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Nguyen Bao Toan

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PRODUCTION OF PROBIOTIC TROPICAL FRUIT JUICES BY FERMENTATION WITH PROBIOTIC BACTERIA

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Nguyen Bao Toan

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The Doctoral School

Name: Doctoral School of Food Science

Discipline: Food Science

Head: **Livia SIMON-SARKADI** DSc
Professor
Department of Nutrition Science
Institute of Food Science and Technology
Hungarian University of Agriculture and Life Sciences

Supervisors: **Quang D. Nguyen** PhD
Professor
Department of Bioengineering and Alcoholic Drink Technology
Institute of Food Science and Technology
Hungarian University of Agriculture and Life Sciences

Erika Bujna PhD
Associate professor
Department of Bioengineering and Alcoholic Drink Technology
Institute of Food Science and Technology
Hungarian University of Agriculture and Life Sciences

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Approval of the Head of Doctoral School Approval of the Supervisors

Table of Contents

1. INTRODUCTION

Nowadays, consumers are aware of the significant correlation between diet and health status (Mark-Herbert, 2004, Nematollahi *et al.*, 2016); thus, the increased demand for functional food is definitely observed. In order to develop nutritionally designed foods that promote health through gut microbial reactions, three different types of food ingredients can be used: living microorganisms (probiotics), non-digestible carbohydrates (dietary fiber and prebiotics), and bioactive plant secondary metabolites such as phenolic compounds (do Espírito Santo *et al.*, 2011). Probiotics are defined as living microbial supplements, which beneficially affect the host by improving its intestinal microbial balance, preventing colon cancer, strengthening the immune system, reducing serum cholesterol level, stimulating calcium absorption, synthesis of vitamins such as vitamin B, nicotinic acid, folic acid (Brown and Valiere, 2004, Kalliomäki *et al.*, 2001). The most commonly used probiotic bacterial genera are *Lactobacillus* and *Bifidobacterium*.

Besides probiotic, the new term "postbiotic" has emerged to denote metabolites and/or cell-wall components, secreted by living bacteria or released after bacterial lysis, with demonstrated beneficial activities in the host (Aguilar-Toalá *et al.*, 2018). The soluble factors secreted by living bacteria including short chain fatty acids (SCFAs), enzymes, peptides, vitamins and organic acids might offer physiological benefits to the host by providing additional bioactivity. Some reports proposed that the administration of postbiotics from *L. plantarum* exerted anti-adhesion and antimicrobial effects in vitro (Gao *et al.*, 2016); *L. casei* B1 has been shown to be effective in anti-oxidative, anti-proliferative and anti-adhesion activity against *S. aureus* (Gao *et al.*, 2016, Merghni *et al.*, 2017); the mixture of *B. breve, B. longum* and *B. infantis* offer anti-inflammatory capacity (Sang *et al.*, 2013).

Several foods are naturally abundant in probiotic and postbiotics (e.g., yoghurt, kefir, pickled vegetables and kombucha). The yoghurt and milk-based products accounted for 65% of the global probiotic drink market in 2019. Due to technological advantages and favourable taste, milk has emerged as the most suitable medium for probiotic products (Heenan *et al.*, 2004). However, these products cannot be consumed by groups who suffer from lactose intolerance or have allergies to milk protein.

Fruits contain many essential nutrients such as vitamins, minerals and antioxidants, which naturally have health-promoting effects for the human body. Therefore, they have been recommended as a suitable medium for the functional health ingredients. Prado *et al.* (2008) revealed that fruit juices could serve as a great alternative carrier in some probiotic products. However, the use of fruit juice may face some challenges that need to be overcome such as the survival and viability of probiotics, potential sensory problems etc.

Fruit juices with a low pH value (pH 4.5 or below) may influence the viability and activity of bacteria during fermentation and storage. Research now focuses on characterizing specific probiotic strains and the growth and survival of probiotic in fruit juices. Lactic acid bacteria show sensitivity to acidic conditions due to the typically low pH, between pH 2.5 and 3.7 (Song *et al.*, 2012). In the study of Nematollahi *et al.* (2016), they used *L. plantarum* ATCC20174, *L. casei*

ATCC 393 and *L. rhamnosus* ATCC 7469 to supplement cornelian cherry juice, and the viability of probiotic bacteria during cold storage was investigated. All strains did not tolerate detrimental conditions of product matrix, especially at very low pH (pH 2.6). They completely lost their viability during the early days of cold storage in such a way that even the most resistant strain (*L. casei* T4) reached an all-dead population state at day 7th.

An easy way to improve probiotic stability in fruit juice could be the fortification of juice with some prebiotics or some ingredients that can exert a protective effect. Another possibility of exposure of probiotics to a sub-lethal stress could induce resistance and an adaptive stress response. Perricone *et al.* (2014) successfully used two different strategies: strain cultivation in a laboratory medium containing different amounts of red fruit juices (which has a strong viability decreasing effect) or added with vanillic acid (phenol stress) or acidified to pH 5.0 (acid stress).

Taste is the primary factor involved in the acceptance and purchasing behaviour of various foods, including functional foods. The off-flavour appears to be related to the presence of probiotics on the sensory characteristics of juices. Luckow *et al.* (2006) have identified that probiotics cause perceptible off-flavours that often contribute to consumer dissatisfaction. Masking is one technique that has been used to reduce the sensations of aversive odours and flavours in foods. It has been performed successfully through the addition of new substances or flavours to juices and is therefore suspected to be capable of reducing the negative sensory attributes contributed by probiotic cultures. Tropical fruit juices (e.g., pineapple, mango, passionfruit) contribute strong, exotic aromatic and flavour contributions that may prevent consumers from identifying the probiotic off-flavours.

The development of new non-dairy probiotic food products was carried out by many researchers. The substrates that they mainly focused on were vegetables and fruits, such as soymilk, the juice of cucumber, potato, carrot or fruit juices (apricot, pineapple, mango, apple, etc.). Some promising results were reported from their studies. However, there are only limited studies on the combination of different probiotic microorganisms in these media. Mixed cultures were believed that can bring more effective health benefits than single strains. Furthermore, it would be interesting to compare the metabolism and growth rate of these bacteria in mono and mixed cultures. Additionally, since fermented fruit juices are known as a novel probiotic product, shelf-life evaluation is important to ensure a quality product during the storage period.

2. OBJECTIVES

Fruits have many beneficial effects on human health, moreover they may serve as a good substrate for probiotic bacteria. However, they are also known as an insufficient source of amino acid as well as low pH media which may give drawbacks to the growth of probiotic bacteria. Therefore, assessing the metabolism and growth of *Lactobacillus* and *Bifidobacterium* strains in tropical fruit juice will be necessary in producing probiotic fruit drinks. Furthermore, the combination of *Lactobacillus* and *Bifidobacterium* in fruit juice fermentation may be a promising idea to develop a probiotic fruit juice as a functional food and nutraceutical with health beneficial effect.

The specific objectives of this work were:

- Investigation of the suitability of some tropical fruit juices for probiotic fruit drink production applying lactic acid bacteria (LAB) and *Bifidobacterium*
- Screening probiotic strains for fermentation of fruit juices
- Production of fermented fruit juice with mixed cultures
- Investigation of viability and production of metabolites, as well as the change of total phenolic content and antioxidant activity of the juices during fermentation and storage
- The survival of probiotics through the simulated gastro-intestinal conditions
- Sensory analysis
- Effects of storage conditions on the stability of fermented products, estimation of the product shelf life.

3. LITERATURE REVIEW

3.1. Probiotics

3.1.1. History and definition of probiotics

The word ‘probiotic’ in the Greek language means ‘for life’. It was initially used by Lilly and Stillwell (1965) to describe “substances secreted by one microorganism which stimulates the growth of another”. According to Schrezenmeir and de Vrese (2001), this definition contrasted with the term “antibiotic” which is “a chemical agent produced by one organism that is harmful to other organisms” (Madigan *et al.*, 1997). Parker (1974) would like to emphasize the role of other substrates, such as antibiotics, in the probiotic definition and give more details of the health benefits of probiotics. He redefined the term of probiotic as “organisms and substances which contribute to intestinal microbial balance”. Fuller (1989) broadened Parker’s probiotic definition as “A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance”. In his definition, the host which was mentioned is animal. Three years later, Haveenar and In't Veld (1992) improved it with the new definition as “A viable mono- or mixed culture of microorganisms which applied to animal or man, beneficially affects the host by improving the properties of the indigenous microflora”. In 1996, two probiotic definitions were given by Salminen and Schaafsma. Salminen (1996) defined probiotic as “a live microbial culture or cultured dairy product which beneficially influences the health and nutrition of the host”. However, there is an observation that nondairy products also contain microorganisms which can improve the properties of the indigenous microflora of the host (Schrezenmeir and de Vrese, 2001). Schaafsma (1996) came with the definition: “Oral probiotics are living microorganisms which upon ingestion in certain numbers, exert health effects beyond inherent basic nutrition”. In his statement, he emphasized the importance of consuming microorganisms with an adequate amount resulting a promoting effect on the host’s health. Guarner and Schaafsma (1998) suggested that probiotic is "live microorganisms, which when consumed in adequate amounts, confer a health effect on the host". Although there are many definitions about probiotics, the definitions have to:

I. restrict the use of the word probiotic to products which contain live microorganisms
II. point out the need for providing an adequate dose of probiotic bacteria in order to exert the desirable effects (FAO and WHO, 2001).

3.1.2. Health-effects of probiotics

Probiotic products have shown to provide numerous health-promoting effects on animals and humans. However, the potential health benefit depends on the characteristic profile of the probiotics and dosage.

- **Preventing the growth of harmful undesirable organisms**

Some *Lactobacillus* species such as *L. acidophilus, L. casei, L. plantarum, L. fermentum,* etc. and *Bifidobacterium* species such as *B. bifidum, B. lactis, B. infantis, B. longum,* etc. can prevent the growth of undesirable microorganisms such as *Salmonella, E. coli, Helicobacter pylori* that might be encountered in the digestive tract (Arboleya *et al.*, 2016, Remacle and Reusens, 2004). The inhibition ability of probiotics against undesirable microorganism may be due to the nutrient competition and large amounts of acid production during their growth (Marth and Steele, 2001). Additionally, several of these probiotic organisms produce bacteriocins (Salminen *et al.*, 2011), which may be related to the antagonistic effect toward these pathogens.

- **Improving the immune response**

L. casei appears to be the one of main microorganisms involved in enhancing the body's immune response (Perdigon *et al.*, 1990). *B. longum* may also stimulate the immune system to control *E. coli* in the digestive tract (Romond *et al.*, 1997). This ability of probiotic bacteria varies greatly between strains of each species. Enhancement the body's immune system may involve activating macrophages thereby destroying pathogenic organisms in the body.

- **Improving the lactose digestion**

People who have trouble in the ability to digest lactose completely are classified as lactose intolerants. The lack of sufficient levels of β-galactosidase in the small intestine causes inadequate lactose digestion (Marth and Steele, 2001). Then, the undigested lactose enters the large intestine where it is fermented by the colonic microbiota that results in symptoms of cramps, flatulence, and diarrhea. *Lactobacillus* and *Bifidobacterium* are effective in improving lactose utilization due to their production of β-galactosidase during fermentation (Gomes *et al.*, 1999, Marth and Steele, 2001).

- **Preventing cancer**

L. acidophilus, L. casei and *L. delbrueckii* subsp. *bulgaricus* are species commonly mentioned as having potential to provide anticancer activities (Marth and Steele, 2001). They reported that consuming a culture of *Lactobacillus* presented an ability in controlling cancer of the colon. The controlling effect may be through the possible mechanisms (Salminen *et al.*, 2011):

- Stimulation of the host's innate immune system
- Limitation of genotoxic reactions by the intestinal microbiota
- Alteration of physicochemical conditions in the colon
- Adsorption or degradation of potential carcinogens
- Nourishment of the intestinal epithelium with macro and micronutrients
- Production of antitumorigenic or antimutagenic compounds

However, further research is required to link purported mechanisms of probiotic action to cancer suppressing effects.

- **Controlling serum cholesterol**

Many researchers revealed that some *Lactobacillus* and *Bifidobacterium* strains can reduce the cholesterol level in laboratory media (Grill *et al.*, 2000, Miremadi *et al.*, 2014, Tahri *et al.*, 1995, Tahri *et al.*, 1996). Probiotic microorganisms may have ability to deconjugate bile acids into free form in the small intestine. The free bile acids are less well absorbed in the small intestine than they are in conjugated form; thus, more are excreted through feces. As a result, the cholesterol level of the body is reduced. This can be explained that cholesterol is a precursor substance for synthesis of the bile acids. The excretion of many bile acids through feces encourages the synthesis of the new ones cause the reduction of the cholesterol level in the body (Marth and Steele, 2001).

In human intestine, as *Lactobacillus, Leuconostoc* and *Bifidobacterium* predominate, they prevent the growth of undesired intestinal pathogen. These microorganisms may be used individually or in combination in probiotics to confer health benefits.

3.1.3. Probiotic microorganisms

Several genera have been used as probiotics, including *Lactobacillus, Bifidobacterium, Propionibacterium* and *Enterococcus*. Other than that, *Bacillus* and yeast members which are considered nonpathogenic are also used (Erkmen and Bozoglu, 2016, Shewale *et al.*, 2014). Roy and Gahlawat (2018) summarized several commercial probiotic strains from species *B. animalis* subsp. *lactis, B. breve, B. lactis, B. longum, L. acidophilus, L. brevis, L. casei, L. crispatus, L. curvatus, L. delbrueckii, L. fermentum, L. gasseri, L. helveticus, L. rhamnosus, L. johnsonii, L. plantarum, L. paracasei, L. reuteri, L. rhamnosus, L. salivarius, Enterococcus faecalis, Enterococcus faecium, Streptococcus cremoris, Streptococcus diacetylactis, Streptococcus intermedius, Streptococcus salivarius, Streptococcus thermophilus*. Among these microorganisms, lactic acid bacteria (LAB) and bifidobacteria have been proposed as the common probiotic culture for human (Conway, 1996, Rivera-Espinoza and Gallardo-Navarro, 2010, Shewale *et al.*, 2014).

Although each strain has its own properties, the microorganism must ensure strict selection criteria for probiotic performance that should be followings (Erkmen and Bozoglu, 2016):

- Appropriateness
    - Taxonomic identification by phylogenetic analysis and rRNA sequencing.
    - Genetically stable to maintain phenotypic properties.
    - Normal inhabitant of the species from a healthy individual.
    - Safety: nontoxic, nonpathogenic, “generally recognized as safe.”
    - Suitable to mass production and storage.
    - Viable at high populations (from 10^7 to 10^9 viable cells/g).
    - Stable during culture preparation, storage, and delivery.

- Provide desirable organoleptic products in foods or fermentation processes.
- Able to survive and metabolically active *in vivo*.
- Resistant to acid and bile.
- Able to compete with microflora.
- Able to adhere, colonize, and remain in the GI tract.

- Functionality
  - Able to exert one or more health benefits.
  - Able to exert antagonistic effect on pathogenic bacteria.
  - Able to produce antimicrobials (bacteriocins, H_2O_2, organic acids, and others).
  - Able to stimulate immunity.
  - Show anti-inflammatory, antimutagenic, and anticarcinogenic effects.
  - Able to produce bioactive compounds (enzymes, vaccines, peptides, etc.)

3.1.3.1. Genus *Lactobacillus*

Lactic acid bacteria (LAB) are characterized as gram-positive, non–spore-forming, catalase-negative, devoid of cytochromes, nonaerobic habit but aerotolerant, fastidious, acid-tolerant, and strictly fermentative, with lactic acid as the major end product during sugar fermentation (Salminen *et al.*, 2011). Considering DNA base composition of the genome, the organism usually show a GC content of lower than 54 mol% (Roy and Gahlawat, 2018). LAB is perfectly adapted to environments rich in nutrients (amino acids, nucleotides and vitamins) and energy sources such as milk, meat, vegetable and beverage. Based on this description, the genera of *Aerococcus, Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus* belong to the LAB group. Classification of LAB can be based on the two main pathways of sugar metabolism which are homofermentative and heterofermentative LAB. Homofermentative LAB catabolize glucose via the glycolysis (Emden-Meyerhof pathway) with at least 85% lactate of the final products (**Figure 3.1 A**) whereas heterofermentative LAB do it through the 6-phosphogluconate/phosphoketolase pathway resulted in significant amounts of other end products such as ethanol, acetate and CO_2 besides lactic acid (**Figure 3.1 B**).

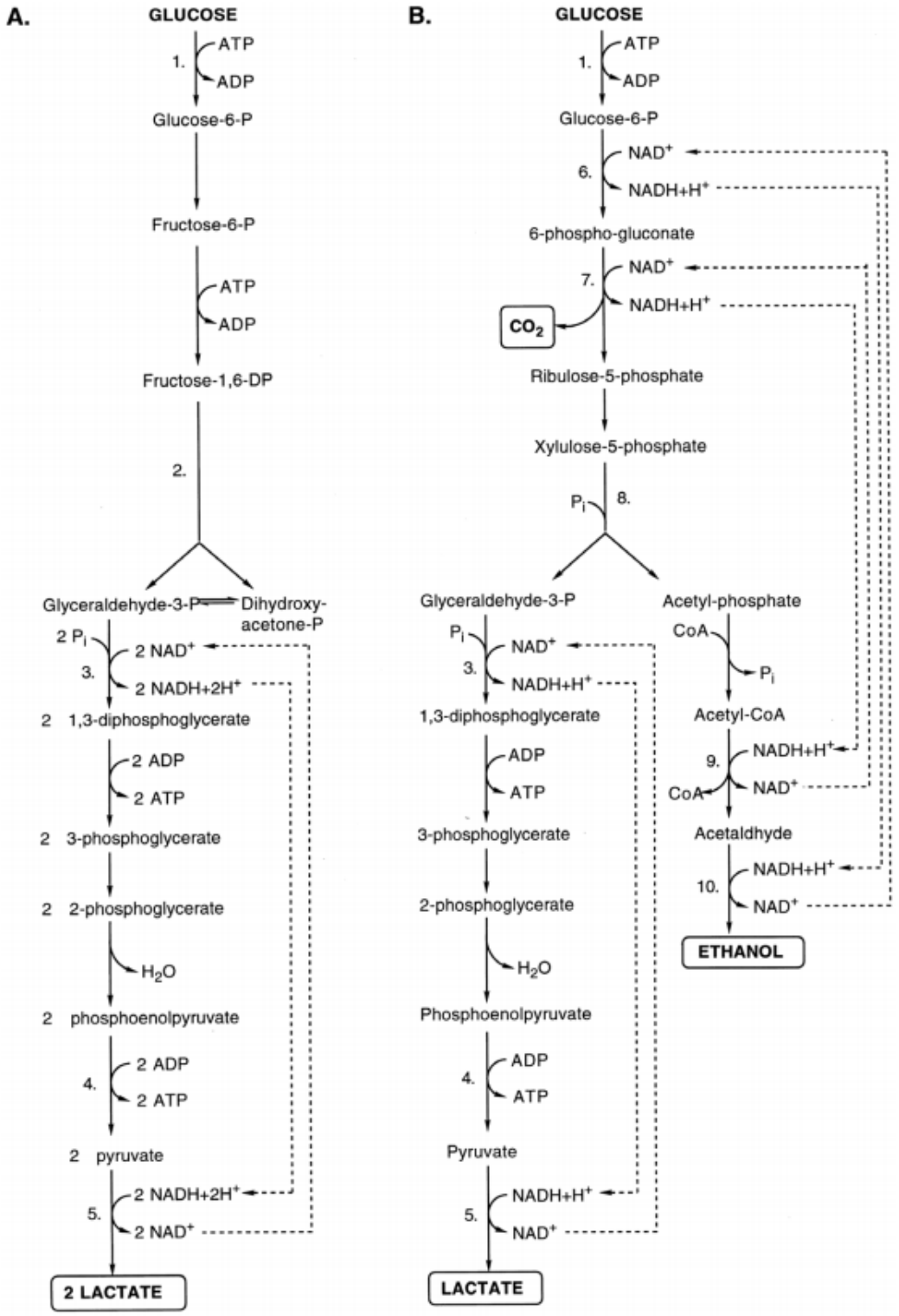


Figure 3.1. Major fermentation pathways of glucose (A) homolactic fermentation (glycolysis, Embden-Meyerhof-Parnas pathway); (B) heterolactic fermentation (6-phosphogluconate/phosphoketolase pathway). Selected enzymes are numbered: 1. Glucokinase; 2. Fructose-1,6-diphosphate aldolase; 3. Glyceraldehyde-3-phosphate dehydrogenase; 4. Pyruvate kinase; 5. Lactate dehydrogenase; 6. Glucose-6-phosphate dehydrogenase; 7. 6-phosphogluconate dehydrogenase; 8. Phosphoketolase; 9. Acetaldehyde dehydrogenase; 10. Alcohol dehydrogenase (Salminen *et al.*, 2011)

Lactobacillus are considered as a major group of LAB. Generally, the members of this genus are anaerobic or aerotolerant, rod-shaped, gram-positive bacteria (Salminen *et al.*, 2011). There are 262 species in the genus *Lactobacillus* and they are very diverse at phenotypic, biochemical, and physiological properties (Zheng *et al.*, 2020). As a member of LAB, *Lactobacillus* can be divided into homofermentative and heterofermentative lactobacilli. However, in some cases, homofermentative lactobacilli can also use the phosphogluconate pathway when metabolizing certain substrates. Genus *Lactobacillus* have been divided into 3 groups which are "obligate homofermentative", "facultative heterofermentative" and "obligate heterofermentative" and could be briefly described as below (Salvetti *et al.*, 2012):

- **Group I (Obligately homofermentative)**: The species metabolize hexoses via the Embden-Meyerhoff pathway to yield lactic acid as the end product.
- **Group II (Facultatively heterofermentative)**: The species metabolize hexoses via the Embden-Meyerhoff pathway to yield lactic acid as the major end product. The organisms can also ferment pentoses and gluconates via the phosphogluconate pathway because they possess both aldolase and phosphoketolase enzyme. As the result, lactic acid, acetic acid, ethanol and formic acid are produced under glucose limitation.
- **Group III (Obligately heterofermentative)**: the species metabolize pentoses and hexoses via the phosphogluconate pathway to yield lactic acid, ethanol (or acetic acid) and CO_2 as end products

Examples of the *Lactobacillus* species belonging to different groups are shown in **Table 3.1**

Table 3.1. Division of selected *Lactobacillus* species (Salminen *et al.*, 2011)

Group I, Obligately homofermentative	Group II, Facultatively heterofermentative	Group III, Obligately heterofermentative
L. acidophilus	*L. casei*	*L. brevis*
L. delbrueckii	*L. curvatus*	*L. buchneri*
L. gasseri	*L. paracasei*	*L. fermentum*
L. helveticus	*L. plantarum*	*L. reuteri*
L. jensenii	*L. sakei*	*L. pontis*
L. salivarius		

Genus *Lactobacillus* also possess proteolytic activity. However, Salminen *et al.* (2011) indicated that the organism exhibited low proteolytic activity. Strains belonging to the genus *Lactobacillus* shows weakly proteolytic compared with those belonging to *Lactococcus* species, but higher activity than propionibacteria (Tobiassen *et al.*, 1997)

3.1.3.2. Genus *Bifidobacterium*

Bifidobacterium presents a globally bacillar form, gram-positive, immobile and non–spore-forming. The V-, Y-, or X-shaped forms may be encountered in some *Bifidobacterium* strains because of the difference of composition of the culture medium (Salminen *et al.*, 2011). The mol% GC content of DNA varies from 42 to 67 (mol%) (Felis and Dellaglio, 2007). The genus *Bifidobacterium* includes 29 species (Sgorbati *et al.*, 1995). Generally, bifidobacteria are obligate anaerobic microorganisms. However, the oxygen tolerance level depends on the species and culture medium (Salminen *et al.*, 2011). According to these authors, there are three types of response of *Bifidobacterium* strains to the presence of oxygen in anaerobic environment:

- Aerobic growth without hydrogen peroxide accumulation. Some strains form small quantities of hydrogen peroxide by NADH oxidation. However, the activity of an unknown peroxidase system which could destroy hydrogen peroxide cause the absence of hydrogen peroxide in the growth medium
- Limited aerobic growth with the accumulation of H_2O_2. Accumulation of H_2O_2 may be considered as an inhibitory factor on the key enzyme fructose-6-phosphate phosphoketolase (F6PPK). Therefore, species without a peroxidase system could soon die due to H_2O_2 accumulation in the cells.
- No growth without accumulation of H_2O_2 in the presence of O_2. Such strains are strictly anaerobic bacteria. They require a low redox potential for growth and fermentation.

