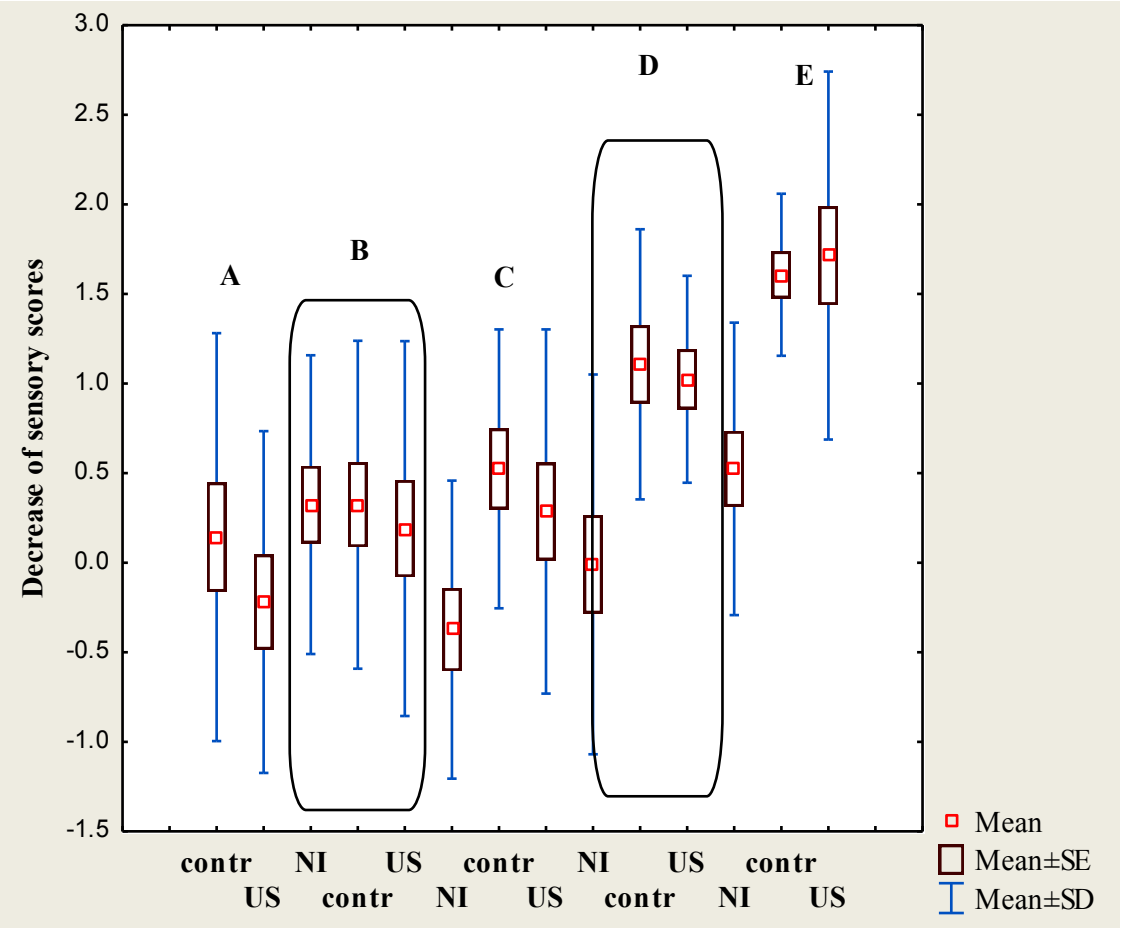


Figure 4.32: Concentration of β-glucans in the organic rice drink stored at 4°C for 11 days; the beverage was inoculated with either *L. casei* LC01 (LC) or *L. plantarum* strain 12 (Lp); US, attenuated microorganisms. Mean values ± standard deviation.