In the genus *Bifidobacterium,* hexoses are degraded via the fructose-6-phosphate pathway (**Figure 3.2**). It leads to a production of three moles of acetate and two moles of lactate from two moles of glucose. However, the proportions of the final fermentation products vary considerably from one strain to another and even within the same species (Salminen *et al.*, 2011).

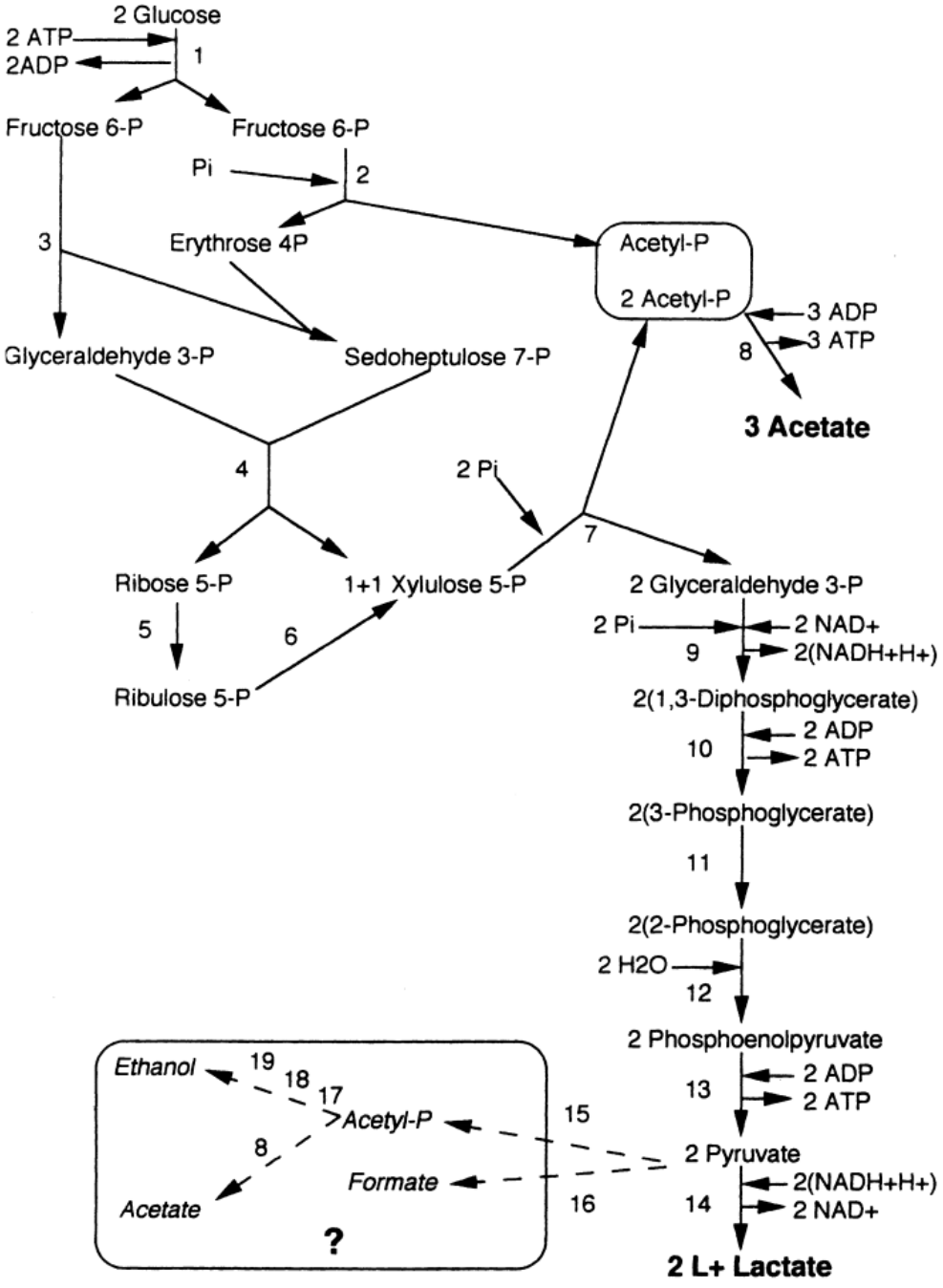


Figure 3.2. Metabolic pathway of *Bifidobacterium*. 1. Hexokinase and glucose-6-phosphate isomerase; 2. Fructose-6-phosphate phosphoketolase; 3. Transaldolase; 5. Ribose-5-phosphate isomerase; 6. Ribulose-5-phosphate epimerase; 7. Xylulose-5-phosphate phosphoketolase; 8. Acetate kinase; 9. Homofermentative pathway enzyme; 10. L (+) lactate dehydrogenase; 11. Phosphoroclastic enzyme; 12. Formate dehydrogenase; 13. Alcohol dehydrogenase (Salminen *et al.*, 2011)

Bifidobacteria are also known to produce vitamins such as: thiamine (B1), riboflavin (B2), pyridoxine (B6), folic acid (B9), cyanocobalamine (B12), nicotinic acid (Fuller, 1989) and ascorbic acid (Holton and Cornish, 1995). Furthermore, several *Bifidobacterium* strains possess β-glucuronidase, β-glucosidase, galactokinase, β-D-galactosidase activity in the final fermentation products (Salminen *et al.*, 2011).

3.2. Tropical fruits

Tropical fruits such as pineapple, mango, banana etc. are fruits produced in the tropical or subtropical regions, and also are known as exotic fruits. They are known as a rich and diverse source of vitamins, minerals and antioxidant compounds (Ellong *et al.*, 2015, Terry, 2011). Therefore, consuming tropical juice confers several health benefits for human. Additionally, tropical fruits possess strong attractive aroma and flavour which may increase consumer consumption and acceptance. Globally, pineapple, mango and banana were among ten kinds of fruits which were produced in the highest quantity in 2018 **(Table 3.2)**.

Table 3.2. The ten, in highest quantity produced fruits in 2018 (FAO, 2018)

Order	Fruit	Quantity (Tons)
1	Bananas	115,737,861
2	Watermelons	103,931,337
3	Apples	86,142,197
4	Grapes	79,125,982
5	Oranges	75,413,374
6	Mangoes, mangosteens, guavas	55,383,785
7	Plantains and others	39,482,164
8	Tangerines, mandarins, clementine, satsumas	34,393,430
9	Fruit, fresh NES*	33,922,019
10	Pineapples	27,924,287

* NES (Not elsewhere specified): Other fresh fruit that are not identified separately because of their minor relevance at the international level based on FAO classification.

These three fruits contain numerous nutrients (**Table 3.3**), as well as free amino acids which have been considered an insufficient component of many fruits (**Table 3.4**).

Table 3.3. Nutrients in pineapple, mango and banana (values/100 g edible portion) (FAO, 2018)

Components	Pineapple	Mango	Banana
Water (g)	86	83.46	74.91
Energy (kcal)	50	60	89
Protein (g)	0.54	0.82	1.09
Total lipid (fat) (g)	0.12	0.38	0.33
Ash (g)	0.22	0.55	1.1
Carbohydrate, by difference (g)	13.12	14.79	22.57
Fiber, total dietary (g)	1.4	1.6	2.6
Sugars, total (g)	9.85	13.66	12.23
Vitamin A (IU)	58	1082	64

Components	Pineapple	Mango	Banana
Vitamin B1 (mg)	0.079	0.028	0.031
Vitamin B2 (mg)	0.032	0.038	0.073
Vitamin B3 (mg)	0.5	0.669	0.665
Vitamin B6 (mg)	0.112	0.119	0.367
Vitamin B9 (µg)	18	43	20
Vitamin C (mg)	47.8	36.4	8.7
Vitamin E (mg)	0.02	0.9	0.1
Vitamin K (µg)	0.7	4.2	0.5
Calcium (mg)	13	11	5
Iron (mg)	0.29	0.16	0.26
Magnesium (mg)	12	10	27
Phosphorus (mg)	8	14	22
Potassium (mg)	109	168	358
Sodium (mg)	1	1	1
Zinc (mg)	0.12	0.09	0.15
Fatty acids, total saturated (g)	0.009	0.092	0.112
Fatty acids, total monounsaturated (g)	0.013	0.14	0.032
Fatty acids, total polyunsaturated (g)	0.04	0.071	0.073

3.2.1. Pineapple

Pineapple (*Ananas comosus L.*) (**Figure 3.3**) is the most economically significant member of the *Bromeliaceae* family (Terry, 2011). For international trade, pineapple cultivars are divided into 4 groups including Cayenne, Abacaxi, Queen and Red Spanish (Ines *et al.*, 2009)

Figure 3.3. Some cultivars of pineapple (Variety: Pineapple Research Station, 2021)

According to FAO (2018), the pineapple is commonly planted and has high production quantity in Costa Rica, Philippines, Brazil, Thailand, China, Indonesia, India, Nigeria, Mexico and Colombia. Among these countries, Costa Rica was responsible for over 11% of the global production. Pineapple has an attractive flavour and refreshing sugar-acid balance resulting in its popularity (Bartolome *et al.*, 1996). Per 100 g edible portion, pineapple contains 13.12 g carbohydrate, variety of vitamin A, B, C, E, K, as well as minerals (**Table 3.3**). Pineapple is also

a rich source of vitamin C (47.8 mg/100 g). Most essential amino acids are found in this fruit (**Table 3.4**). Additionally, the fruit contains a variety of polyphenol compounds, such as gallic acid, gentisic acid, syringic acid, vanillin, ferulic acid, sinapic acid, isoferulic acid, chlorogenic acid, epicatechin, quercitrin and o-coumaric acid (Sopie *et al.*, 2011). These components are responsible for the antioxidant activity which confers many health benefits to the consumer. Terry (2011) reported that the antioxidant capacity in the fruit varies between 0.32 mg GAE/g and 0.52 mg GAE/g. Moreover, Mohd Ali *et al.* (2020) revealed that the most prominent fibre components in pineapple are hemicellulose, cellulose, and pectin.

Table 3.4. Free amino acids in pineapple, mango and banana

Amino acid (mg/L)	**Pineapple (mg/L)** (Pimentel, 2017)	**Mango (mg/L)** (Pimentel, 2017)	**Banana (µmol/100g)** (Ito *et al.*, 2017)
Histidine	22	8	228.20
Arginine	17	358	58.14
Methionine	11	6	1.26
Valine	24	16	20.63
Phenylalanine	20	19	18.21
Isoleucine	12	10	9.79
Leucine	13	9.84	59.80
Lysine	14	73	11.36
Serine	83	147	65.38
Glycine	13	12	27.86
Alanine	111	86	9.84
Tyrosine	26	50	14.57
Aspartate	51	370	3.76
Glutamate	106	96	2.29

3.2.2. Mango

Mango (*Mangifera indica L.*) belongs to the *Anacardiaceae* family which consists of numerous species of tropical fruit (Terry, 2011). India is documented the top position among mango, mangosteen and guava producing countries in the world with over 36% of the total quantity in 2018. The following countries are China, Thailand, Indonesia, Pakistan, Mexico, Brazil, Malawi and Bangladesh (FAO, 2018).

Mango (**Figure 3.4**) is known as a rich source of vitamin A and C, containing 1082 mg and 36.4 mg of these vitamins per 100 g edible portion of this fruit, respectively (**Table 3.3**). Arginine (358 mg/L) is the most prominent of the essential amino acid found in mango juice (**Table 3.4**). Besides the nutrient components, mango contains high concentration of pectin (8.2 g/100 g of pulp) which is the most important healthy prebiotic fiber (Maldonado-Celis *et al.*, 2019). Furthermore, mango presented several important phytochemicals such as cryptoxanthin, lutein,

gallic acid and anacardic acid, etc. (Kasa, 2017, Maldonado-Celis *et al.*, 2019). Gallic acid and hydrolysable tannins are reported as the major antioxidant polyphenolics in mango (Terry, 2011).

Figure 3.4. Some cultivars of mango (Mango cultivar differences, 2016)

3.2.3. Banana

Banana *(*species of genus *Musa)* (**Figure 3.5**) is important edible fruit of the family *Musacea* (Terry, 2011), and is one of the highest consumption fruit in the world. India with over 24% total production quantity of the world in 2018 was considered as the leading producers of banana, followed by China, Indonesia, Brazil, Ecuador, Philippines, Guatemala, Colombo and Angola (FAO, 2018).

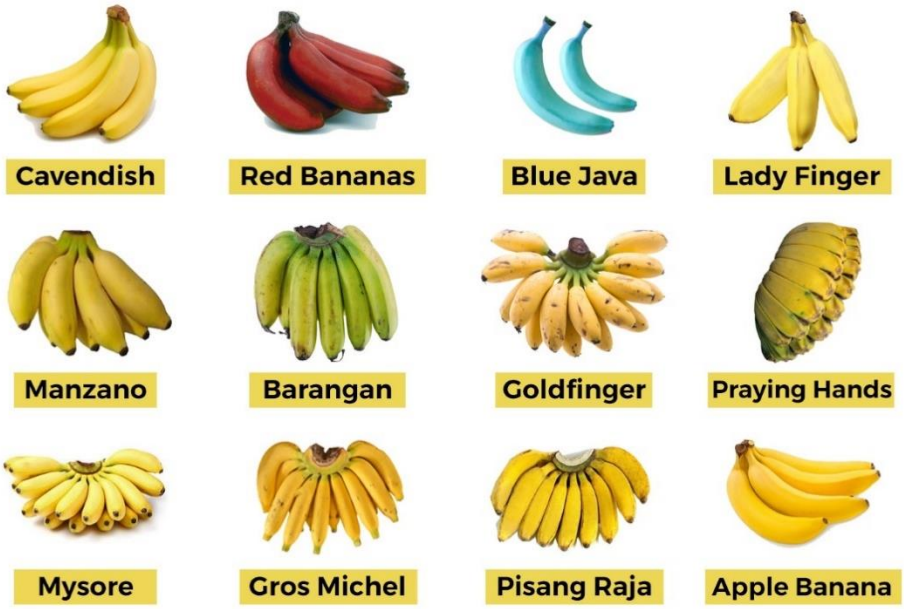


Figure 3.5. Some cultivars of banana (Only foods, 2020)

This fruit provides a good amount of carbohydrate, mineral, vitamin and many other important phytochemicals. Each 100 g edible portion of banana contains 22.57 g carbohydrate, 358 mg potassium, 27 mg magnesium and 22 mg phosphorus (**Table 3.4**). Terry (2011) reported that catechin, gallocatechin, epicatechin and condensed tannins are responsible for antioxidant activity of this fruit. Someya *et al*. (2002) revealed that gallocatechin concentration in banana pulp was 0.3 mg/g. Additionally, other antioxidant compounds, such as lutein, zea-xanthin and α-carotenes, also presented in banana (Sidhu and Zafar, 2018). According to Alothman *et al*. (2009) and Lim *et al*. (2007), the TPC value ranged between 0.24 and 0.72 mg GAE/g depending on the extraction method. The fruit is also known as a rich source of fructooligosaccharides (Terry, 2011).

3.3. Probiotic beverage

3.3.1. Probiotic fruit drink actual market

The probiotic drink market is growing globally and represents one of the most promising areas of investigation and innovation in the food sector (Patel, 2017). According to the forecasts of Grand View Research (2020), the global probiotic drink market size was valued at US $13.65 billion in 2019 and will reach $21.95 billion by 2027. The increased demand for probiotic products resulted from the rising consumers' awareness of the connection between food and health. The health benefits of using probiotic products were introduced, such as reduction in gastrointestinal infections, antimicrobial activity, improvement in lactose metabolism, reduction in serum cholesterol, stimulation of the immune system, antimutagenic, anticarcinogenic and antidiarrheal properties, improvement in symptoms of irritable bowel syndrome, suppression of *Helicobacter pylori* infections, and reduction of obesity and atopic dermatitis (Pimentel, 2017). In a survey of Chaiyasut *et al*. (2017) on 235 people who have consumed probiotic products, all the consumers reported that they consume the probiotic products to improve health conditions and treat the health problems.

Plant-based products have been concerned by consumer nowadays because of their given health benefits, such as low content of cholesterol, allergenic proteins, as well as ongoing trend of vegetarianism. In probiotic drinks market, plant-based products held the revenue share of 45% in 2019 while the dairy-based one took the largest revenue (55%). However, plant-based products are expected to experience the fastest growth over the forecast period. Over the past few years, the demand for plant-based drinks, including probiotic fruit and vegetable juices, is increasing significantly (Grand View Research, 2020).

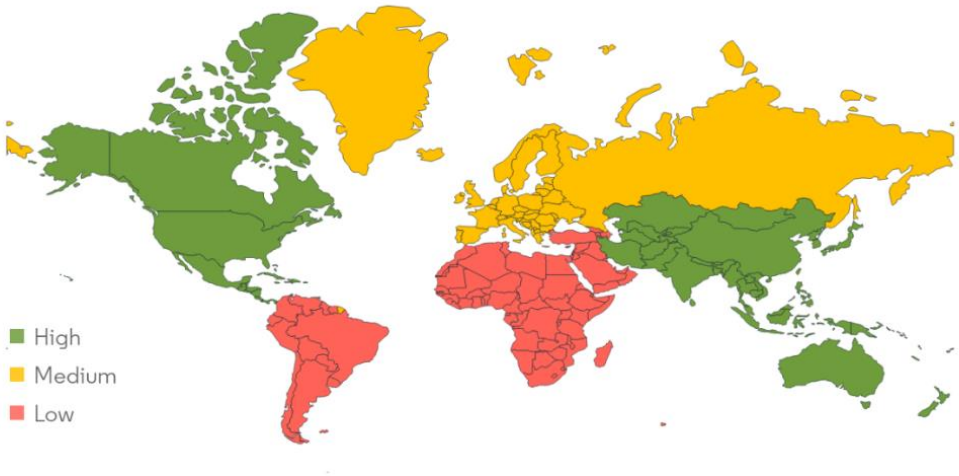


Figure 3.6. Probiotic drinks market in 2018 (Grand View Research, 2020)

The Asia Pacific region presented as a huge market for probiotic drink products (**Figure 3.6**). In this region, China, India and Japan are the dominant markets. The rising health concerns of consumers along with the expanding availability of the ready-to-drink products may cause the fast growth of the plant-based probiotic product in the upcoming years. Some probiotics juices and related beverages available in market are shown in **Table 3.5.**

Table 3.5. Commercially available probiotic juices

Product label	**Company**	**Substrate**	**Probiotic microorganisms**
Plant-based drinkable yoghurts	Califia Farms, USA	Almond, coconut, oat, strawberry and mango	*B. lactis* Bb12
Obi probiotic organic soda	Organic Soda Pops, USA	Fruit juice	*Kefir culture*
Rela	Biogaia, Sweden	Fruit juice	*L. reuteri* MM53
Bravo Friscus	Skanemajerier, Sweden	Orange, apple juice	*L. plantarum* HEAL 9, *L. paracasei* 8700:2
Wild probiotic	Sujajuice, USA	Lemon, cayenne, maqui berry	*Bacillus coagulans* GBI-30 6086
Tropicana essentials probiotics	PepsiCo, USA	Strawberry, banana, pineapple, mango and peach passion	*B. lactis*
Biola	Tine, Norway	Orange, mango, apple	*L. rhamnosus* GG
Kevita, Sparkling probiotic drink	PepsiCo, USA	Mango, coconut	Mixture of *Bacillus coagulans* GBI-306086, *L. paracasei* 8700:2, *L. plantarum* HEAL 9
Gefilus fruit drinks	Valio, Finland	Berries	*L. rhamnosus* GG, *Propionibacterium fredenreichii* ssp *shermanii* JS

Product label	Company	Substrate	Probiotic microorganisms
Bioprofit	Valio, Finland	Fruit juice	*L. rhamnosus* GG, *Probionibacterium freudenreichii shermanii* JS
Malee probiotics	Malee enterprise, Thailand	Prune, grape and orange	*L. paracasei*
Goodbelly probiotics	NextFoods, UK	Fruit juices	*L. plantarum* 299v
Healthy life probiotics	Golden circle, Australia	Fruit juices	*L. paracasei* 8700:2, *L. plantarum* HEAl 9
Proviva	Skane Dairy, Sweden	Fruit juice with oatmeal	*L. plantarum* 299v

3.3.2. Recent development of probiotic fruit drinks

Traditionally, most probiotics products available commercial are dairy-based, such as yoghurt, cheese, cultured milks. Indeed, yoghurt is responsible for 78% of current probiotic sales in the world today (Granato *et al.*, 2010). Using milk as a substrate for fermentation began for thousands of years ago because milk exhibited a potential matrix for inoculation of probiotic microorganisms. However, up to 70% of the world population is affected by lactose-intolerance (Perricone *et al.*, 2015). Moreover, high content of cholesterol, allergenic milk proteins in milk, as well as ongoing trend of vegetarianism limited the dairy based probiotic consumption. Nowadays, along with increase of consumers needs and technological advances, many substrates have been studied to alter milk in probiotic production. Fruit juices, vegetable, soy, and cereal are probably suitable media for delivering probiotics (Granato *et al.*, 2010). They already contain minerals, vitamins, dietary fibers, and antioxidants which support the growth of probiotic microorganisms while lacking the dairy allergens that affect consumer's health.

3.3.2.1 Probiotic fruit drinks with monoculture

Garcia *et al*. (2020) observed a noticeable increase in article number of scientific publications about fermented fruit and vegetable beverages on during the last 20 years. The scientific interest is growing for beverages such as fermented fruit and vegetable juices, as they seem to be new substrate for probiotic delivery, confer numerous nutritional advantages and win consumer preference. Some probiotic fruit drink products recently developed are shown in **Table 3.6**, which also suggests suitability of various fruit juices for the growth of probiotic bacteria. *Lactobacillus* and *Bifidobacterium* were the most commonly used probiotic strains in these food products. Although, these researches indicated the well growth of probiotic strains in juices, further studies are still needed to be carried out in order to obtain a well-accepted beverage, because the presence of probiotic in juice may affect sensory characteristics of products (Luckow and Delahunty, 2004). Hence, Garcia *et al*. (2020) suggested that the quality of products can be improved by adjustment of the mix of fruit or vegetables or choice of starters. In this way, the amounts of undesirable compounds, flavours or biogenic amines may reduce.

Table 3.6. Some probiotic fruit drinks

Juice	Strain	Results	Reference
Grapefruit	*L. plantarum* 01, *L. fermentum* D13, *L. rhamnosus* B01725, *B. bifidum* B7.5	In all cases, cell counts were 10^8–10^9 CFU/mL after 24 hours of fermentation	Tran *et al.* (2020)
Bergamot	*L. plantarum* AF1	After 72 hours fermentation at 37 °C, viability of probiotic bacteria increased to 8.9 ± 0.1 log CFU/mL	Hashemi and Jafarpour (2020)
Sohiong	*L. plantarum* MCC 2974	After fermentation at 37 °C for 72 h, the number cell counts increased up to 10 log CFU/mL. The probiotic microbe population was higher than 6 log CFU/mL after four weeks of storage at 4 ± 1 °C	Vivek *et al.* (2019)
Cocoa pulp	*L. casei*	The pH value and microbial population of the juice after fermentation at the optimum conditions of initial pH 6.2, temperature of 33°C and fermentation time of 12 hours reached 4.32± 0.01, 8.76 ± 0.05 log CFU/mL, respectively	Santos Filho *et al.* (2019)
Fig	*L. delbrueckii*	Viability of *L. delbrueckii* was increased during incubation (8.41 log CFU/mL). But a significant reduction was observed during storage time (6.59 log CFU/mL)	Khezri *et al.* (2018)
Pomegranate	*L. plantarum* ATCC 14917	Cell viability retained in high levels after the 24 h of fermentation and storage for 4 weeks (above 8.8 log CFU/mL)	Mantzourani *et al.* (2018)
Cupuassu	*L. casei*	The population reached 9.34 log CFU/mL after fermentation	Pereira *et al.* (2017)
Sweet lemon	*L. plantarum* LS5	The cell counts of the *L. plantarum* LS5 increased from 7.0 ± 0.1 to 8.63 ± 0.38 log CFU/mL during fermentation (37°C for 48 h) and decreased from 8.63 ± 0.38 to 7.14 ± 0.21 log CFU/mL after storage (4°C for 28 d)	Hashemi *et al.* (2017)

Juice	**Strain**	**Results**	**Reference**
Carrot-orange	*L. acidophilus*	The population reached 8.34 log CFU/mL. After 40 days of cold storage, the value decreased to 7.77 log CFU/mL	Valero-Cases and Frutos (2017a)
Apricot	*B. lactis* Bb12, *B. longum* Bb46, *L. casei* 01, *L. acidophilus* La5	All tested strains exhibited good growth properties on apricot juice without any nutrient supplementation	Bujna *et al.* (2017)
Conelian cherry	*L. casei* T4	The bacterium can grow well in juice (reached 8.67 log CFU/mL after fermentation). It remained their population during 28 days of cold storage at pH 3.5 but could not withstand at pH 2.6 after 7 days	Nematollahi *et al.* (2016)
Peach	*L. delbrueckii, L. casei*	The bacteria grew well in peach juice, reached nearly 10×10^9 CFU/mL. After four weeks of cold storage at 4 °C, the viable cell counts of *L. delbrueckii* were 1.72×10^7 CFU/mL while *L. casei* could not survive in fermented juice after the cold storage	Pakbin *et al.* (2014)
Watermelon and tomato	*L. fermentum, L.casei.*	The population reached 2.3-9.4 × 10^8 CFU/mL after fermentation. *L. fermentum* had a significant decrease after storage (3×10^4 CFU/mL), while *L. casei* remained 1.7×10^6 CFU/mL	Seelam *et al.* (2014)
Pomegranate	*L. plantarum, L. acidophilus*	Both bacteria were able to grow in the juice and their viable cells reached to 3.9×10^8 CFU/mL after 72 h of fermentation	Mousavi *et al.* (2013)
Pineapple	*L. casei* NRRL B442	Maximal microbial viability was 8.65 log CFU/mL. After storage at 4°C/42 days, the number was 6.03 log CFU/mL	Costa *et al.* (2013)
Cashew apple	*L. casei*	The population reached 8.41 log CFU/mL at the end of fermentation process. After 35 days of storage, viable cell count was 8.62 log CFU/mL	Pereira *et al.* (2011)
Noni	*L. casei, L. plantarum, B. longum*	All tested strains grew well in noni juice, reaching nearly 10^9 CFU/mL after 48 h fermentation. After 4 weeks of cold storage at 4°C, *B. longum* and *L. plantarum* survived while *L. casei* exhibited no cell viability in fermented noni juice after 3 weeks	Wang *et al.* (2009)

3.3.2.2 Probiotic fruit drinks with mixed cultures

The combination of different strains results in improved qualities of product such as aroma, texture, sensory properties, as well as enhance the viability and survival of probiotic microorganisms in harsh conditions, such as the low pH of the digestion system (Bujna *et al.*, 2017, Soni *et al.*, 2020). However, it should be noted that not all strains can be mixed because they might be synergistic or antagonistic effect on each other (Soni *et al.*, 2020). There is a lack of research on mixed cultures for probiotic product because such research is more difficult to conduct and thus more expensive. Recently, some mixed culture probiotic products were developed and the benefits of combination in strains were reported.

From the health aspect, the combination of *Lactobacillus* and *Bifidobacterium* confers some positive effects on the human digestive system. Rinninella *et al.* (2019) introduced that gut microbiota are composed of several phyla, including: *Actinobacteria, Firmicutes, Bacteroidetes, Proteobacteria*, *Fusobacteria* and *Verrucomicrobia*. The *Actinobacteria* phylum mainly represented by the *Bifidobacterium* genus. The *Firmicutes* phylum is composed of more than 200 different genera such as *Lactobacillus*, *Bacillus*, *Clostridium*, *Enterococcus,* and *Ruminicoccus*. *Bacteroidetes* consists of predominant genera such as *Bacteroides* and *Prevotella*. Odamaki *et al.* (2016) reported that the composition of human gut microbiota changes with age. These authors carried out an experiment on faecal samples from 367 healthy Japanese persons between the ages of 0 and 104 years. The results showed that the gut microbiota was predominant by four phyla: *Firmicutes, Bacteroidetes, Actinobacteria* and *Proteobacteria*. *Actinobacteria* were the predominant phylum in gut during the first years of age, then decreased after weaning and continued to decrease with age. *Firmicutes* was the most predominant phylum after weaning. This phylum decreases with age but keep predominating in gut microbiota afterward. *Bacteroidetes* and *Proteobacteria* increased in subjects over 70 years of age. Therefore, in order to maintain a healthy gut, the supplement of genus in *Actinobacteria* and *Firmicutes* phylum are necessary. Indeed, Alard *et al.* (2018) reported that the change of intestinal microbiome may be related to inflammatory bowel disease. The reduced diversity of the gut microbiota in inflammatory bowel disease patients is largely due to a low abundance of probiotics belonging to the *Lactobacillus* and *Bifidobacterium* genera. In a study of Li *et al.* (2019), they revealed that the combination of *L. acidophilus* and *B. animalis* subsp. *lactis* exerted a potent anti-inflammatory effect in the gut, while a weaker anti-inflammatory effect was observed in case of individual strains. The benefit of *Lactobacillus* and *Bifidobacterium* combination was also demonstrated in a study of Pilarczyk-Zurek *et al.* (2017). Their results show that consuming the mixture of *L. plantarum, L. rhamnosus,* and *B. longum* once a day for at least 2 months was efficacious in inducing and maintaining remission in ulcerative colitis.

From technological aspect, the first advantage of the combination is enhancement of growth. Wang *et al.* (2003) noted that the cell counts of *B. longum* in combination with *L. acidophilus* were significantly higher than that in soymilk fermented by an individual organism after 24 h of fermentation. Bujna *et al.* (2017) reported the fermentation of apricot juice with the combination of *Bifidobacterium* and LAB resulted in higher levels in microbial cell counts than

found in monocultures. Murti *et al.* (2006) reported that the presence of bifidobacteria in soymilk fermentation stimulated the growth of yoghurt bacteria. The improved growth of bacteria results in shorter the fermentation time, as well as improved nutrient content and increased palatability. Indeed, Božanić *et al.* (2011) added *B. lactis* subsp. *animalis* Bb12 with yoghurt culture YCX11 (*Streptococcus thermophilus* and *L. delbrueckii* ssp. *bulgaricus*) to shorten the fermentation time of soy yoghurt production to 4 hours, instead of 12-17 hours of fermentation with individual strains. Wang *et al.* (2002) revealed that the combination of *L. acidophilus* and *Bifidobacterium* resulted maximum cell counts sooner than when growing alone in soymilk. Bujna *et al.* (2017) reported a significant decline of pH from 6.6 to 4.6-4.9 after the fermentation of apricot juice with combinations of *Bifidobacterium* and *Lactobacillus* strains. These authors claimed that the drop of pH was due to the intensive growth and metabolic activity of probiotic bacteria. Soni *et al.* (2020) produced yoghurt with the mixed cultures of *L. plantarum* - *L. casei*, *L. acidophilus* - *B. bifidum.* Their results showed that yoghurt with the mixed cultures of *L. plantarum* - *L. casei* contained more protein, carbohydrate, calcium, higher viscosity and lower syneresis, exhibited higher acid tolerance and consumer acceptability with more sourness and less sweetness. While in yoghurt with the combination of *L. acidophilus* - *B. bifidum* increased total soluble solids. It also reduced the pH and syneresis as compared to yoghurt with a single bacterial probiotic source.

3.4. Fermentation

3.4.1. Fermentation conditions

The growth of probiotic microorganisms is affected by fermentation conditions, such as nutrients, pH, temperature and others (Costa *et al.*, 2013). The probiotic bacteria, in particular *Lactobacillus* and *Bifidobacterium*, require fastidious media which are rich in nutrients (amino acids, nucleotides and vitamins). Although fruit juices have been considered as a good matrix for delivery probiotic, they have presented the insufficient amounts of peptides and free amino acids required for probiotics (Pimentel, 2017). Compared to other substrates (milk, soymilk, cereal milk), the free amino acid in fruit juice is significant lower (Dove *et al.*, 2009). Furthermore, plant-based substrate contains glycosides that gives detrimental effects on microorganisms. Anandharaj *et al.* (2014) reported that the high solanine content in unripe vegetable (such as tomato) might inhibit the growth of LAB. *L. reuteri* strains was inhibited in the presence of stevia glycosides (Deniņa *et al.*, 2013). Quercetin can inhibit *Lactobacillus* and *Bifidobacterium* with concentration of over 50 µg/mL while naringenin with 250 µg/mL. Over 250 µg/mL of hesperetin or catechin has negative effect on *Bifidobacterium* (Duda-Chodak, 2012). *B. longum* B7254 can grow well in milk but has a low growth in legume milks with high concentration of flavonoids (Di Gioia *et al.*, 2014).

The pH is considered as the most important factor effecting the survival of probiotic bacteria (Perricone *et al.*, 2015, Song *et al.*, 2012). According to Salminen *et al.* (2011), the initial optimum growth pH is between 6.5 and 7.0. The *Lactobacillus* organisms grow in slightly acidic media at pH of 6.4-4.5, but growth stops at a pH of 4.0-3.6 (Rivera-Espinoza and Gallardo-

Navarro, 2010, Song *et al.*, 2012). In fruits, pH is usually range from pH 2 to pH 4.5. Moreover, fruits contain high level of organic acid. The combined biological effect of low pH and organic acid presenting in an environment may expose acid stress on micro-organism. In this sense, Sheehan *et al.* (2007) assessing survival of *Lactobacillus* and *Bifidobacterium* strains in orange juice (OJ), pineapple juice (PJ) and cranberry juice (CJ). *L. salivarius* ssp. *salivarius* UCC118, *L. salivarius* ssp. *salivarius* UCC500 survived in OJ and PJ in 2 weeks. *L. paracasei* ssp. *paracasei* NFBC43338, *L. rhamnosus* GG, *L. casei* DN-114, *B. animalis* ssp. *lactis* Bb12 survived for longer in OJ and PJ compared to CJ. The results showed that *L. casei* DN-114 001, *L. rhamnosus* GG and *L. paracasei* NFBC43338 displayed the greatest robustness surviving at levels above 10^7 CFU/mL in OJ and above 10^6 CFU/mL in PJ for at least 12 weeks at 4°C. Notably, the lower pH of PJ (pH 3.4) in comparison to OJ (pH 3.65) resulted in a faster rate of decline in the viability of probiotic strains. Nematollahi *et al.* (2016) reported that Iranian native probiotic strain (*L. casei* T4) could not withstand the conditions of cornelian cherry juice at pH 2.6 for more than 7 days of storage. However, the effect depends on the strains and substrates, also. Nualkaekul *et al.* (2011) reported that *B. longum* NCIMB 8809 can survive after 6 weeks of storage in orange and pineapple juice, which had a pH of about 3.8. Although the pH was similar (pH 3.2) in grapefruit and blackcurrant, the log decrease of the organism population was quite different (0.4 and 0.7 log, respectively). The highest loss of cell number (8 log) of the bacterium was observed in pomegranate and strawberry juice (Nualkaekul *et al.* (2011).

Besides pH factor, temperature is also the critical parameter for probiotic growth and survival. *Lactobacillus* and *Bifidobacterium* have the optimum temperature range of 30°C-40°C. *Lactobacillus* can grow at a temperature of 45°C; however, the optimum is found between 35 and 40°C (Song *et al.*, 2012). For *Bifidobacterium* species from human, the optimal growth temperature is in the range of 36°C and 38°C while, that is slightly higher, about 41°C-43°C for the animal species (Salminen *et al.*, 2011). Song *et al.* (2012) also revealed that *L. rhamnosus, L. casei* and *L. plantarum* can grow well in plant-based substrates during fermentation when the fermentation temperature is adjusted at 37°C. Similar reports were found in other studies such as Kun *et al.* (2008) fermented the carrot juice with *Bifidobacterium* strains, Champagne and Gardner (2008) developed the commercial fruit drinks fermented by *L. acidophilus* and *L. rhamnosus*, Bujna *et al.* (2017) produced the apricot juice using *Lactobacillus* and *Bifidobacterium* strains. A lower temperature can reduce or inhibit the growth of probiotic bacteria. Pereira *et al.* (2011) optimized the fermentation conditions of *L. casei* NRRL B442 in cashew apple juice. They revealed that the bacteria cannot grow under 15°C during 24 h of fermentation.

3.4.2. Metabolisms of probiotic bacteria in fermentation

Fermentation occurring in juice leads to a bioconversion process, along with the release of the metabolites into the culture medium. Generally, probiotic fermentation confers several advantages, such as enhancement of the bioavailability of nutritive compounds, degradation of toxic and anti-nutritional compounds, generation of bioactive molecules (Garcia *et al.*, 2020).

2.4.2.1. Acid and sugar

Theoretically, homolactic fermentation of glucose results in 2 mol of lactic acid per mol glucose consumed. Meanwhile heterolactic fermentation of 1 mol glucose gives 1 mol each of lactic acid, ethanol and CO_2, whereas the *Bifidobacterium* ferments 2 mol glucose and results an acetic acid/lactic acid ratio of about 3/2 at the end. Thus, lactic acid is recognized as the main metabolite of LAB as well as *Bifidobacterium* metabolism. Mousavi *et al.* (2013) revealed that lactic acid production is more noticeable than other acids (acetic, formic and propionic) in the fermentation of pomegranate juice using *L. plantarum* and *L. acidophilus*. The lactic acid level also varied depending on strains, substrates, fermentation conditions. Herein, they reported that the lactic acid concentration in fermented pomegranate juice with *L. plantarum* was significantly higher than that using *L. acidophilus*. In terms of quantity, formic and propionic acids were the next main organic acid produced by both investigated bacteria, while acetic acid constituted the least proportion of the produced organic acids.

The acid accumulation in fermented juice is due to sugar metabolism of bacteria. Mousavi *et al.* (2013) observed the reduction of concentration of both sugars (glucose and fructose) during fermentation process. The rate of glucose consumption was significantly higher than fructose. The level of both sugars consumed by *L. acidophilus* was significantly lower than by *L. plantarum*. Besides sugars, these selected probiotic bacteria were able to metabolize citric acid as a carbon source in the juice. As mentioned above, LAB produce more lactic acid than acetic acid and bacteria which utilize glucose through heterofermentative way produce a higher level of acetic acid than homofermentative bacteria during fermentation. However, Zalán *et al.* (2011) revealed that the ratio of acetic acid and lactic acid were not only affected by heterofermentative nature of strains, but by carbon source too. These authors fermented *Jerusalem artichoke* juice containing the well-known carbohydrate inulin, using ten *Lactobacillus* strains. They reported that *L. casei* subsp. *casei* 154 and *L. paracasei* subsp. *paracasei* 2750 produced significant amounts of lactic, succinic and acetic acids during fermentation. The acetic acid production of these strains was greater than that of lactic acid. They also revealed that the high acetic acid strains produced succinic acid and the amounts of these acids were in correlation of each other. They also indicated that all the investigated strains could utilize inulin and its derivatives for growth. Chen *et al.* (2018) reported that the concentration of lactic acid, formic acid, pyruvic acid, malic acid and acetic acid significantly increased after fermentation of papaya juice by *L. acidophilus* and *L. plantarum*. *L. plantarum* produced more lactic and acetic acid than *L. acidophilus* did. They also mentioned that tartaric acid may be used for microbial metabolism during the fermentation process since there was significantly decreasing in its concentration.

2.4.2.2. Polyphenol and antioxidant

Besides the production of organic acids, probiotic bacteria also have the ability to enhance the activity of some bioactive compounds through fermentation. In case of beverages fermented with various probiotic strains have been largely reported to increase the antioxidant activity,

sometimes together with an increase in total phenols and flavonoids content (Garcia *et al.*, 2020). Mousavi *et al.* (2013) reported that delphinidine 3-glucoside, cyanidin 3,5-diglucosides, pelargonidin 3,5-diglucosides and ellagic acids recognized as the main phenolic compounds present in pomegranate juice, were metabolized by *Lactobacillus* and *Bifidobacterium* strains. These compounds significantly decreased after fermentation with *Bifidobacterium* and *Lactobacillus*. 3-Glucoside compounds were observed as the highest loss in the bioactive compounds. It was explained that those bacteria had β-glucosidase activity and participated in the hydrolysis of plant β-glycosides which change glycosylated anthocyanins form into aglycone. Then, the free sugar could be metabolized and consumed by probiotic lactic acid bacteria. Regarding to antioxidant activity, it was indicated that lactic acid fermentation was performed by both probiotic strains improving the antioxidant activity of pomegranate juice. The samples fermented by *L. acidophilus* had significantly higher antioxidant activity compared to ones by *L. plantarum.* The change from glycoside form into aglycone form of anthocyanin compounds resulted the higher radical scavenging effect. Chen *et al.* (2018) reported that the total phenolic, carotenoid and vitamin C contents in fermented papaya juice with *L. acidophilus* and *L. plantarum* decreased significantly after fermentation while total flavonoids content showed opposite trend. Because the juice contains various antioxidant compounds which may act against oxidizing agents through different mechanisms, these authors used four methods of assessing antioxidants, including 2,2-Diphenyl-1-Picrylhydrazyl (DPPH), 2,2-Azinobis-(3-Ethylbenzothiazoline-6-Sulfonate) (ABTS), Ferric Reducing Antioxidant Power (FRAP) and Cupric Ion Reducing Antioxidant Capacity (CUPRAC). A significant decrease in antioxidant activity of the fermented papaya juice using *L. acidophilus* was observed, whereas in the case of *L. plantarum* a slight increase of these values was reported. The decrease in the phenolic compounds in the papaya juices during probiotics fermentation was due to their precipitation or oxidation during the process, the combination or adsorption of phenolic compounds with solids, proteins and polymerization. The increase in total flavonoids content was explained by enzymatic degradation and acid production by the strain that facilitated the release of phenolics and flavanones from their complexed forms into freely soluble forms. Liu *et al.* (2018) reported that the ABTS and DPPH inhibition values, as well as the FRAP and total phenolic content, significantly increased in fermented tomato juice using *L. plantarum* and *L. casei*. They also revealed that all analysed probiotic bacteria can produce exopolysaccharides (EPS) under laboratory conditions, which have strong antibacterial abilities and scavenging activities. For *L. plantarum*, r-EPS1 and r-EPS2 represent potent antioxidative activities for hydroxyl and DPPH radical scavenging and for reducing power assays. For *L. casei*, the bio-surfactants from the strains exhibit considerable antioxidant and antiproliferative potencies.

2.4.2.3. Hydrogen peroxide production

In the presence of oxygen, lactic acid bacteria are able to generate hydrogen peroxide (Zalán *et al.*, 2011), however, Fontaine *et al.* (2009) argue that hydrogen peroxide does not accumulate to significant amounts *in vivo*, because it is decomposed by peroxidases, flavoproteins

and pseudocatalase. Hydrogen peroxide is a strong oxidizing agent and thus a well-known antibacterial component. Hydrogen peroxide affects the bacterial cell through oxidizing sulfhydryl groups of cell proteins and membrane lipids, however the oxidizing effect also can cause bleaching of coloured components and in this way causes undesired loss of colour of the product, besides degradation of the antioxidant components. Zalán *et al.* (2011) reported that *Lactobacillus* strain produced H_2O_2 in the range of 0.25-1.77 (mg/L) during the fermentation of *Jerusalem artichoke* juice. They also found that the *Lactobacillus* strains, including *L. plantarum* 2142, *L. curvatus* 2770, *L. casei* subsp. *pseudoplantarum* 2750 and *L. casei* Shirota, accumulated hydrogen peroxide in tomato juice broth, however, the levels of H_2O_2 in juice were significantly lower than that in MRS medium. *L. casei* subsp. *pseudoplantarum* 2750 seemed to be the best peroxide producer of hydrogen peroxide in the juice (Zalán *et al.*, 2005). Awojobi *et al.* (2016) revealed that LAB extended the shelf life of pineapple juice due to their high antimicrobial effect on foodborne contaminants. The antimicrobial compounds produced by *L. fermentum, L. plantarum* and *L. lactis* including lactic acid, diacetyl and hydrogen peroxide were investigated by these authors. Their results show that the concentration of hydrogen peroxide produced by *L. fermentum* and *L. plantarum* was 0.03 g/L. A light lower concentration was reported in the case of *L. lactis* (0.02 g/L).

Other factors such as polyphenol concentration, glucose concentration, pH of the medium also affect the production of hydrogen peroxide of probiotic bacteria. Piekarska-Radzik and Klewicka (2021) indicated that polyphenols which are particularly abundant in plant-based food can form hydrogen peroxide through their oxidation process. The quantity of H_2O_2 produced by *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 was lower at pH 5.0 than at pH 6.5 (Kot *et al.*, 1996). H_2O_2 production was 288 nmol/mL after 60 min of incubation at pH 6.5. At pH 5.0, a significant lower value was reported. Furthermore, these authors indicated that in the absence of glucose, only very less amounts of H_2O_2 were produced.