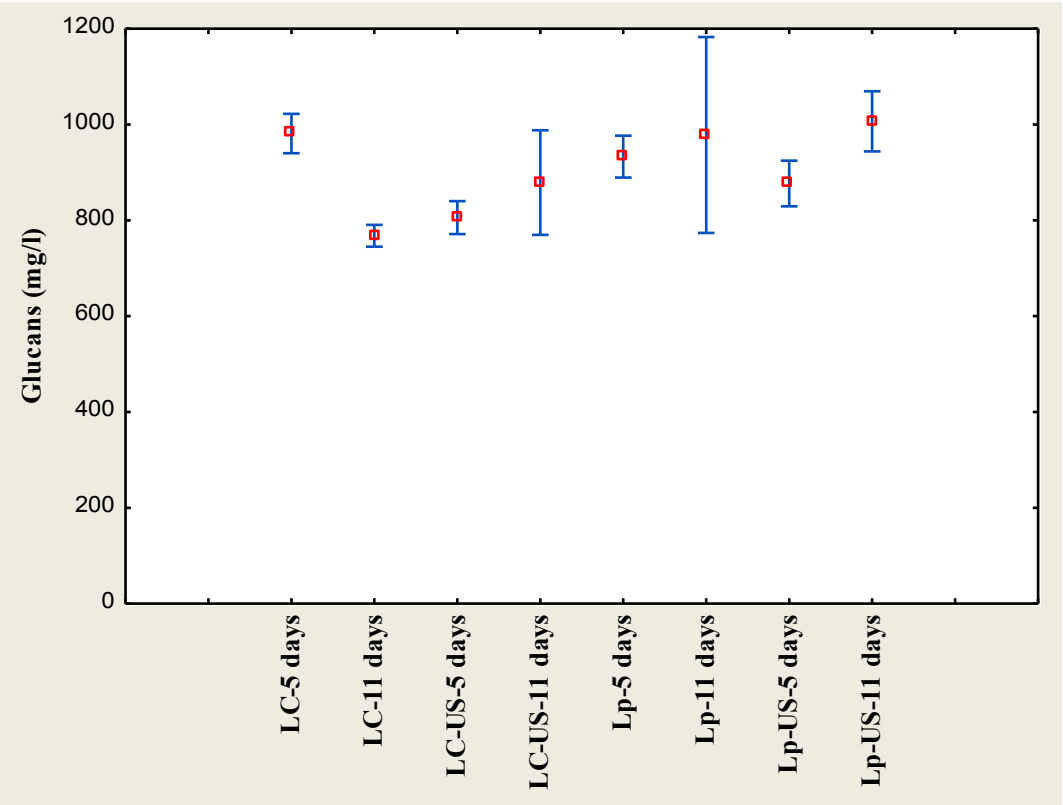
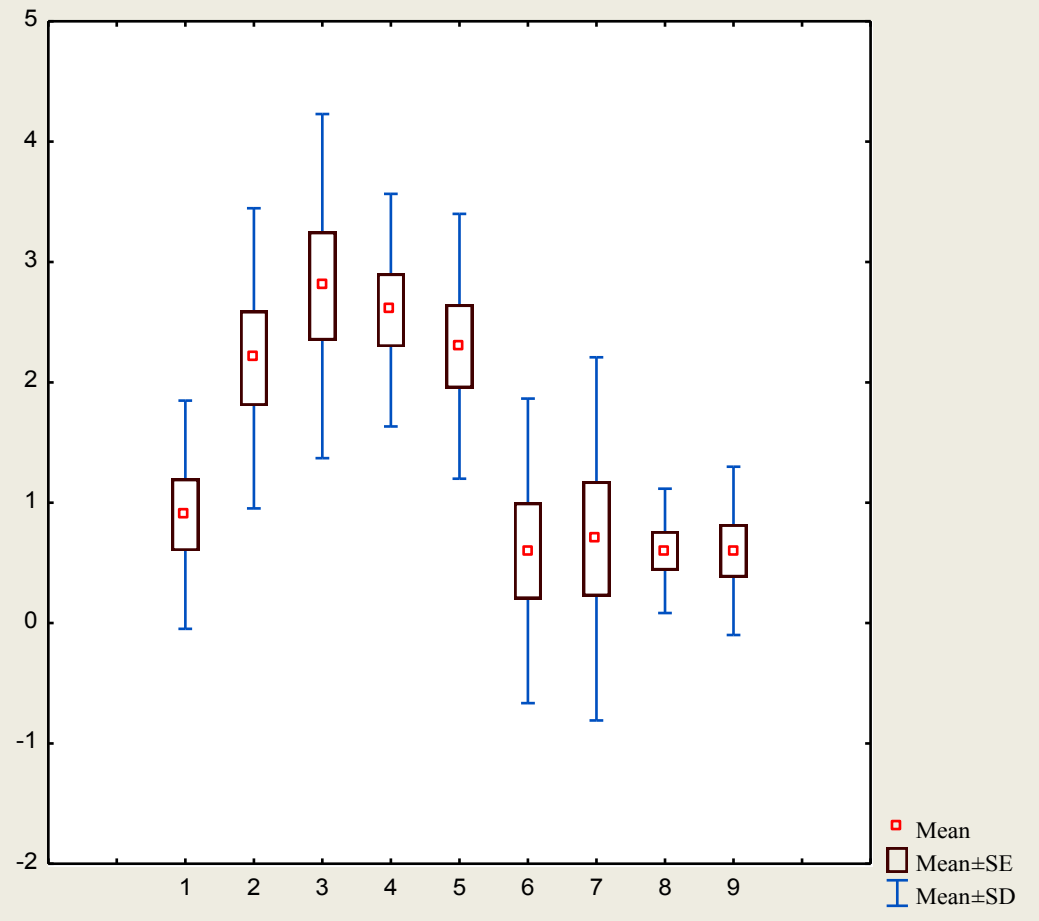


Figure 4.33: Sensory scores of organic drink inoculated with *L. casei* LC01 or *L. plantarum* and added with β-glucans after 11 days of storage at 4°C; data were modeled as decrease of score referred to uninoculated beverage, stored at 4°C. 1, β-glucans; 2, *L. casei*; 3, *L. plantarum*; 4, β-glucans+*L. casei*; 5, β-glucans+*L. plantarum*; 6, US-attenuated *L. casei*; 7, US-attenuated *L. plantarum*; 8, β-glucans+US-attenuated *L. casei*; 9, β-glucans+US-attenuated *L. plantarum*.



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