2.4.2.4. Aroma formation

The sensory properties of a product are important quality parameters that affect the status of the finished product in the market and whether the consumer will like and buy the product (Panghal *et al.*, 2018). Flavours and aroma are important sensory parameters of food products. Depending on the enzymes present in the starter cultures used, different flavours can develop and thus different products from the point of view of their aroma (Zalán *et al.*, 2011). For fermented fruit juice, the impact of microorganisms on organoleptic parameters of the product has not been deeply studied yet, however, some reports assumed that the presence of probiotic bacteria in a fruit juice would confer different taste profiles compared to the juice (Luckow and Delahunty, 2004, Mantzourani *et al.*, 2018, Pimentel *et al.*, 2015). In LAB, pyruvate is a starting molecule for the formation of short-chain flavour compounds such as acetaldehyde, acetate, acetoin, diacetyl and ethanol. *Lactobacillus* can also metabolize citrate to produce acetoin, acetolactate and diacetyl. Amino acids of the substrate may contribute to the production of flavour and aroma substances such as aldehydes, acids, alcohols, esters and sulphur compounds. Alves Filho *et al.* (2017)

evaluated the volatile profile of probiotic melon and probiotic cashew juice fermented by *L. casei*. Originally, total of 38 and 11 volatile compounds were identified in melon and cashew apple juice, respectively. They mentioned that the lactic acid fermentation of fruits showed a volatile profile with slightly formation or degradation of aroma compounds. In the case of melon juice, decanal, 2-decanal, 3-phenylpropyl acetate, methyl diethyl carbamodi-thioic acid and hexadecanal were found only after the fermentation while the compounds 2,5-octanedione, hexyl acetate, eucalyptol, phenylacetaldehyde and nonanal were disappeared. In the case of cashew juice samples, the compounds ethyl 2-methylbutanoate, benzaldehyde, α-ocimene and decanal were found only before fermentation, while ethyl benzoate was detected only after the fermentation. Interestingly, the compounds acetaldehyde, diacetyl, propanoic acid, acetic acid, acetone, acetoin, formic acid, butanoic acid, dimethylsulfide, benzaldehyde and 2,3-pentanodione, which usually present in yoghurts' character, were not found in both fermented juices. Mantzourani *et al.* (2018) determined 11 alcohols, 11 aldehydes, 11 ketones, 10 esters, 7 terpenoids and furfural in pomegranate beverage. The fermentation process mainly affected on the groups of volatile compounds, including aldehydes, ketones, alcohols and esters. Fermentation of the pomegranate by *Lactobacillus paracasei* K5 certainly enhanced the aromatic profile of the pomegranate beverage through the production of desirable volatile compounds. Liu *et al.* (2018) also found the influence of fermentation on formation of aromatic compounds on tomato juice using *L. casei* and *L. plantarum*. An increase in alcohols, acids, and ketones level and decrease in hydrocarbons, aldehydes and esters were observed. They claimed that these changes were probably due to the activity and variety of enzymes related to *L. plantarum* and *L. casei*. Chen *et al.* (2018) reported that papaya juice contains acids, alcohols, esters, aldehydes, ketones and phenols as volatile compounds and esters and alcohols are the main aroma components. After fermentation papaya juice using *L. plantarum* and *L. acidophilus*, they observed that the aroma components were similar after fermentation but in different proportions. The volatile profiles given by *L. acidophilus* and *L. plantarum* were quite similar, although more alcohols and aldehydes were globally found with *L. plantarum.*

The change of flavour and aroma components in final products leads to the change of sensory attribute. In a study of Luckow and Delahunty (2004) on consumer acceptance of functional orange juice, they revealed that the consumer recognized the "dirty", "earthy", "medicine" aroma and flavour in orange juice fortified with probiotic. And finally, for consumers were completely unacceptable these attributes presented in orange juice. The study of Tuorila and Cardello (2002) showed that consumer would not be willing to consume a functional beverage, if they recognize off-flavours, even though information about health benefits was provided. In order to eliminate the "medicinal" off-flavours of the probiotic cultures, Luckow *et al.* (2006) masked functional orange juice with 10% of a tropical juice concentrate (containing pineapple, mango and passionfruit juice). The results show that tropical juice was successful in masking the off-flavours associated with probiotic ingredients. Furthermore, the consumers showed a high acceptance of the product with 10% supplement of tropical juice. These authors also revealed that providing

consumers with health benefit information associated with probiotic cultures can have a positive effect on the perceived sensory quality of probiotic juices.

3.5. Storage

3.5.1. Storage conditions

Fermentation process in production of probiotic drinks reduces the pH of substrate which may affect the stability of product, including the survival of probiotic bacteria - one of the most important requirement of the probiotic product. Generally, this value is not lower than 10^6 CFU/mL. Champagne and Gardner (2008) reported that the pH expected of fermented milks is in the range of pH 4 and pH 5 which can cause the loss of viable cells during storage period. In the fruit juice substrate, the pH is even lower (around pH 3.5) due to the stronger acid environment. Therefore, it is essential to investigate the stability of probiotics during storage. Numerous studies have been carried out to monitor the viability of microorganism in probiotic fruit drinks (**Table 3.7)**. Besides pH, temperature has been also concerned. Most storage studies were set up at 4^{o}C, which might be considered an optimal storage temperature for this product. The quality changes of probiotic products have a strong relationship with temperature (Zhi *et al.*, 2018). Moreover, it is not always ensured that the product will be kept per manufacturer's storage instructions with the temperature at 4^{o}C during distribution chain of product. Therefore, evaluation of quality changes and model the effect of variable storage conditions on shelf life are essential to provide additional information for develop probiotic fruit drink products commercially.

Table 3.7. The viability of probiotics in some fermented fruit juices during storage

Fruit juice	Microorganisms	Viability at the end of storage	Storage conditions	References
Lemon juice	*L. plantarum* LS5	7.14 ± 0.21 log CFU/mL	4^{o}C, 28 days	Hashemi *et al.* (2017)
Carrot blended with orange juice	*L. plantarum* CECT 220	10^8-10^9 CFU/mL	4^{o}C, 30 days	Valero-Cases and Frutos (2017b)
Carrot blended with orange juice	*L. acidophilus*	10^6-10^7 CFU/mL	4^{o}C, 40 days	Valero-Cases and Frutos (2017a)
Carrot, beet and apple juice	*L. casei*	1.5 x 10^6 CFU/mL	4^{o}C, 4 weeks	Zandi *et al.* (2016)
Peach juice	*L. casei*, *L. delbrueckii*	1.72 x 10^7 CFU/mL	4^{o}C, 4 weeks	Pakbin *et al.* (2014)
Pineapple juice	*L. casei*	10^6 CFU/mL	4^{o}C, 42 days	Costa *et al.* (2013)
Red and green smoothies	*Weissella cibaria*, *L. plantarum*, and *L. pentosus*	Approx. 9.0 log CFU/g	4^{o}C, 30 days	Di Cagno *et al.* (2011)

Fruit juice	Microorganisms	Viability at the end of storage	Storage conditions	References
Cashew apple juice	*L. casei*	over 8.00 Log CFU/mL	4°C, 42 days	Pereira *et al.* (2011)
Noni juice	*L. casei*, *L. plantarum*, *B. longum*	10^8 CFU/mL	4°C, 4 weeks	Wang *et al.* (2009)
Commercial fruit juice drink	*L. acidophilus* LB2, LB3 and LB45, *L. brevis* LB6, *L. rhamnosus* LB11 and LB24, *L. fermentum* LB32, *L. plantarum* LB42 and *L. reuteri* LB38	Highest loss in *L. acidophilus* viability (approx. 10^2 CFU/mL) High viabilities in the rest strains (approx. 10^7 CFU/mL)	4°C, 80 days	Champagne and Gardner (2008)
Orange, pineapple and cranberry juice	*L. casei*, *L. paracasei*, and *L. rhamnosus*	10^8 CFU/mL in orange juice, 10^7 CFU/mL in pineapple juice and very low cell viability in cranberry juice	4°C, 12 weeks	Sheehan *et al.* (2007)

3.5.2. Shelf-life of probiotic plant juice products

The shelf-life of food was defined as the period during which the food retains an acceptable quality from a safety and organoleptic point of view. It depends on four main factors, namely formulation, processing, packaging and storage conditions (Gallagher *et al.*, 2011). Food are very complex systems, in which microbiological, enzymatic, and physicochemical reaction can take place simultaneously. Therefore, there are many factors may affect to deteriorate the quality and safety of food products during storage and distribution. Valero-Cases and Frutos (2017a) categorized these factors into intrinsic and extrinsic groups.

Intrinsic factors are the properties of the final product, such as water activity, pH value and total acidity, available oxygen, redox potential, nutrients, natural microflora and surviving microbiological counts, natural biochemistry of the product formulation (enzymes, chemical reactants).

Extrinsic factors are those factors that the final product encounters as it moves through the food chain, such as time and temperature profile during processing, relative humidity, exposure to light during processing/storage/distribution, temperature control during storage/distribution, composition of atmosphere within packaging, pressure in the headspace, subsequent heat treatment and consumer handling.

Establishment of shelf-life of product are necessary to ensure food safety and high quality. Shelf-life assessment can be approached by two different methods, including direct and indirect method.

- **Direct method**: Direct method, also known as real time study. This method involves storing the product under preselected conditions for period longer than the expected shelf-life and monitor the product at regular interval of time until it begins to failure.
- **Indirect method**: Indirect methods predict the shelf-life of a product without running a real time storage trial. Therefore, they can be useful for products with long shelf-lives. The common testing used in this method is accelerated shelf life testing (Mizrahi, 2011).

The indirect method using accelerating factors is the most commonly used methodology in establishment of shelf-life prediction model of food (Labuza, 2000). Since the period of time needed for this method is much shorter than the direct method. Among accelerating factors such as temperature, humidity, light, etc., temperature is the most often used parameter. Because temperature affects significantly on reaction rates, so that it can speed up the products spoilage in a short time. This methodology has been used in several studies to determine the shelf-life of fermented food products. Aini *et al.* (2021) estimated shelf life of corn yoghurt using zero-order, first order reaction and Arrhenius equation. These authors stored the products at different temperature (25°C, 30°C, and 35°C). During storage period, parameters including pH, viable cell counts, dissolved solids, total acid, viscosity and protein were monitored. After calculation, the product shelf life stored at 5^{o}C, 10^{o}C, 15^{o}C, and 20^{o}C were estimated 41, 40, 39, and 38 days, respectively. Two kinds of yoghurt which were supplied by manufacturer were used to develop a shelf-life prediction model by Zhi *et al.* (2018). First order reaction model and Arrhenius equation were used. The yoghurt samples were stored at various temperatures (5°C, 15°C, 25°C and 35°C). Then the acidity, viscosity and sensory evaluation were quantified. The microbial population of LAB in the products was excluded because it can still fulfill the minimum population standard. The results showed that product shelf-life prediction was 15.5 and 18.5 days at 5^{o}C for product 1 and 2, respectively. Bambara groundnut probiotic beverage fermented with *L. bulgaricus* alone and mixture of *L. bulgaricus* and *L. plantarum* had shelf-life estimation for 28 days at 5^{o}C; 18 days and 10 days at 15^{o}C, respectively; and 2 days at 25^{o}C for both products (Murevanhema and Jideani, 2020). To predict the product shelf life, Murevanhema and Jideani (2020) stored the fermented juice at 5^{o}C, 15^{o}C and 25^{o}C, then pH values were monitored and calculated using the Mitscherlich's law of diminishing returns model and Arrhenius equation. In both cases of starter cultures, the microbial population dropped from approx. 7.5-7.8 (log CFU/mL) to approx. 7 log CFU/mL during stored at all the investigated temperatures.

2.5.2.1 Prediction of quality loss

Even if food product was stored in good conditions, the loss of quality or nutritional value can take place. Labuza (1984) summarized some general modes of food deterioration, including microbial decay of foods, senescence, non-enzymatic browning, lipid oxidation, vitamin loss, colour changes, enzymatic activity, sensory changes, physical deterioration.

Some information needs to be regarded in order to make useful shelf-life predictions (Labuza, 1984):

- The potential major modes for loss of quality of the product
- The factors which control the initial quality or nutritional value during manufacture
- The environmental conditions the food will be exposed to including temperature, relative humidity and light.

The key to the application of kinetics to prediction of quality loss is selection of the major mode of deterioration, measurement of some quality factors related to this mode, and then application of mathematical models to make the needed predictions.

The most often used methodology to predict the loss of food quality can be represented by the equation bellow (Gallagher *et al.*, 2011):

$$\frac{dC}{dt} = k.C^n$$

where, C is the quality factor measured, t is a storage time, k is a constant, n is a power factor called the order of the reaction, and $\frac{dC}{dt}$ is the rate of change of C in time. A negative sign is used if the deterioration is a loss of C and a positive sign if it is for production of an undesirable end-product.

Table 3.8. Reaction order model equations (Savva, 2019)

Reaction order	Equation	Integration	Explanation
0	$\frac{dC}{dt} = k$	$C_o - C = kt$	Reaction rate is independent of reactant concentration. It means that the reactant concentration increases or decreases with time by a constant amount.
1	$\frac{dC}{dt} = kC$	$\ln(\frac{C_o}{C}) = kt$	The rate is directly proportional to the reactant concentration. It means that the rate of change of reactant concentration with time is high when the amount of reactant is high, and it decreases as the amount or concentration of the reactant remaining decreases.
n>1	$\frac{dC}{dt} = k.C^n$	$\frac{1}{C^{n-1}} - \frac{1}{{C_o}^{n-1}} = (n-1)kt$	The rate is directly proportional to the reactant concentration power of reaction order.

The mathematical expression for order reaction is shown in **Table 3.8**. In food processing, zero-order and first-order reaction were widely used to estimate the quality changes of product during time (Zhi *et al.*, 2018).

The graphs of reaction kinetic for zero-, first- and the higher-order reactions were shown in **Figure 3.7.** Zero- and first-order reactions involve only one kind of molecule, and can be described with linear or exponential relationships. Second- and higher-order reactions involve multiple interactions of two or more kinds of molecules and are characteristic of most biological materials that consist of large and complex molecular structures (Magari, 2003).

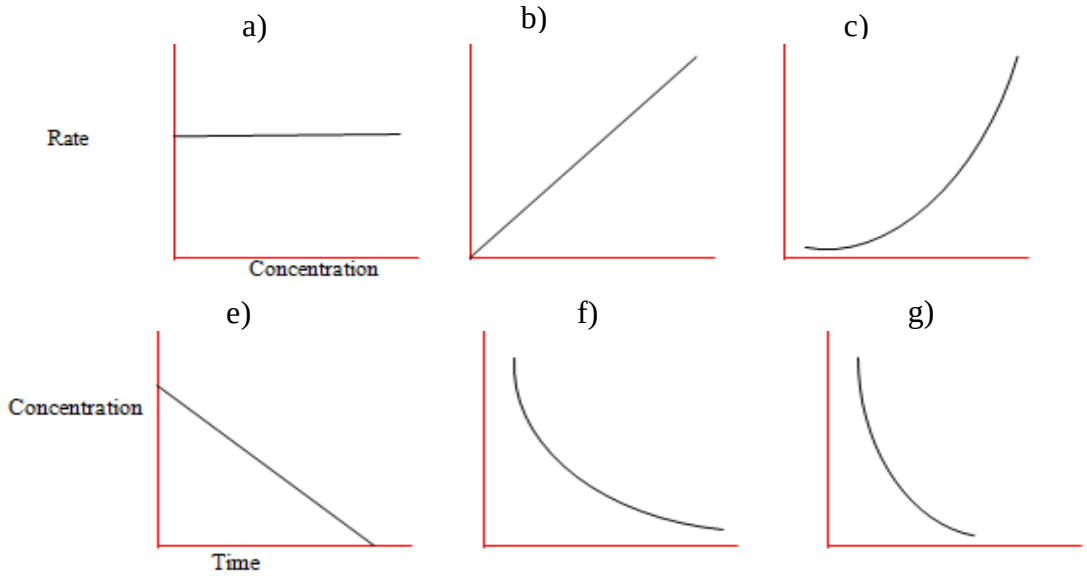


Figure 3.7. Rate against concentration of reactant for a) zero-order, b) first-order and c) second-order; and **the change of concentration of reactant against time for** e) zero-order, f) first-order and g) second-order (OCR: Chemistry A-level, 2020)

3.5.2.2 Arrhenius equation

The degradation rate depends on the conditions where the chemical reaction takes place. Products degrade faster when subjected to acceleration factors such as temperature, humidity, pH and radiation. Temperature affects significant on reaction rates. One of the most accepted models demonstrated the dependence of k on temperature is Arrhenius (Mizrahi, 2011).

$$k = k_o e^{-\frac{Ea}{RT}}$$

where, k is the constant pre-exponential or absolute rate, Ea is the activation energy (kJ/mol), R is the gas constant (1.986 Cal/mol), k_o is the reaction rate constant, and T is the absolute reaction temperature (°K).

Theoretically, the Arrhenius equation does not apply when more than one kind of molecule is involved in reactions. However, if the degradation rate and temperature are linearly related, the prediction of shelf-life can be approximated by the Arrhenius equation (Magari, 2003).

4. MATERIALS AND METHODS

4.1. Fruit juices

Commercial pineapple, mango and banana juice (named Rauch Happy Day - Rauch Hungaria company, Hungary) were purchased from the local market. The fruit contents in the products are 100% pineapple, 23% mango and 30% banana, respectively. The juice pH was adjusted to pH 6.4 with 4 N NaOH before fermentation.

4.2. Microorganisms

Three *Lactobacillus* strains: *Lactobacillus acidophilus* 150 (from Exquim SA, Spain), *Lactobacillus casei* 01 (from Chr. Hansen, Denmark), *Lactobacillus plantarum* 299v (from Probi, Sweden) and two *Bifidobacterium* strains: *Bifidobacterium lactis* Bb12 (from Chr. Hansen, Denmark) and *Bifidobacterium longum* DSM16603 (from Probiotical, Italy) were used in this research.

The stock cultures were prepared by suspending the lyophilized *Lactobacillus* and *Bifidobacterium* strains in MRS and TPY broth, respectively. After that, the cultures were incubated for 24 h at 37^{o}C. For *Bifidobacterium*, the incubation took place in an anaerobic jar gas-pak system. Then 1 mL of the cultures was transferred into 10 mL of the corresponding media and incubated at the same previous method. The viable cell count of *Lactobacillus* strains in the MRS broth after 24 hour was in range of 8.5-9.5 log CFU/mL, and of *Bifidobacterium* strains in TPY was a little bit lower which was around 8.5 log CFU/mL These cultures were used as a starter to the juice fermentation.

4.3. Chemicals

4.3.1. Media

De Man, Rogosa, and Sharpe (MRS) broth contained (per liter) proteose peptone 10 g, yeast extract 8 g, meat extract 8 g, glucose 20 g, sodium acetate 5 g, tri-ammonium citrate 2 g, K_2HPO_4 2 g, $MgSO_4$ 0.2 g, $MnSO_4$ 0.05 g and Tween80 1 mL.

Trypticase-phytone-yeast extract (TPY) broth contained (per liter) trypticase peptone 10 g, Phytone peptone 5 g, glucose 5 g, yeast extract 2.5 g, Tween80 1 mL, cysteine-HCl 0.5g, K_2PO_4 2 g, $MgCl_2.6H_2O$ 0.5 g, $ZnSO_4.H_2O$ 0.25 g, $CaCl_2$ 0.15 g, $FeCl_3$ 0.03 g.

MRS-bile agar was obtained from the addition of 0.3% bile salts into the MRS medium (Sohrabvandi *et al.*, 2012)

Agar medium is the medium supplemented by agar in a concentration of 15 g/L.

4.3.2. Other chemicals

Folin-Ciocalteu reagent, tripyridyltriazine (TPTZ), Iron (II) sulfate ($FeSO_4$), gallic acid and other standards (glucose, fructose, lactic acid, and acetic acid) for HPLC analysis, as well as pepsin and bile salts, were purchased from Sigma–Aldrich (Hungary). Other chemicals were supplied from Reanal (Hungary) and VWR (Hungary).

4.4. Fermentation of fruit juices using monocultures

150 mL Erlenmeyer flasks containing 50 mL juices were inoculated with 1% of individual culture from MRS and TPY broth, so finally, the initial cell concentration in juices was around 6.5-7.5 log CFU/mL in the case of *Lactobacillus* strains, and around 6-6.5 log CFU/mL in the case of *Bifidobacterium* strains. The inoculated samples with *Lactobacillus* strains were conducted in an incubator for 16 h at 37°C. In the case of *Bifidobacterium*, the juices had been placed in an anaerobic jar gas-pack system before being incubated at 37°C for 24 h. After incubation, the fermented fruit juices were stored at 4°C for four weeks. The samples were taken interval every 4 hours of fermentation and two weeks of storage. The stability of the products during fermentation and storage was investigated through the changes of cell number, pH, quantity of acid, carbohydrates, total phenolic content and antioxidant activity. The survival of microorganisms through simulated gastro-intestinal conditions was also evaluated.

4.5. Fermentation of mixed fruit juice using monocultures

The probiotic bacteria screened in the session 4.4 were used to ferment mixed fruit juice composed of an equal proportion of pineapple, mango and banana. The fermentation was conducted in 150 mL Erlenmeyer flasks containing 50 mL mixed juices. After that, 1% of monoculture was inoculated into the juices. All the flasks were placed into an incubator set at 37°C for 16 hours. The samples with *Bifidobacterium* strains were put into an anaerobic jar before incubation. Further 4 weeks of storage at 4°C of fermented juices was conducted after fermentation. The parameters including cell number, pH, acid, sugar concentration, total phenolic content and antioxidant activity of the mixed juice during fermentation and storage were investigated.

4.6. Fermentation of fruit juices using mixed cultures

In the present study, individual juice of pineapple, mango and banana and mixed fruit juices with a combination of the three juices at different ratios were used to produce probiotic fruit drink products. The strains of bacteria obtained from the experiment using monoculture were mixed in an equal proportion and then used as a starter for fermentation.

When fermentation was performed, 50 mL of individual fruit juices or mixed fruit juices were placed in 150 mL Erlenmeyer flasks. Then, 1% of the mixed culture was inoculated the

media. Fruit juices containing probiotic bacteria were incubated at 37°C for 16 h when the pH of the products was lower than 4.6, then further storage of the fermented products at 4°C for four weeks was taken place. The samples were taken in every 4 hours of fermentation and two weeks of storage, and change of cell number, pH, the quantity of acid, carbohydrates, total phenolic content and antioxidant activity were investigated. The survival of microorganisms through simulated gastro-intestinal conditions was also evaluated before and after storage.

4.7. Sensory evaluation

After fermentation, the fruit juices were refrigerated at 4°C for one day before evaluation. Sensory analysis was carried out by seven panellists (3 females and 4 males), ranging in age from 25 to 45. The appearance, aroma, taste, texture, and overall attributes of the fermented fruit juice formulations were chosen for acceptance testing using a 9-points hedonic scale (9-like extremely and 1-dislike extremely). The formulations (25 mL) were served at a temperature of 4°C in 50 mL plastic cups coded with 3-digit random numbers, one by one, in random order. Drinking water and sandwich were provided to clean the mouth during the testing process. The tests were performed in individual tables under white light.

4.8. Storage study

The influence of storage temperatures on the change of product quality was carried out through maintaining the products at different temperatures of 5°C, 15°C, 25°C and 35°C. Samples stored at 5°C were measured every 6 days at the initial storage time and every 3 days at the end of storage. The samples stored at 15°C were tested every 3 days. Those at 25°C were sampled every day, while samples stored at 35°C were evaluated every 12 hours due to their short shelf-life. The parameters were investigated, including the change of cell number, pH, the quantity of acid, carbohydrates, moreover total phenolic content and antioxidant activity of samples.

Table 4.1. Nonlinear and linear equations of zero-order, first-order, second-order and third-order model

Models	Nonlinear equation	Linear equation	Equation name
0 order	$\frac{dA_t}{dt} = -k_o$	$A = -k_o t + A_o$	**(Eq. 4.1)**
1st order	$\frac{dA_t}{dt} = -k_1 A_t$	$Ln(A) = -k_1 t + Ln(A_o)$	**(Eq. 4.2)**
2nd order	$\frac{dA_t}{dt} = k_2 A_t^2$	$\frac{1}{A} = k_2 t + \frac{1}{A_o}$	**(Eq 4.3)**
3rd order	$\frac{dA_t}{dt} = k_3 A_t^3$	$\frac{1}{A^2} = 2k_3 t + \frac{1}{A_o^2}$	**(Eq. 4.4)**

where, k is the rate of the change of pH, A_o is the pH value before storage and A_t is pH value at the storage time t

Additionally, accelerated shelf-life testing was conducted to estimate the shelf-life of product based on the rate law and Arrhenius equation. Four rate reaction models were used to predict the product shelf-life, including zero-order, first-order, second-order and third-order. The rate reaction model equations and their integration form were shown in **Table 4.1.** In the current study, the changes of pH during the different storage temperatures were used to establish models.

The Arrhenius equation is shown in equation bellow:

$$k = k_o e^{-\frac{Ea}{RT}} \qquad \textbf{(Eq. 4.5)}$$

where, k is the constant pre-exponential or absolute rate, Ea is the activation energy (kJ/mol), R is the gas constant (1.986 Cal/mol), k_o is the reaction rate constant, and T is the absolute reaction temperature (°K).

When we converted the natural logarithm of the above equation, the following equation was obtained:

$$Lnk = -\frac{Ea}{R}\frac{1}{T} + Lnk_o \qquad \textbf{(Eq. 4.6)}$$

So based on these equations, the shelf-life prediction model can be established.

In order to assess the fit of regression models, a prediction of shelf-life of the product at 30°C was carried out. Simultaneously, an experiment of storage fermented juice at 30°C was conducted. Root mean square error (RMSE) was used to evaluated how close the observed data points to the model's predicted values. The lower values of RMSE indicate the better model fit (Nunes *et al.*, 2015).

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}(X_{obs,i} - X_{model,i})^2}{n}} \qquad \textbf{(Eq. 4.7)}$$

where, $X_{obs,i}$ is the observation value, $X_{model,i}$ is the predicted value and n is the total number of observations in a set of data.

4.9. Analytical methods

4.9.1. Viability of probiotic strains

The cell number of *Lactobacillus* and *Bifidobacterium* strains were determined by the plate count method. In detail, 10-fold dilution series was started with transferring 0.5 mL of sample into a test tube containing 4.5 mL saline (0.85%). Once the dilution was made, 50 µL aliquot was transferred into the 60 mm dishes. Afterward, MRS or TPY agar medium was poured into the dishes. Then all the plates were incubated at 37°C for 48h-72h. The petri dishes with TPY agar were placed into the anaerobic jar gas-pack system before incubating.

To estimate the counts of *Bifidobacterium* in mixed cultures by subtraction method, counts

of *Lactobacillus* on MRS-bile agar at 37°C for 48 h under aerobic incubation could be subtracted from total counts of *Lactobacillus* and *Bifidobacterium* enumerated on MRS agar at 37°C for 72 h under aerobic incubation (Sohrabvandi *et al.*, 2012).

4.9.2. Measurement of Brix and pH

The total soluble solids (Brix) and pH were measured by using a refractometer (Atago, Japan) and a pH meter (Mettler Toledo, Switzerland), respectively.

4.9.3. Organic acid and sugar quantification

Carbohydrate and organic acid content were determined by HPLC method (Bujna *et al.*, 2017). Briefly, the fermented juices were centrifuged at 14,000 rpm for 10 minutes before the supernatants were separated and filtered through 0.45 µm membrane. The analysis was performed using the surveyor HPLC system (Thermo Scientific Corporation, USA). An Aminex HPX-87H ion exclusion column with refractor index (RI) and photodiode array (PDA) detectors were used to detect sugars and organic acids, respectively. 5 mM H_2SO_4 was used as the mobile phase. The temperature of the column was maintained at 45°C, and the running time was 25 min. The data acquisition and integration were performed using the ChromQuest 5.0 software package. Results were calculated using standard curves of corresponding sugar and organic acid.

4.9.4. Total phenolic content

The content of total polyphenols in fermented juices was determined using Folin's phenol reagent method (ISO:14502-1:2005) which has some modifications. Briefly, samples from the fermented juices were centrifuged at 14,000 rpm for 10 mins. 0.2 mL of properly diluted samples were mixed thoroughly with 1mL of 10% Folin-Ciocalteu reagent. Afterward, the mixtures were incubated for 8 mins at room temperature before adding 0.8 mL of 7.5% solution of Na_2CO_3. After 15 mins of heating at 50°C, the mixtures' absorbances were measured by spectrophotometer (Unicam Helios UV/Vis) at a wavelength of 765 nm. Results were calculated using a gallic acid standard curve and expressed as a mg gallic acid equivalent (GAE) per 100 g fermented fruit juice.

4.9.5. Antioxidant capacity

The antioxidant activity was evaluated following ferric reducing antioxidant power (FRAP) assays (Benzie and Strain, 1996). Five-fold dilution of supernatant fluids (14,000 rpm for 10 mins) was mixed with 1.5 mL of FRAP reagent, which was prepared by mixing 0.3 M acetate buffer (pH 3.6), 10 mM tripyridyltriazine (TPTZ) prepared in 40 mM HCl, and 20 mM ferric chloride solution ($FeCl_3$) with a ratio of 10:1:1. The mixtures were maintained at 37°C in a water bath for 10 mins, and the absorbances were measured at 593 nm. The results were compared to

a $FeSO_4$ calibration curve and expressed as a ferrous equivalent per 100 g fermented fruit juice.

4.9.6. Survival of probiotic strains through simulated gastro-intestinal conditions

To examine the survival of probiotics they will be exposed for 135 min in 0.5% NaCl (pH 2.0) containing pepsin in the concentration of 0.3%, followed 150 min incubation in the presence of 0.6% bile salts prepared in potassium phosphate buffers (pH 7.4). Briefly, 20 mL of fermented juice was incubated in 100 mL of 0.3% pepsin at 37°C for 135 min. The cell number before and after incubation was evaluated using the plate count method. Then, 10 mL of the incubated mixture was centrifuged and washed by 10 mL of phosphate-buffered saline. The pellet was mixed well with 0.6% bile salt solution and incubated for 150 min at 37°C before the cell number was recorded. The survival rate of microorganism was calculated to follow equation (Santos *et al.*, 2017):

$$Survival\ rate\ (\%) = \frac{N_1}{N_o} x100 \quad \textbf{(Eq. 4.8)}$$

where, N_o is the initial viable cell count (log CFU/mL) before treatment and N_1 is the final viable cell count (log CFU/mL) through simulated gastric juice and bile solutions.

4.10. Statistical analysis

Results obtained were presented as mean ± SD. Values were performed from the average of triplicated experiments. One-way ANOVA was used to evaluate statistically significant difference between the variables.

5. RESULTS AND DISCUSSION

5.1. Fermentation of fruit juice using monocultures

Fermentations of individual tropical fruit juices (pineapple, mango and banana) using monocultures, including *L. acidophilus* 150, *L. casei* 01, *L. plantarum* 299v, *B. lactis* Bb12 and *B. longum* DSM16603, were set up. The fermentation time was last until the final pH of fermented juices reached under 4.6 which can control the growth of some pathogens in food. Thus, in the case of the inoculated samples with *Lactobacillus* strains, the fermentation time were 16 hours, while the time of 24 hours was set for the samples inoculated with *Bifidobacterium*. In order to evaluate the stability of the products during fermentation and storage, the change of cell number, pH, quantity of acid, carbohydrates, total phenolic content and antioxidant activity of samples which were taken interval every 4 hours of fermentation and two weeks of storage were investigated. The survival of microorganisms through simulated gastro-intestinal conditions before and after storage was also evaluated.

5.1.1. Changes of pH and viability

The change of pH and microbial population in the juices during fermentation and storage were presented in **Figure 5.1**. The pH of the fermented juices decreased with the rising fermentation and storage time. In detail, after 16 h of fermentation, the pH of the juices inoculated with *Lactobacillus* strains dropped from the initial pH 6.4 to approx. pH 4.0 in pineapple juice, pH 4.2 in mango juice and pH 4.3 in banana juice. The pH values in fermented pineapple juice were recorded the lowest among the products.

For *Bifidobacterium* strains, after 24 h of fermentation, the pH values of pineapple juice decreased from pH 6.4 to 3.8. Fermented mango and banana juice demonstrated a slightly higher pH value (approx. pH 4.1) than that in fermented pineapple juice. Interestingly, the juices inoculated with *B. lactis* Bb12 showed a slighter decrease in pH value during the fermentation period from hour 8^{th} to 20^{th} compared to *B. longum* DSM16603. However, the rising fermentation time minimized the gap between the pH values in the fermented juices.

In all investigated *Lactobacillus* and *Bifidobacterium* strains, *B. lactis* Bb12 introduced the lowest pH reduction rate. Indeed, the pH value of juice inoculated with this strain decreased slowly during fermentation. For example, at the fermentation time 16^{th}, the pH values of pineapple, mango and banana with *B. lactis* Bb12 were 4.95, 5.56 and 5.2, respectively while the pH of the juices with other strains dropped below pH 4.6

During storage, the pH in fermented juices slightly decreased in the cases of *Lactobacillus* and *Bifidobacterium* strains.

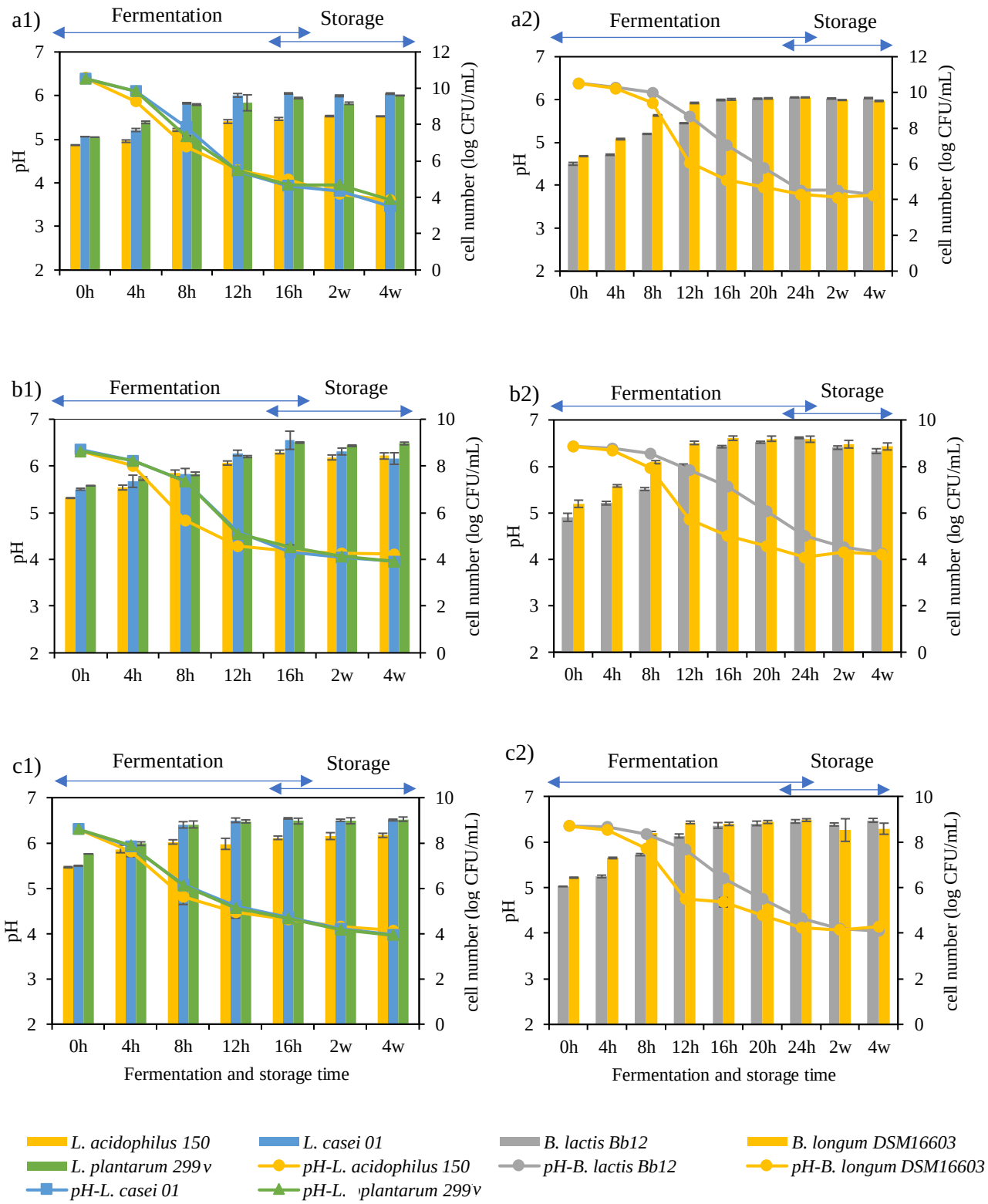


Figure 5.1. pH values and microbial population of tropical fruit juice: a1), a2) pineapple; b1), b2) mango; and c1), c2) banana fermented at 37°C by *Lactobacillus* 1) and *Bifidobacterium* 20 strains, and further stored for 4 weeks at 4°C

The viable cell number of microorganisms is one of the most important criteria for probiotic products. According to Pereira *et al.* (2017), the probiotic products should contain a significant

number of probiotic bacteria over 10^7 CFU/mL for health benefits. The data illustrated that *Lactobacillus* and *Bifidobacterium* strains could grow well in all investigated juices without any supplementation of nutrients. The microbial population of the juices after fermentation was not lower than 8 log CFU/mL.

For the *Lactobacillus* strains, the juices inoculated with *L. casei* 01 and *L. plantarum* 299v presented the highest viable cell counts (approx. 10 log CFU/mL in pineapple juice, and approx. 9 log CFU/mL in mango and banana juice). The *L. acidophilus* 150 strains showed the poorest growth in fruit juices. The cell counts of this strain reached only 8.22 log CFU/mL-8.63 log CFU/mL in all juices.

In the case of *Bifidobacterium* strains, although *B. lactis* Bb12 showed a slighter growth than *B. longum* DSM16603 at initial fermentation time (hour 0^{th}-12^{th}), the population of these strains were similar at the end of fermentation period. The viable cell counts reached approx. 10 log CFU/mL in pineapple juice and 9 log CFU/mL in mango and banana juice.

During 4 weeks of storage at 4^oC, most bacteria remained stable in population.

Fermentation of pineapple, mango and banana juice using probiotic LAB as starters were also carried out by other researchers. Their results indicated that these juices supported the growth of the bacteria which is consistent with our finding. Three LAB strains *Pediococcus pentosaceus* LaG1, *Lactobacillus rhamnosus* GG, *Pediococcus pentosaceus* LBF2 were used for pineapple fermentation at 37^oC for 72 h (AdebayoTayo and Akpeji, 2016). After fermentation, the viability of probiotic bacteria in juice ranged from $1.05\text{-}1.10\times10^9$ CFU/mL. Reddy *et al.* (2015) reported a well growth of *L. plantarum*, *L. delbrueckii*, *L. acidophilus*, and *L. casei* during mango juice fermentation at 30^oC for 72h. The final cell number exhibited in fermented juice was in a range of $1.5\text{-}2.2\times10^9$ CFU/mL. An increase viability of *L. acidophilus* from around 10^5 to 10^6 CFU/mL after fermentation of banana juice for 80 h at 37^oC was reported by Tsen *et al.* (2004). In these studies, the fermentation time is much longer than ours (16-24 h). This is because of the difference in the initial pH of juices. These authors used the juices without adjusting the pH value which ranged from 4.5-5.5, while in our study, the pH was adjusted to around 6.4. Reddy *et al.* (2015) observed that the bacteria had to pass a longer lag phase (in the first 12 h) due to the stress induced from the differences between the pre-culture medium and the fermentation medium, as well as the low pH condition of the juice. Reddy *et al.* (2015) also investigated the stability of fermented mango juice during 4 weeks of storage. The result indicated a significant decrease in cell number of *L. plantarum* as well as of other strains after storage. They explained that the reduction in the sugar level, an accumulation of organic acid and storage temperature resulted in the reduction of probiotic viability. However, in our finding, the viability of LAB strains remained stable during 4 weeks of storage at 4^oC. A significantly lower pH value (pH 3.2) of fermented juice in the study of Reddy *et al.* (2015) compared to ours (pH 4.26) may give a detrimental condition to the viability of probiotic bacteria during cold storage. Hence, pH adjustment of juice before fermentation may offer a positive effect on the growth and survival of probiotic bacteria during fermentation and storage.

Other authors also reported that probiotic bacteria can grow well in fruit juices. Ellendersen *et al.* (2012) conducted apple beverage fermented with *L. casei* and *L. acidophilus* for 20 h. Their

results showed that the products presented enough cell growth to be considered as a probiotic product (10^8 log CFU/mL). The authors also indicated that *L. casei* in fermented apple juice showed higher cell counts as compared to *L. acidophilus*. According to Lean (2011), *L. acidophilus* exists in the gastrointestinal tract and vagina of humans and animals while, *L. casei* can be found in both plant and animal origin (Batt and Tortorello, 2014), and *L. plantarum* is most frequently found in the plant-derived materials (O'sullivan *et al.*, 2011). The non-plant origin of *L. acidophilus* bacteria may impair its growth in juice while the plant-derived strains can grow well in this familiar substrate.

Bujna *et al.* (2017) fermented apricot juice with *Bifidobacterium* strains (*B. lactis* Bb12 and *B. longum* Bb46) and *Lactobacillus* strains (*L. casei* 01 and *L. acidophilus* 5) for 24 h and they revealed that the cell yield after fermentation using all investigated strains varied from 2.7 x 10^9 CFU/L.h to 1.78 x 10^{10} CFU/L.h. The pH value of fermented apricot juices varied by strains and was approx. pH 4.8 - 5.1 that was by far higher than our results. It can be explained that the acid accumulation in our fermentation process was significantly higher than in their study. Furthermore, the sugar contents in our investigated juices were higher than in apricot juice. According to İçier *et al.* (2015) the higher carbohydrate source provided as energy source led to an increase in the metabolic activity of bacteria, and thus contributed to the decrease in pH value. Furthermore, the decrease in pH not only depends on the amounts of acids, but on the kind of acids produced too. Acetic acid and lactic acid are known as the main products of the fermentation. Comparing to acetic acid with pKa = 4.73, lactic acid registers as a stronger one with pKa = 3.86 (Zalán *et al.*, 2011). Meanwhile, the amounts of acetic acid produced in their study was higher, whereas the lactic acid content was significantly lower than our findings.

Kun *et al.* (2008) found that *B. lactis* Bb12 reached the maximum cell counts (8.22 log CFU/mL) after 12h of fermentation of carrot juice. In our study, after 12 hours of fermentation, the cell counts of *B. lactis* Bb12 reached higher values (8.85-9.4 (log CFU/mL)) and continued increase in population approx. 8.9-9.7 log CFU/mL after 24 h. İçier *et al.* (2015) reported that viable cell counts of *Lactobacillus* in soymilk supplemented with a 15%-25% apple juice reached 8.98-9.1 log CFU/mL after one day fermentation at 37°C. They also mentioned that the addition of apple juice to soymilk increased slightly the number of *L. acidophilus* of about 0.25 and 0.35 (log units). It can be explained that fruit juice contains diversity of nutrients such as free amino acids, peptides, vitamins and fermentable carbohydrates which meet the complex nutrient requirements of *Bifidobacterium* as well as *Lactobacillus* (Bujna *et al.*, 2017).

In conclusion, pineapple, mango and banana juice showed promising substrates for all the investigated strains. Pineapple juice was recorded as the best substrate. The population of all the strains after fermentation and storage were not lower than 8 log CFU/mL, which was fulfilled the requirement of a probiotic product. The *L. acidophilus* 150 strain showed the slowest growth in all investigated *Lactobacillus* strains.

5.1.2. Changes of sugars and organic acids

Generally, fruit juices are a rich source of sugar. Mango juice contains the highest sugar quantity with 7.75% w/v glucose and 5.45% w/v fructose, followed by banana with 7.61% w/v glucose and 4.26% w/v fructose. Pineapple juice was registered as the lowest sugar content with 5.9% w/v glucose and 3.43% w/v fructose.

The changes of sugar content in juices during fermentation and storage are shown in **Figure 5.2**. The concentration of glucose and fructose in juices decreased significantly during fermentation and storage.

In the cases of *Lactobacillus* strains, after 16 hours of fermentation, sugar content in pineapple juice decreased by around 1.6% w/v in glucose and 0.6% w/v in fructose. In mango juice, the concentration dropped by in a range of 1.6-2.9 (% w/v) in glucose and in a range of 0.8-1.2 (% w/v) in fructose. *Lactobacillus* strains utilized around 1.3% w/v glucose and 0.6% w/v fructose in banana juice after fermentation. Glucose was observed as the preferable carbohydrate source for these strains.

Bifidobacterium strains utilized both glucose and fructose for their metabolism. *B. longum* DSM16603 used 1.96% w/v glucose and 2.1% w/v fructose for pineapple juice fermentation, 2.01% w/v glucose and 0.8% w/v fructose for mango fermentation and 1.14% w/v glucose and 2.19% w/v fructose for mango fermentation. Whereas *B. lactis* Bb12 consumed 1.8% w/v glucose and 1.21% w/v fructose in pineapple, 1.32% w/v glucose and 0.59% w/v fructose in mango and 1.45% w/v glucose and 0.66% w/v fructose in banana juice. These results indicated that higher amounts of sugars were consumed by *B. longum* DSM16603 than *B. lactis* Bb12.

The sugar utilization properties of *Lactobacillus* strains were also reported in a study of Mousavi *et al.* (2013). They revealed that the rate of glucose consumption of *Lactobacillus* strains (*L. plantarum* and *L. acidophilus*) was significantly higher than that of fructose in pomegranate juice. After fermentation, the glucose and fructose concentration of the juice inoculated with *L. plantarum* reduced by 26.8% and 16.22%, respectively. These results are very close to a report of Hashemi *et al.* (2017). The authors fermented lemon juice with *L. plantarum* LS5. After fermentation, they observed a reduction of a 26.09% glucose and 13.11% fructose in the juice. However, an opposite observation was reported by Pereira *et al.* (2017). They presented that fructose was the most consumed carbohydrate source of *L. casei* (84.76%), followed by glucose (62.1%) and sucrose (34.52%) during fermentation of cupuassu beverage. This can be explained that the metabolism of carbohydrates by probiotic bacteria varies from strain to strain and depends on the substrate and also on the fermentation conditions (Mousavi *et al.*, 2013).

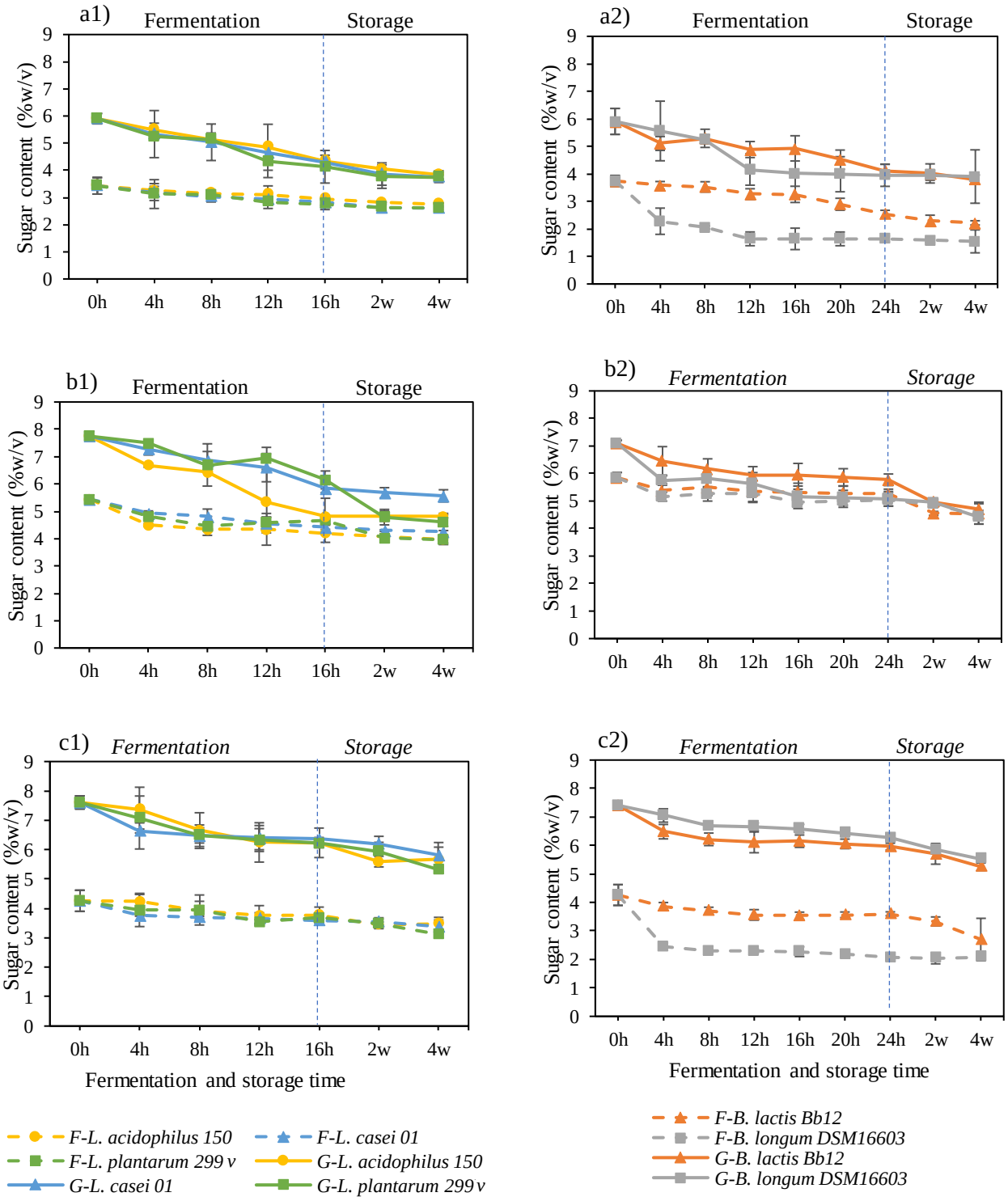


Figure 5.2. Sugar (glucose - G and fructose - F) concentration of tropical fruit juice: a1), a2) pineapple; b1), b2) mango; and c1), c2) banana fermented at 37°C by *Lactobacillus* 1) and *Bifidobacterium* 2) strains, and further stored for 4 weeks at 4°C

Organic acid, including lactic acid and acetic acid, are accumulated during carbohydrate metabolism of LAB and bifidobacteria (Axelsson, 2004, Salminen *et al.*, 2011). They have an essential effect on the stability of the product by undesirable microbe restriction/inhibition (Bujna

et al., 2017, Pereira *et al.*, 2017). In comparison to acetic acid, lactic acid was registered as a major product of sugar metabolism of both *Lactobacillus* and *Bifidobacterium* during fermentation, resulted a decrease in the pH value of medium.

The change of lactic acid concentration of juice during fermentation and storage are shown in **Figure 5.3**. In the case of *Lactobacillus* strains, the quantity of lactic acid increased significantly during fermentation and storage. After 16 h of fermentation, the lactic acid concentration of juices was determined to be in a range of 1.7-2.01 (% v/v) in pineapple juice, 0.9-1.39 (% v/v) in mango juice and 1.47-1.77 (% v/v) in banana juice. Fermented pineapple juice had the highest lactic acid concentration in all investigated juice. After four weeks of storage, a significant increase in lactic acid quantity was observed. The lactic acid concentration of pineapple juice inoculated with *Lactobacillus* strains was in a range of 2.01-2.85 (% v/v) in pineapple juice, 1.23-1.75 (% v/v) in mango juice and 1.59-1.93 (% v/v) in banana juice.

For *Bifidobacterium* strains, lactic acid concentration of juices increased during fermentation. In detail, the lactic acid content of fermented pineapple was 2.7-3.06 (% v/v). A lower value was recorded in the case of mango juice and banana juice which were in a range of 1.75-2.09 (% v/v) and 1.33-1.77 (% v/v), respectively. Lactic acid concentration of fermented pineapple juice continued increasing during storage and reached 2.97-3.44 (% v/v) at the end while the quantity of this acid remained unchanged in mango and banana juice.

Besides lactic acid, acetic acid was also reported as a product of sugar metabolism. However, our data showed that the concentration of this acid in the fermented fruit juices was minor (**Table 5.1**). The quantities of acetic acid were range in 0.04-0.21 (% v/v) depending on the strains and juices. Those values were stable during storage.

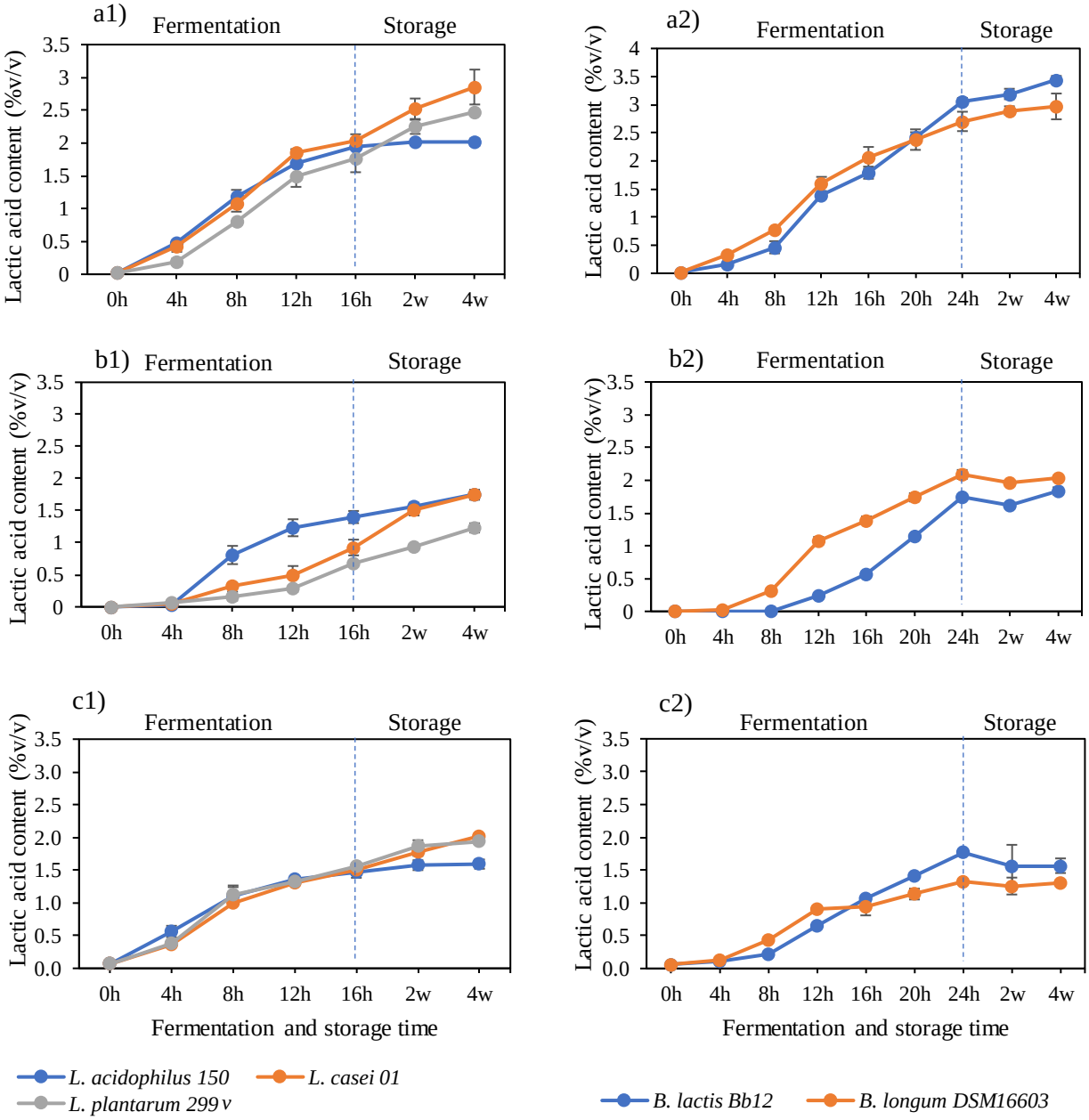


Figure 5.3. Lactic acid concentrations of tropical fruit juice: a1), a2) pineapple; b1), b2) mango; and c1), c2) banana fermented at 37°C by *Lactobacillus* 1) and *Bifidobacterium* 2) strains, and further stored for 4 weeks at 4°C

The similar finding was reported by Kun *et al.* (2008). They revealed that the production of lactic acid was more evident compared with acetic acid in carrot juice fermentation using *Bifidobacterium*. Lactic acid content in fermented juice reached 15-17 (mg/mL) while acetic acid concentration was in the range of 3.3-5.3 (mg/mL). Valero-Cases and Frutos (2017a) reported that lactic acid concentration of the fermented carrot-orange juice with *L. acidophilus* increased after storage (14.33%).

Regarding acetic acid, it could be the predominant acid in the product fermented by *Bifidobacterium*. Theoretically, fermentation of glucose by *Bifidobacterium* results in a molar ratio of 3/2 of acetic acid/lactic acid. However, the results showed a different trend in our cases.

Bifidobacterium produced significant amount of lactic acid during fermentation compared to acetic acid. Other researchers, Kun *et al.* (2008), Bujna *et al.* (2017), Tran *et al.* (2020), also reported the similar results.

Table 5.1. Acetic acid concentration of pineapple, mango and banana fermented at 37°C by *Lactobacillus* and *Bifidobacterium* strains, and further stored for 4 weeks at 4°C

Juice	Bacteria	Acetic acid concentration (% v/v)		
		0h	After fermentation	After storage
Pineapple	*L. acidophilus* 150	0	0.11 ± 0.01	0.11 ± 0.01
	L. casei 01	0	0.07	0.09 ± 0.02
	L. plantarum 299v	0	0.09 ± 0.04	0.07
	B. lactis Bb12	0	0.21 ± 0.01	0.26 ± 0.01
	B. longum DSM16603	0	0.12 ± 0.01	0.16 ± 0.03
Mango	*L. acidophilus* 150	0.03	0.14 ± 0.01	0.13 ± 0.01
	L. casei 01	0.03	0.12	0.17 ± 0.01
	L. plantarum 299v	0.03	0.04 ± 0.01	0.02
	B. lactis Bb12	0.03	0.08 ± 0.01	0.12 ± 0.01
	B. longum DSM16603	0.03	0.1 ± 0.01	0.09 ± 0.01
Banana	*L. acidophilus* 150	0.01	0.11 ± 0.01	0.1 ± 0.01
	L. casei 01	0.01	0.09	0.1 ± 0
	L. plantarum 299v	0.01	0.1 ± 0.02	0.09
	B. lactis Bb12	0.01	0.12	0.09 ± 0.01
	B. longum DSM16603	0.01	0.07	0.08 ± 0.01

In short, sugar metabolism of both *Lactobacillus* and *Bifidobacterium* strains in juices produced lactic acid as the main final product. Fermented pineapple juices had the highest value of lactic acid. Acetic acid appeared with a small amount is a promising result because acetic acid may give an off-flavour in beverage products (Bujna *et al.*, 2017).

5.1.3. Changes of total phenolic content and antioxidant capacity

Polyphenols are beneficial compounds found in fruits, vegetables and cereals. They strongly correlate with antioxidant properties that may be involved in health improvement or pathogen restriction (Pandey and Rizvi, 2009). In the present study, total phenolic content (TPC) and antioxidant activity (FRAP) of juices were investigated during fermentation and storage. The change of TPC and FRAP values are shown in **Figure 5.4**. Both TPC and FRAP values of fermented juices showed a slight decrease during fermentation and a significant reduction during storage. The data presented that pineapple juice had a highest TPC value which was 0.45-0.46 (µg/mL gallic acid), followed by mango juice with 0.38-0.4 (µg/mL gallic acid) and banana juice with 0.33-0.37 (µg/mL gallic acid). In the juices inoculated with *Lactobacillus* strains, TPC content decreased by approx. 1.4%-14.2% after fermentation and reached 7.8%-26.26% after storage. In the case of the juices fermented with *Bifidobacterium*, TPC concentration decreased

more significantly than with *Lactobacillus*. The value dropped by 8.28%-22.54% after fermentation and 11.36%-31.63% after storage.

Regarding antioxidant activity, the highest FRAP value was observed in mango juice (4.05 mM $FeSO_4$), followed by banana (3.49 mM $FeSO_4$). The antioxidant activity of pineapple juice was the lowest with 2.55 mM $FeSO_4$. After 16 h of fermentation, the antioxidant activity of samples with *Lactobacillus* strains dropped by approx. 1.6%-13.9%. After four weeks of storage at 4^oC, antioxidant capacity of fermented pineapple juice decreased in a range of 14.32%-21.04%. A more significant decrease in FRAP was observed in mango and banana juice. The FRAP values reduced 16.28%-31.65% in the case of mango juice and 27.28%-42.76% in banana juice at the end of storage period.

For *Bifidobacterium* strains, FRAP values of juices reduced by 10.89%-23.82% after 24 h of fermentation. After storage, FRAP values of pineapple, mango and banana juice dropped by 12.1%-18.07%, 33.98%-44.86% and 28.15%-36.63%, respectively. Interestingly, although pineapple juice showed the highest TPC, its antioxidant activity was the lowest in all investigated juices. And, decrease rate of FRAP of this juice during fermentation and storage was also lowest.

A similar trend of the change of TPC and FRAP of juice during lactic acid fermentation was found in a study of Tran *et al.* (2020). A reduction in phenolic content and antioxidant activity of grapefruit fermented with *L. plantarum* after fermentation was reported. The TPC and FRAP values decreased by 3.75% and 7.82%, respectively. Panda *et al.* (2017) revealed that the total phenolic content of prickly pear decreased from 0.45 µg/mL to 0.41 µg/mL after fermentation with *L. fermentum*. These authors also mentioned a decrease in antioxidant activity of the lacto-juice (22.22%). Khezri *et al.* (2018) observed a significant decrease in the TPC and antioxidant activity of fig juice fermented with *L. delbrueckii* during 28 days of storage. They recorded an approx. 30% and 17% reduction of these parameters, respectively. Kim *et al.* (2012) showed a decrease in antioxidant property of potato juice fermented with *L. casei* during 72h of fermentation. Jaiswal and Abu-Ghannam (2013) revealed that fermentation might affect negatively on the polyphenolic content. They found an approx. 15% and 24% TPC reduction in cabbage juice fermented with *L. plantarum* and *L. rhamnosus*, respectively. A loss of 5%-13% antioxidant activity in the juice after 24 h of fermentation was also observed in their study. These authors claimed that enzymes such as β-glucosidase, p-coumaric acid decarboxylase, decarboxylase produced by LAB may be responsible for the breakdown of certain phenolic compounds. Moreover, the decrease of antioxidant activity showed greatly influenced by the phenolic composition of the sample.

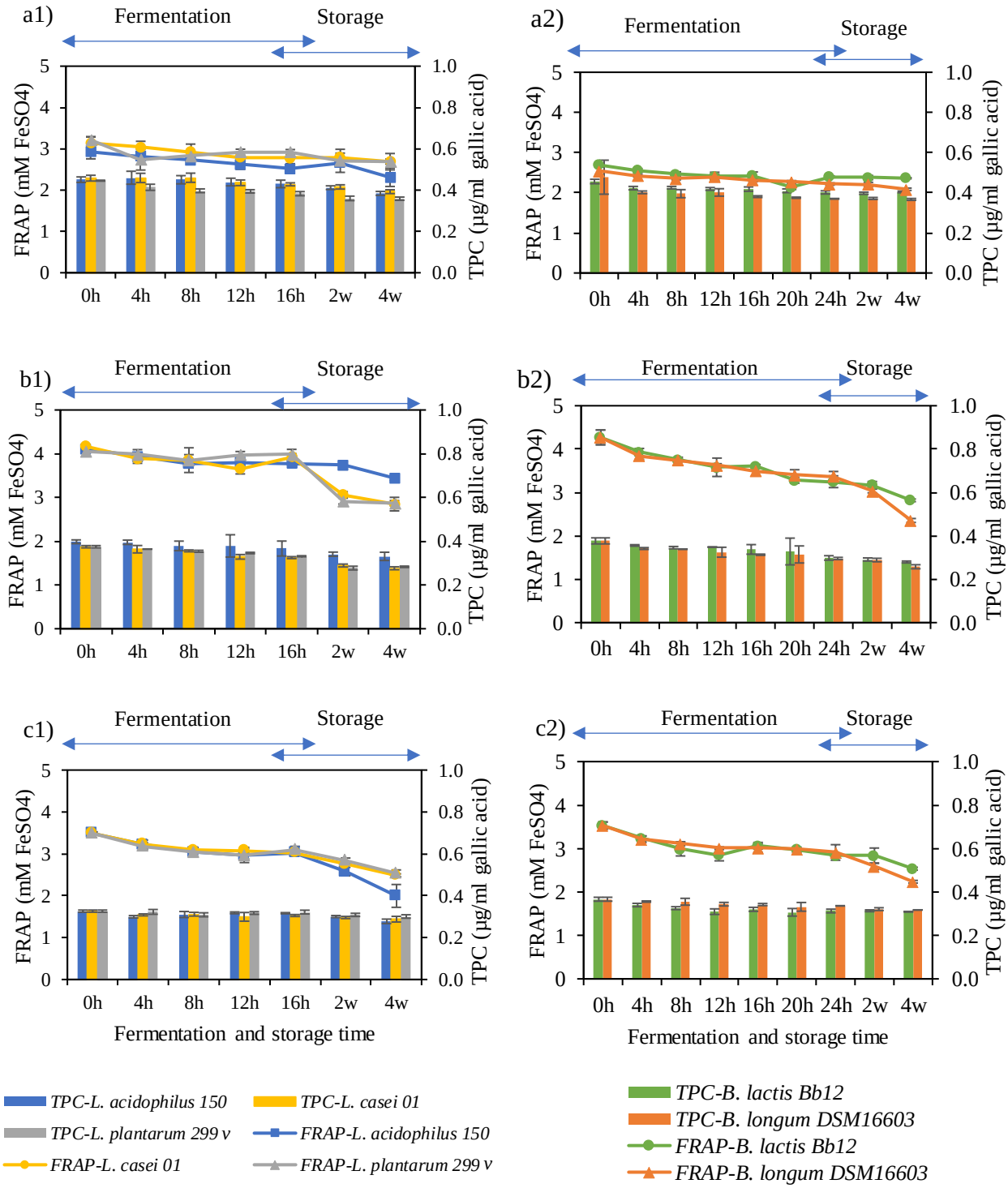


Figure 5.4. Total phenolic content and antioxidant activity of tropical fruit juice: a1), a2) pineapple; b1), b2) mango; and c1), c2) banana fermented at 37°C by *Lactobacillus* 1) and *Bifidobacterium* 2) strains, and further stored for 4 weeks at 4°C

5.1.4. The survival of bacteria through the simulated gastro-intestinal model

One of the main places for activity of probiotic bacteria should be the large intestine (Gilliland *et al.*, 1984), thus the tolerance of microorganisms to gastro-intestinal conditions is

generally considered to be a crucial criteria for probiotic selection (Lee *et al.*, 2004). Before reaching the colon tract, probiotic bacteria must survive passage through the stomach and small intestine. In this part of the tract, the secretion of gastric juice, and then bile salt is the primary defense mechanism against the majority of ingested microorganisms. The pH of the stomach is generally in the range from pH 1.0 during fasting to pH 4.5 after a meal (Soni *et al.*, 2020) and the concentration of bile salts in the small intestine is from 0.2% to 2% varied to the individual, type and amount of food consumed (Gunn, 2000). The food ingestion also can take up for 3 hours. In the current study, a simulated gastro-intestinal condition was carried out. These selected strains were exposed in 0.5% NaCl (pH 2.0) containing 0.3% pepsin for 135 min. Then, the incubated mixture was centrifuged and washed by phosphate-buffered saline. The pellet was incubated with the 0.6% bile salts pH 7.4 solution for 150 mins at 37^{o}C.

The survival of *Lactobacillus* strains in all fermented fruit juices after exposure to pepsin and bile salts solution was over 80% (**Table 5.2**). Storage condition did not affect significantly on the survival of these bacteria through the test.

The similar trend was found in the case of *Bifidobacterium* which obtained the survival through simulated gastro-intestinal conditions except *B. lactis* Bb12 in banana juice which had lower survival rate than *B. longum* DSM16603 (86.2% in pepsin and 69.07% in bile salts). However, after four weeks of storage, their survival improved, which reached 99.48% and 93.11%, respectively. *B. longum* DSM16603 in mango juice showed an opposite trend. Their survival percentage decreased in both pepsin and bile salts conditions after 4 weeks of storage. A 70.69 % and 56.42% of survival of *B. longum* DSM16603 through pepsin and bile salts solution, respectively, were observed.

Kim *et al.* (2012) presented a similar result that LAB in potato juice had high tolerance to gastric juice and bile salts conditions. They indicated that the survival rate of *L. casei* after 3 h exposure to gastric juice (pH 2.5 and 0.3% pepsin) and bile salts (0.3%) was 90%. Ashraf and Smith (2016) reported that the survival of *B. lactis* Bb12 strain was not much affected during simulated gastric transition (survivability to pepsin and bile salt was >95% and >60%, respectively). Andriantsoanirina *et al.* (2013) mentioned that *B. longum* and *B. breve* presented the best tolerance to oxygen, bile and acid stresses among the bifidobacteria tested. Champagne and Gardner (2008) conducted the resistant tests of four strains (*L. acidophilus* LB3, *L. rhamnosus* LB11, *L. reuteri* LB38 and *L. plantarum* LB42) in simulated gastro-intestinal condition. They reported that none of these strains was significantly affected by 0.3 % bile salt fluid during 2 hours of incubation. Storage condition (4^{o}C/35 days) also did not affect this characteristic. This report was in agreement with our results, but in the case of acid tolerance of the bacteria, different result was observed. There was a significant loss in viability of these bacteria (by 2.6 log CFU/mL-2.8 log CFU/mL) when exposed at 37^{o}C for 2h incubation at pH 2 without pepsin supplement. Furthermore, storage for 35 days in fruit juice enhanced the loss by 3.2 log CFU/mL-5.0 log CFU/mL. In our cases, the investigated bacteria showed a high tolerance to low pH stress (pH 2 with 0.3% pepsin). It can be explained that incubation in low pH media (approx. pH 4 at the end of fermentation) for a long time induced the acid tolerance response of bacteria (Lorca and Font

de Valdez, 2001). Mättö *et al.* (2006) and Guo *et al.* (2009) also revealed that the presence of pepsin in simulated gastric juice improved the survival of some probiotics under acidic conditions. However, the protective effect of pepsin definitely depends on species and strains. For example, in the case of *B. animalis* subsp. *lactis*, pepsin helps in maintaining the pH homeostasis in the bacterium and supports the role of enzyme H^+-ATPase (Mättö *et al.*, 2006).

Table 5.2. Effect of storage (at 4ºC for 4 weeks) on survival of *Lactobacillus* and *Bifidobacterium* strains in fermented tropical fruit juices through the simulated gastro-intestinal conditions

Fruit juice	Bacteria	The survival percentage of bacteria (%)			
		Pepsin pH 2		Pepsin pH 2 + Bile salts	
		After fermentation	After storage	After fermentation	After storage
Pineapple	*L. acidophilus* 150	96.92 ± 3.58	98.42 ± 0.83	80.41 ± 2.54	100.02 ± 0.56
	L. casei 01	99.76 ± 1.01	99.81 ± 0.75	98.43 ± 0.5	97.56 ± 1.29
	L. plantarum 299v	99.91 ± 0.81	99.29 ± 0.83	102.12 ± 0.79	95.23 ± 5.74
	B. lactis Bb12	99.68 ± 0.31	99.21 ± 0.83	98.56 ± 0.23	97.77 ± 0.72
	B. longum DSM16603	99.09 ± 0.78	99.51 ± 0.69	99.23 ± 0.73	98.77 ± 0.55
Mango	*L. acidophilus* 150	101.82 ± 7.29	101.61 ± 1.85	101.31 ± 9.3	93.71 ± 6
	L. casei 01	107.05 ± 4.66	108.37 ± 3.57	111.12 ± 5.01	106.28 ± 3.91
	L. plantarum 299v	101.35 ± 1.01	97.68 ± 1.25	99.71 ± 0.77	97.63 ± 1.09
	B. lactis Bb12	100.46 ± 0.4	102.04 ± 1.6	98.15 ± 1.79	98.08 ± 2.78
	B. longum DSM16603	101.53 ± 1.71	70.69 ± 3.55	98.05 ± 2.31	56.42 ± 4.68
Banana	*L. acidophilus* 150	100.96 ± 1.89	97.77 ± 0.82	101.92 ± 1.69	87.96 ± 1.8
	L. casei 01	97.64 ± 0.53	96.65 ± 1.86	97.56 ± 0.61	93.51 ± 1.33
	L. plantarum 299v	102.13 ± 5.59	96.11 ± 0.9	97.91 ± 1.7	91.19 ± 1.18
	B. lactis Bb12	86.2 ± 6.56	99.48 ± 0.98	69.07 ± 2.91	93.11 ± 1.29
	B. longum DSM16603	97.25 ± 1.37	96.95 ± 1.35	94.49 ± 3.23	96.83 ± 1.74

In summary, all tested strains can grow well in three kinds of juices with the viable cell counts over than 8 log CFU/mL. *L. acidophilus* presented a lower growth than the rest of *Lactobacillus* strains. Furthermore, these selected strains can survive over 80% through simulated gastro intestinal conditions except *B. longum* DSM16603, thus, *L. casei* 01, *L. plantarum* 299v and *B. lactis* Bb12 were selected for further experiments. Pineapple juice seemed to be the best substrate among the juices for growth of probiotic bacteria. Furthermore, the addition of mango and banana can improve the flavour as well as the nutritional value of the product.

5.2. Fermentation of mixed fruit juice using monocultures

Mixed fruit juice of pineapple, mango and banana in equal proportion was fermented using single strain of *L. casei* 01, *L. plantarum* 299v and *B. lactis* Bb12. The fermentation was carried out in an incubator at 37°C for 16 h. Then, the fermented samples were stored at 4°C for 4 weeks.

5.2.1. Change of viability and pH

The change of viable cell number and pH value of mixed juice during fermentation and storage are shown in **Figure 5.5.** The three probiotic bacteria, *L. casei* 01, *L. plantarum* 299v and *B. lactis* Bb12, grew rapidly in the juices and reached around 9 log CFU/mL after 16 h of fermentation. The *L. casei* 01 strain had greater growth rate than other strains and recorded the highest microbial population in the juice after fermentation. Storage time did not result in a decrease in viable cell counts of the three strains.

A declining trend was observed in pH values. The pH values of the juices dropped from pH 6.4 at initial to around pH 4.3 at the end of the fermentation period. The lowest value was recorded in the case of juice fermented with *Lactobacillus* strains (around pH 4.0). The pH decrease rate of juice inoculated with *Bifidobacterium* strain was significantly slower than the juice with *Lactobacillus* strain. After 16 h of fermentation, the pH of the juice containing *B. lactis* Bb12 was pH 4.35.

In the previous experiment, pineapple juice demonstrated the most suitable substrate for probiotic bacteria, followed by mango and banana juice. In this experiment, the growth of *Lactobacillus* and *Bifidobacterium* strains in the mixed juice of pineapple, mango and banana was better than in single mango and banana juice. Additionally, the substrate shortened the fermentation time of *B. lactis* Bb12 from 24 h to 16 h based on the pH value (<4.5).

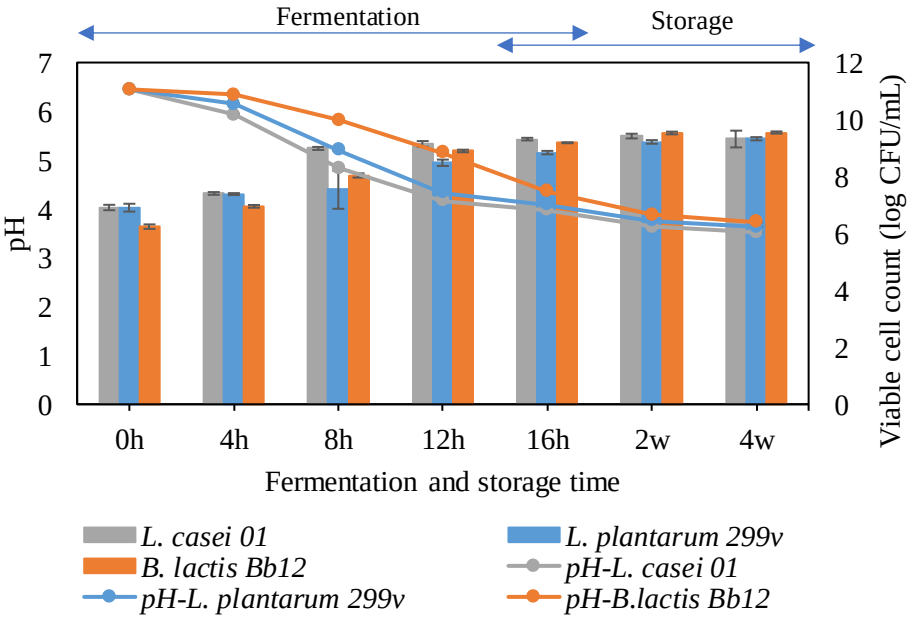


Figure 5.5. Change of viable cell count and pH value of mixed juice of pineapple, mango and banana during fermentation and storage using *Lactobacillus* and *Bifidobacterium* strains

5.2.2. Change of sugar and acid content

Contents of sugar (glucose and fructose) and lactic acid of the mixed juice fermented with probiotic bacteria during fermentation and storage are presented in **Figure 5.6 a)** and **Figure 5.6 b)**, respectively. The glucose content in the juice (7.29% w/v) was significantly higher than fructose (3.39% w/v). Both kinds of sugars were utilized by the probiotic bacteria during fermentation and storage. Upon completion of fermentation, the concentration of glucose and fructose dropped by in a range of 0.53-1.09 (% w/v) and in a range of 0.22-0.6 (% w/v), respectively. A continuous decrease in the sugar contents was also observed during the storage period.

The three probiotic strains utilized the sugars for cell synthesis and lactic acid production. The production of lactic acid was more intensive in the juices fermented with *Lactobacillus* strains (around 2.2% v/v) compared to *Bifidobacterium* strain (1.78% v/v). After 4 weeks of storage, the highest lactic acid concentration was observed in the case of *L. casei* 01 (3.84% v/v), followed by *L. plantarum* 299v (3.33% v/v) and *B. lactis* Bb12 (2.73% v/v).

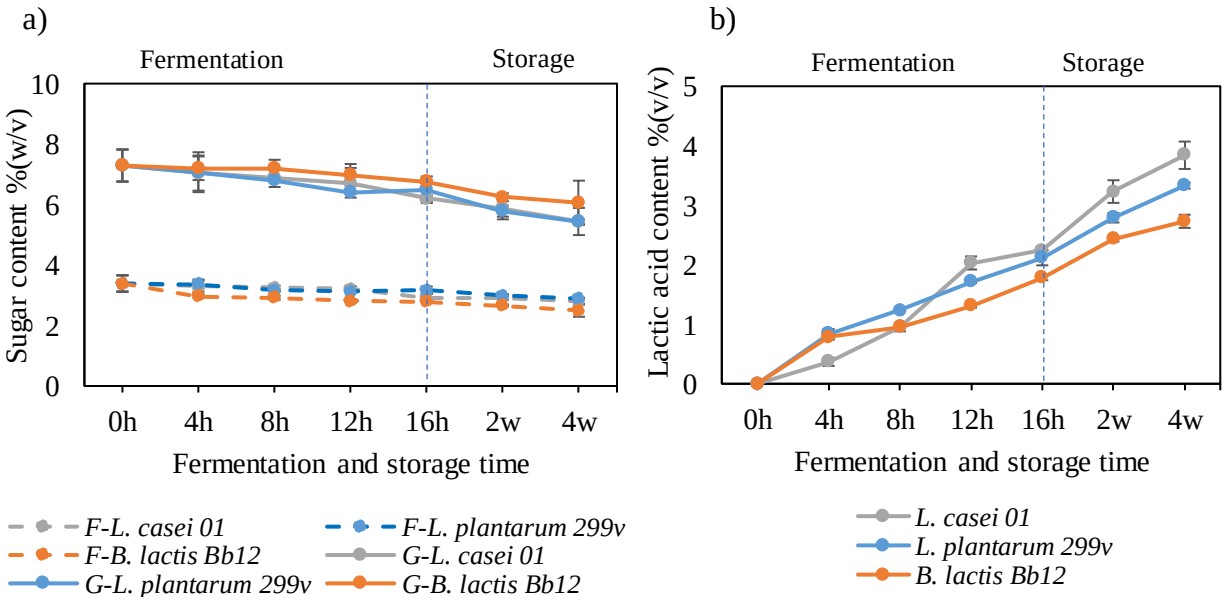


Figure 5.6. Change of chemical properties: a) sugar (glucose - G and fructose - F) and b) lactic acid content of the mixed juice fermented by *Lactobacillus* and *Bifidobacterium* strains during fermentation and storage

5.2.3. Change of total phenolic content and antioxidant activity

Figure 5.7 shows the change of total phenolic content and antioxidant activity of the mixed juice during fermentation and storage. The initial phenolic content of the juice was 0.37 µg/mL gallic acid, and the antioxidant activity was 3.81 mM $FeSO_4$. These parameters decreased during fermentation. After fermentation, a lower value of FRAP was recorded in the juice inoculated with *B. lactis* Bb12 compared to the juice with *Lactobacillus* strains. In detail, the FRAP value of juice fermented with *B. lactis* Bb12 was 3.16 mM $FeSO_4$, with both *Lactobacillus* strains was around 3.47 mM $FeSO_4$. A slight continuous decline of TPC and FRAP was observed during storage.

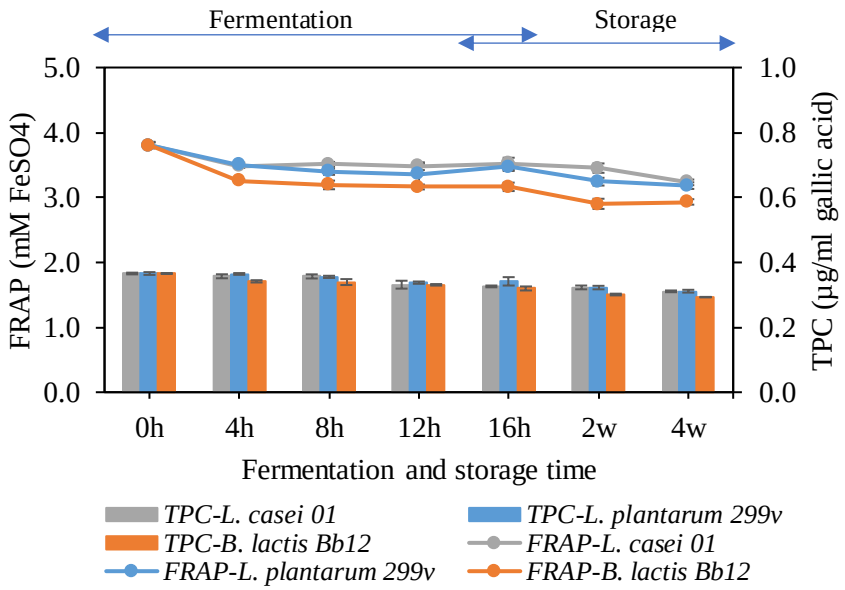


Figure 5.7. Change of total phenolic content and antioxidant activity of the mixed juice fermented with single strain of *Lactobacillus* and *Bifidobacterium* during fermentation and storage

5.3. Fermentation of fruit juices using mixed culture

Fermentation of each fruit juice (pineapple-P, mango-M, banana-B) and their mixture with ratio of 70P:15M:15B, 50P:25M:25B and 33P:33M:33B with mixed culture of *L. casei* 01*: L. plantarum* 299v*: B. lactis* Bb12 (1:1:1) were conducted at 37^{o}C for 16 hours. Then, the fermented samples were stored at 4^{o}C for 4 weeks.

5.3.1. Change of viability and pH

The data (**Figure 5.8)** showed that pH values of the juices decreased significantly during fermentation and storage. After 16 h of fermentation, meanwhile pH values of all mono fruit juices (pineapple, mango and banana) dropped slightly from pH 6.5 to pH 4.25, pH 4.33 and pH 4.49, respectively, whereas bigger decrease in pH values (to pH 4) of the mixed samples were observed. The pH values of all fermented juices continued the decline tendency during storage. In the first two weeks, the pH values decreased significantly from pH 4 to approx. pH 3.6 in the case of juice mixtures and from a range of pH 4.25-4.49 to a range of pH 3.67-3.92 in the case of individual juices. In the next two weeks, the reduction rate of pH value decreased. The pH of mixed fruit juices after storage was approx. pH 3.5. The pH values of fermented pineapple, mango and banana after storage were pH 3.51, pH 3.64 and pH 3.76, respectively. Wang *et al.* (2003) observed the pH values of 5.98 and 6.24 in fermented soymilk with the mixed culture of *L. acidophilus* + *B. infantis* and *L. acidophilus* + *B. longum* after 24 h of fermentation, respectively. These values were lower than the pH of soymilk fermented with mono strain of *L. acidophilus* (pH 6.45). Bujna *et al.* (2017) reported a significant decline of pH from pH 6.6 to a range of pH 4.6-4.9 after fermentation

of apricot juice with combinations of *Bifidobacterium* and *Lactobacillus* strains. These authors claimed that the drop of pH due to the intensive growth and metabolic activity of probiotic bacteria.

Changes of cell number in juice samples during fermentation and storage are shown in **Table 5.3**. The mixed culture of *Lactobacillus* and *Bifidobacterium* increased their population during fermentation and remained quite stable through the storage period. The total microbial viability in most fermented substrates after fermentation obtained in a range of 9.18-9.41 (log CFU/mL), except in banana juice with lower viable cell count of 8.92 log CFU/mL. During storage for four weeks, the cell counts of bacteria in fermented juices had a little fluctuation but still remained more than 9 log CFU/mL. These results illustrated that mixed culture could improve the growth of each microorganism in all fruit juices and mixed juices. The microbial population in pineapple juice, mixed fruit juice with the ratio of 70P:15M:15B and 50P:25M:25B after fermentation were higher than in other substrates. Wang *et al.* (2003) noted that the cell counts of *B. longum* in combination with *L. acidophilus* was significantly higher than that in soymilk fermented by an individual organism after 24 h of fermentation. Interestingly, they also reported that the growth of *L. acidophilus* was inhibited when it was inoculated into soymilk alone. Fermentation of apricot juice with the combination of bifidobacteria and LAB presented the higher levels in cell counts than in case of monocultures (Bujna *et al.*, 2017). These authors also revealed that the best combination was the mixture of *B. lactis* Bb12 with *L. casei* 01. Božanić *et al.* (2011) added *B. lactis* Bb12 with yoghurt culture YCX11 (*Streptococcus thermophilus* and *L. delbrueckii* ssp. *bulgaricus*) to shorten the fermentation time of soy yoghurt production. They also addressed that the fermentation time was shortened to 4 hours, compared to 12-17 (hours) of fermentation with individual *L. acidophilus* La5, *Lactobacillus casei* 01, and *B. lactis* Bb12.

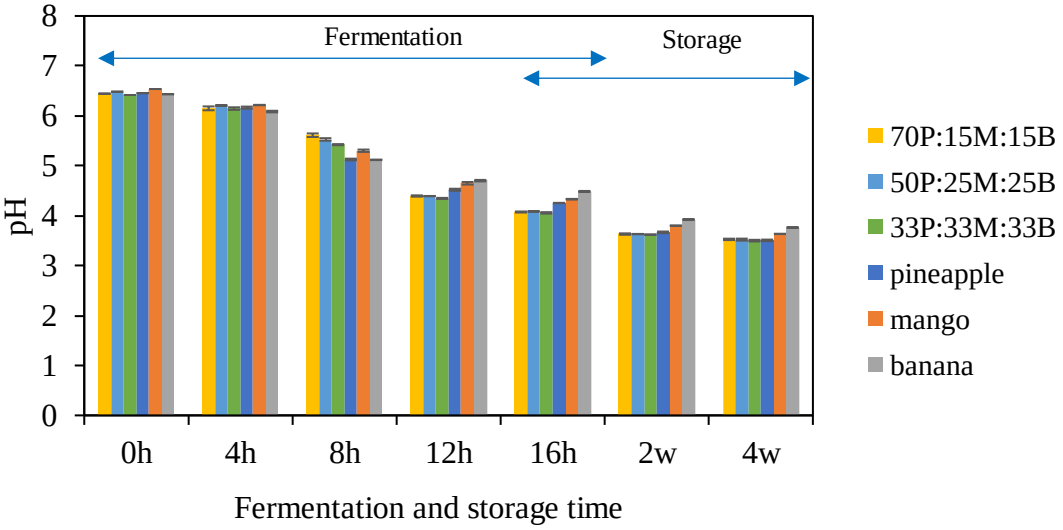


Figure 5.8. pH values of individual fruit juice (pineapple-P, mango-M, banana-B) and mixed fruit juice with ratio of 70P:15M:15B, 50P:25M:25B and 33P:33M:33B fermented at 37°C by mixed culture of *L. casei* 01:*L. plantarum* 299v:*B. lactis* Bb12 (1:1:1), and further stored at 4°C for 4 weeks

Wang *et al.* (2002) presented the similar result that *L. acidophilus* in combination with *Bifidobacterium* strain obtained maximum cell counts sooner than when growing alone in soymilk. Murti *et al.* (2006) reported that the presence of bifidobacteria in soymilk fermentation stimulated the growth of yoghurt bacteria. It is commonly believed that LAB has a minimal ability to synthesize amino acids when using inorganic nitrogen sources. Therefore, they depend on the presence of available amino acids in the growth medium as a nitrogen source (Salminen *et al.*, 2011). Belkaaloul *et al.* (2010) mentioned that the proteolytic system of *Lactobacillus* was enhanced when it combined with bifidobacteria. Cinquin *et al.* (2004) noted that acidic pH accelerated the growth of bifidobacteria. In this way, LAB produces lactic acid at the beginning leading to a decrease in pH value that can stimulate bifidobacteria growth in mixed culture media. On the other hand, Deguchi *et al.* (1985) indicated that *Bifidobacterium* of human origin take part in synthesis of six vitamins by thiamine (B1), riboflavin (B2), pyridoxine (B6), folic acid (B9), cyanocobalamin (B12), and nicotinic acid (B3). Three of these vitamins (B1, B2 and B9) are the required vitamins for some of *Lactobacillus* strains (Franklin and Sharpe, 1964).

Table 5.3. Microbial population of individual fruit juice and mixed fruit juices fermented at 37°C by mixed culture of *L. casei* 01*: L. plantarum* 299v*: B. lactis* Bb12 (1:1:1), and further stored at 4°C for 4 weeks

Fruit juices	Bacteria	Microbial population (log CFU/mL)						
		Fermentation period					Storage period	
		0h	4h	8h	12h	16h	2w	4w
Pineapple (P)	*Lactobacillus*	6.82 ± 0.09	7.35 ± 0.05	8.82 ± 0.04	9.23 ± 0.05	9.18 ± 0.07	9.28 ± 0.03	9.06 ± 0.05
	Bifidobacterium	6.02 ± 0.02	6.51 ± 0.36	7.88 ± 0.14	8.24 ± 0.16	8.81 ± 0.24	8.83 ± 0.14	8.64 ± 0.04
	Total	**6.93 ± 0.11**	**7.42 ± 0.07**	**8.87 ± 0.04**	**9.27 ± 0.03**	**9.35 ± 0.06**	**9.41 ± 0.05**	**9.2 ± 0.05**
Mango (M)	*Lactobacillus*	6.64 ± 0.09	7.17 ± 0.11	8.55 ± 0.05	8.91 ± 0.09	9.05 ± 0.06	8.88 ± 0.12	8.88 ± 0.08
	Bifidobacterium	5.55 ± 0.02	6.7 ± 0.2	7.73 ± 0.19	8.24 ± 0.05	8.59 ± 0.17	8.58 ± 0.1	8.53 ± 0.06
	Total	**6.93 ± 0.11**	**7.31 ± 0.04**	**8.62 ± 0.05**	**9 ± 0.08**	**9.18 ± 0.08**	**9.06 ± 0.07**	**9.05 ± 0.05**
Banana (B)	*Lactobacillus*	6.64 ± 0.09	7.29 ± 0.07	8.59 ± 0.06	8.68 ± 0.08	8.64 ± 0.11	8.88 ± 0.08	9.24 ± 0.05
	Bifidobacterium	5.55 ± 0.02	6.68 ± 0.12	8.03 ± 0.23	8.39 ± 0.15	8.56 ± 0.14	8.6 ± 0.25	8.12 ± 0.5
	Total	**6.93 ± 0.11**	**7.39 ± 0.07**	**8.71 ± 0.01**	**8.87 ± 0.05**	**8.92 ± 0.06**	**9.09 ± 0.05**	**9.29 ± 0.04**
70P:15M:15B	*Lactobacillus*	6.96 ± 0.03	7.37 ± 0.04	8.33 ± 0.48	9.22 ± 0.05	9.25 ± 0.11	9.34 ± 0.07	9.33 ± 0.07
	Bifidobacterium	6.27 ± 0.09	6.7 ± 0.21	8.35 ± 0.37	8.56 ± 0.15	8.81 ± 0.27	8.74 ± 0.12	8.78 ± 0.17
	Total	**7.1 ± 0.09**	**7.46 ± 0.04**	**8.77 ± 0.14**	**9.31 ± 0.04**	**9.41 ± 0.06**	**9.44 ± 0.04**	**9.44 ± 0.07**
50P:25M:25B	*Lactobacillus*	6.96 ± 0.03	7.32 ± 0.06	8.62 ± 0.16	9.22 ± 0.09	9.23 ± 0.05	9.26 ± 0.06	9.32 ± 0.05
	Bifidobacterium	6.27 ± 0.09	6.52 ± 0.24	7.93 ± 0.35	8.32 ± 0.29	8.77 ± 0.29	8.71 ± 0.23	8.72 ± 0.21
	Total	**7.1 ± 0.09**	**7.39 ± 0.07**	**8.73 ± 0.08**	**9.28 ± 0.06**	**9.38 ± 0.06**	**9.38 ± 0.05**	**9.43 ± 0.06**
33P:33M:33B	*Lactobacillus*	6.96 ± 0.03	7.31 ± 0.15	8.72 ± 0.09	9.26 ± 0.06	9.06 ± 0.08	9.16 ± 0.07	9.19 ± 0.12
	Bifidobacterium	6.27 ± 0.09	6.52 ± 0.2	7.55 ± 0.16	8.1 ± 0.19	8.69 ± 0.21	8.45 ± 0.14	8.44 ± 0.32
	Total	**7.1 ± 0.09**	**7.38 ± 0.14**	**8.75 ± 0.08**	**9.29 ± 0.05**	**9.23 ± 0.07**	**9.24 ± 0.06**	**9.28 ± 0.10**

5.3.2. Change of sugar and organic acid

During fermentation and storage, an increase in organic acid content was observed in all media (**Figure 5.9)**. After fermentation, the highest concentration of lactic acid was recorded in the case of mixed fruit juice (2.68% v/v) with ratio of 70P:15M:15B, followed by pineapple juice (2.33% v/v), mixed juice with ratio of 50P:25M:25B (2.30% v/v), mixed fruit juice with ratio of 33P:33M:33B (2.03% v/v) and banana juice (0.98% v/v). Mango juice had the lowest quantity of lactic acid concentration (0.56% v/v).

The concentration of lactic acid rose dramatically during storage for 4 weeks. At the end of the storage period, mixed fruit juice with ratio of 70P:15M:15B and pineapple juice obtained the highest lactic acid concentration (approx. 4.2% v/v). Fermented mango and banana juice had by far the lowest lactic acid concentration after storage. The lactic acid quantity of these juices was less than half of the concentration of others (1.67-1.82 (% v/v)).

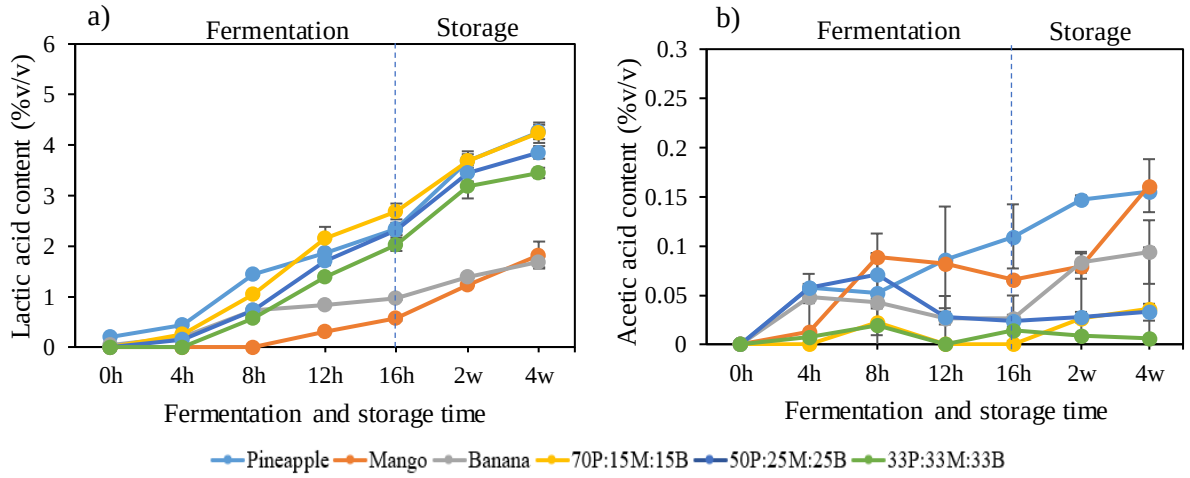


Figure 5.9. Concentration of a) lactic acid and b) acetic acid concentration of mono fruit juice and mixed fruit juices fermented at 37°C by mixed culture of *L. casei* 01:*L. plantarum* 299v:*B. lactis* Bb12 (1:1:1), and further stored at 4°C for 4 weeks

Acetic acid was found with small amount in all fermented juices and mixed juices. The individual pineapple, mango and banana juice contained more acetic acid after fermentation than mixed fruit juices. The highest concentration was observed in the case of pineapple juice. The values were 0.11% v/v and 0.16% v/v after fermentation and storage, respectively. The mixed juices were observed with very small quantity of acetic acid during fermentation and storage.

Combination starter of *Lactobacillus* and *Bifidobacterium* were used in studies of other researches. Bujna *et al.* (2017) observed the range of 70 mM-89 mM lactic acid in fermented apricot juice inoculated with different mixed cultures of bifidobacteria and LAB. The titers of 13.93 mmol/L and 12.36 mmol/L acid lactic concentration were found in soymilk after 32 h of fermentation with the mixed culture of *L. acidophilus* + *B. infantis* and *L. acidophilus* + *B. longum*, respectively (Wang *et al.*, 2003). Matsuyama *et al.* (1992) indicated that higher acid amount could be obtained when inoculating the mixture of LAB and bifidobacteria compared to the

corresponding individual LAB. Wang *et al.* (2003), however, stated that fermentation by culturing a mixture of *L. acidophilus* and *Bifidobacterium* resulted higher or lower amounts of acid, depending on the species of *Bifidobacterium*, compared to fermentation by mono *L. acidophilus*. In our study, the amount of acid production by mixed culture were quite similar to the concentration of acid found in the juices fermented by monoculture, and these result are consistent to the findings of Bujna *et al.* (2017).

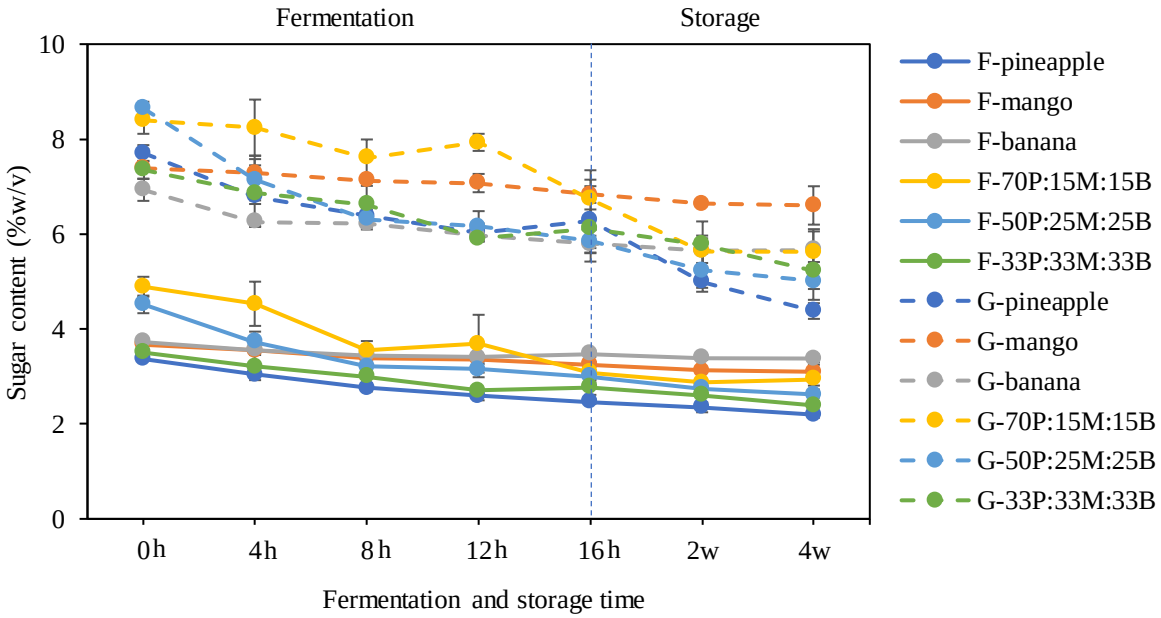


Figure 5.10. Sugar (glucose-G and fructose-F) concentration of individual fruit juice and mixed fruit juices fermented at 37°C by mixed culture of *L. casei* 01:*L. plantarum* 299v:*B. lactis* Bb12 (1:1:1), and further stored at 4°C for 4 weeks

Sugar consumption by mixed cultures during fermentation and storage was presented in **Figure 5.10.** A decline sharp of glucose and fructose concentration was observed in all the juices and mixed juices. The sugar (glucose and fructose) in the pineapple juice and mixed fruit juices were utilized more rapidly than in mango and banana. After 16 h of fermentation, the glucose and fructose content in pineapple juice decreased by 1.42% w/v and 0.9% w/v, respectively. In the mixed juice with ratio of 70P:15M:15B, these values were 1.66% w/v and 1.81% w/v, respectively. In the mixed juice with ratio of 50P:25M:25B, glucose concentration reduced by 2.8% w/v and fructose content dropped by 1.52% w/v. In the mixed juice with ratio of 33P:33M:33B, glucose and fructose quantity decreased by 1.24% w/v and 0.73% w/v, respectively. Meanwhile, in mango juices, these values were 0.55% w/v and 0.42% w/v, and in banana juice, the numbers were 1.14% w/v and 0.24% w/v, respectively.

During storage process, the glucose and fructose concentration of the fermented juices continued decreasing. The highest reduction of these sugar contents was observed in the case of pineapple and mixed juices. After fermentation and storage, the probiotic bacteria used a range of 2.13-3.63 (% w/v) glucose and a range of 1.11-1.96 (% w/v) fructose. A lower sugar consumption

of probiotic bacteria was recorded in mango and banana juice which were in a range of 0.78-1.26 (% w/v) glucose and in a range of 0.35-0.57 (% w/v) fructose.

5.3.3. Change of total phenolic content and antioxidant activity

Based on data presented in **Figure 5.11**, pineapple juice was found as the richest TPC source (0.48 µg/mL gallic acid), and mango and banana juice had lower TPC (0.35 µg/mL gallic acid and 0.37 µg/mL gallic acid, respectively). The mixture of three kinds of juice including 70P:15M:15B, 50P:25M:25B and 33P:33M:33B contained 0.39 µg/mL gallic acid, 0.36 µg/mL gallic acid and 0.33 µg/mL gallic acid, respectively. The TPC of these media decreased slightly during fermentation and storage. After fermentation, the highest decline (15.4%) in TPC was observed in the case of mango juice, followed in the cases of pineapple juice, banana juice and the combination of 50P:25M:25B (approx. 11%). The TPC of mixed juices with ratio of 70P:15M:15B and 33P:33M:33B decreased by 9.08% and 5.08%, respectively.

During four weeks of storage, the concentration of TPC kept reducing slightly. Fermented mango juice registered as the highest decline in TPC (19.57%) while the mixed fruit juices with ratio of 33P:33M:33B was recorded as the lowest (10.01%).

Regarding antioxidant activity, the highest FRAP values were observed (approx. 3.6 mM $FeSO_4$) in mango, banana juice and mixed fruit juice with ratio of 33P:33M:33B, whereas the smallest value was in pineapple juice (2.73 mM $FeSO_4$). The data (**Figure 5.11**) showed that antioxidant capacity dropped significantly after first four hours of fermentation in all mono and mixed juices. From the hour 4, this parameter decreased gradually until the end of the storage period in the cases of mango, banana and mixed fruit juice with ratio 33P:33M:33B as well as unchanged sharp in the cases of pineapple, mixed fruit juice with the ratios of 70P:15M:15B and 50P:25M:25B. After four weeks of storage, the highest decrease of FRAP was observed in fermented banana juice (28.64%), followed ones in the mixed fruit juices (70P:15M:15B and 33P:33M:33B) (approx. 17.5%), in mango juice (16.15%) and in pineapple juice (13.78%). In the case of the mixed fruit juice with ratio 50P:25M:25B, the lowest change in FRAP (7.2%) was recorded.

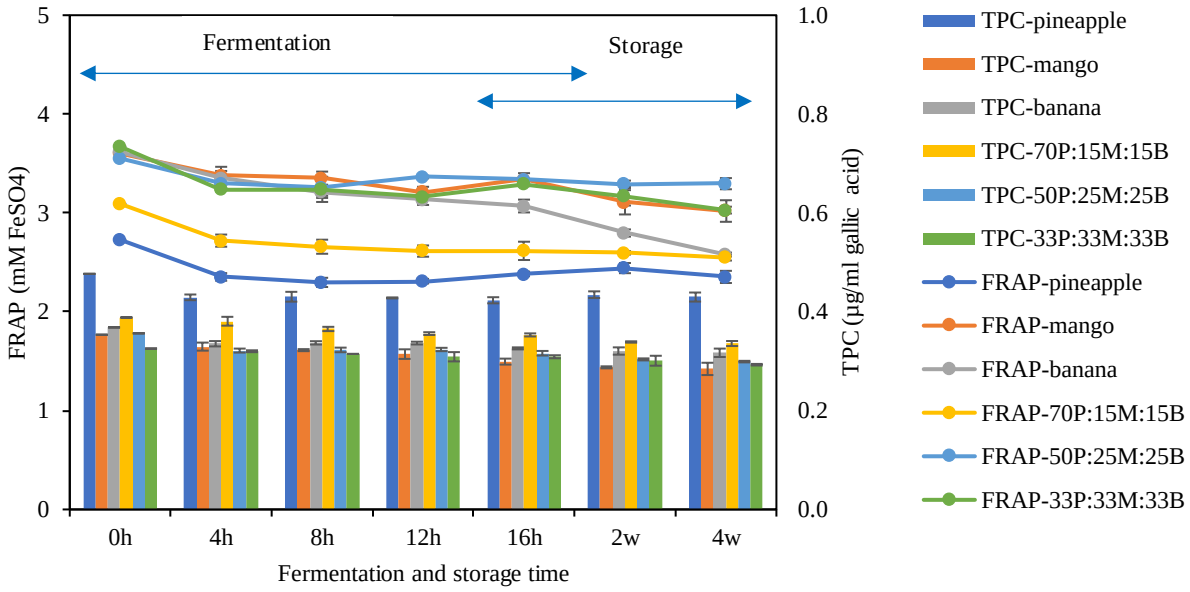


Figure 5.11. FRAP and TPC content of mono fruit juice and mixed fruit juices fermented at 37°C by mixed culture of *L. casei* 01:*L. plantarum* 299v:*B. lactis* Bb12 (1:1:1), and further stored at 4°C for 4 weeks

The antioxidative properties depend on both quality and quantity of antioxidant compounds. For example, the contribution of vitamin C to the antioxidant activity of mango, papaya and banana varied widely from 4.4 % to 62.3% (Songsermsakul *et al.*, 2012). Therefore, although pineapple juice had the highest TPC contents, the antioxidant activity was the lowest among three kinds of investigated fruit juices.

The decrease of antioxidant activity of fermented juice was also confirmed by Tien *et al.* (2005). They revealed a decrease in the antioxidant activity of apple puree and juice fermented with mono and mixed starter of *L. delbrueckii* subsp. *lactis* ATCC 7830, *L. paracasei* subsp. *paracasei* ATCC 25598 and *L. casei* subsp. *casei* ATCC 393 strains. Bujna *et al.* (2017), however, reported that the combination of *L. casei* 01 with *Bifidobacterium* did not affect significantly on antioxidant activity of apricot juice during the fermentation. In contrast, a slight increase was observed in the combination of *L. acidophilus* La5 and *Bifidobacterium*. Therefore, antioxidant capacity (production of antioxidative metabolites and/or metal ion chelating ability etc.) of probiotic microorganisms is strain-depending and may not be affected synergistically.

5.3.4. Probiotic survival through the simulated gastro-intestinal model

Mixed culture was not significantly affected by artificial gastric fluid and bile salt through the model. The survival of bacteria in both *Lactobacillus* and *Bifidobacterium* strains were over 90% (**Table 5.4**) after four weeks of storage at 4°C. The results indicated that the bacteria would not enhance the sensitivity to gastric fluid and bile salt of each other when growing in the media simultaneously. Sahadeva *et al.* (2011) investigated the survival of five brands (A to E) of commercially cultured milk drinks through simulating the human gastro-intestinal pH and bile

concentration. Brand E contained *L. acidophilus, L. casei* and *Bifidobacterium*. Their results showed that the microbial viability in brand E significantly decreased when exposed to simulated gastric juice of pH 3 after 3 hours of incubation. However, the cell number was still high after exposed to bile, even when subjected to 2% of bile. They also observed some increases in microbial viability of other brands after the treatment. The rise of cell number could be explained that the bacteria had a stress adaptation mechanism along with the long incubation time, which was for 3 hours of gastric juice and for 24 hours of bile salt. The enhanced survival capabilities were due to the acclimatization of the bacteria to the low pH environment, therefore minimizing the relative toxicity to glycoconjugates in the intestine (Begley *et al.*, 2005, Martoni *et al.*, 2007). The protective effect of food matrix also may prevent the bacteria from bile exposure and hence, giving rise to the increased bile resistance of the strains (Begley *et al.*, 2005).

Table 5.4. Survival of mixed cultures of *L. casei* 01*:L. plantarum* 299v*:B. lactis* Bb12 (1:1:1) in fermented individual fruit juices and mixed fruit juices through simulated gastro-intestinal conditions before and after 4 weeks of storage

Juice	Strain	0 week		4 weeks	
		Pepsin pH 2	Bile salts	Pepsin pH 2	Bile salts
Pineapple	*Lactobacillus*	99.43 ± 0.35	95.66 ± 0.39	99.3 ± 0.15	95.01 ± 0.46
	Bifidobacterium	100.56 ± 1.66	95.58 ± 3.03	99.87 ± 1.21	92.66 ± 2.45
	Total	**99.86 ± 0.4**	**95.84 ± 0.54**	**99.48 ± 0.34**	**94.66 ± 0.75**
Mango	*Lactobacillus*	99.43 ± 0.39	93.66 ± 0.53	99.45 ± 0.45	95.52 ± 0.9
	Bifidobacterium	99.69 ± 3.3	94.51 ± 4.71	95.87 ± 1.41	89.47 ± 1.21
	Total	**99.55 ± 0.89**	**94.26 ± 1.31**	**98.68 ± 0.41**	**94.47 ± 0.79**
Banana	*Lactobacillus*	99.32 ± 0.44	93.82 ± 0.68	98.99 ± 0.44	92.53 ± 0.67
	Bifidobacterium	94.3 ± 4.86	92.44 ± 3.4	100.38 ± 2.3	97.41 ± 4.26
	Total	**97.95 ± 1.3**	**93.69 ± 1.39**	**99.14 ± 0.47**	**93.84 ± 1**
70P:15M:15B	*Lactobacillus*	99.63 ± 0.21	96 ± 0.15	99.63 ± 0.19	95.39 ± 0.23
	Bifidobacterium	100.78 ± 1.69	95.54 ± 2.64	98.12 ± 1.71	95.93 ± 1.26
	Total	**99.86 ± 0.2**	**96.21 ± 0.25**	**99.36 ± 0.24**	**95.63 ± 0.3**
50P:25M:25B	*Lactobacillus*	99.51 ± 0.43	95.62 ± 0.57	99.52 ± 0.31	95.86 ± 0.23
	Bifidobacterium	100.8 ± 1.92	96.84 ± 1.32	100.68 ± 1.09	96.53 ± 2.54
	Total	**99.88 ± 0.3**	**96.06 ± 0.2**	**99.85 ± 0.09**	**96.25 ± 0.19**
33P:33M:33B	*Lactobacillus*	99.74 ± 0.53	95.67 ± 0.31	98.96 ± 0.58	94.44 ± 0.63
	Bifidobacterium	98.66 ± 2.02	95.59 ± 0.88	102.19 ± 1.86	94.7 ± 2.84
	Total	**99.61 ± 0.19**	**95.81 ± 0.1**	**99.82 ± 0.44**	**94.76 ± 0.84**

5.3.5. Sensory evaluation

Generally, growth of bacteria in fruit juice may cause the change of some characteristics of the product. Some compounds will be consumed, and others are produced. Such as while the sugar consumption provides the maintenance of the cell, whereas the production of organic acids will take place, or the presence of biomass causes the thick texture of fermented beverage (Ellendersen *et al.*, 2012). Hence, the sensory evaluation of fermented fruit juice is important. The

acceptance of sensory attributes (appearance, aroma, taste, texture and overall) of fermented fruit juices was carried out. The results are shown in **Figure 5.12.** It concluded that the consumer accepted most of the formulations in the range of like moderately to like very much (7-8 (points)) except fermented pineapple juice with overall score of 6.43 points (based on hedonic scale of 9 points). Consumers highly accepted the fermented mango and banana juices while pineapple juice had the lowest acceptance. Both attributes received moderately liked scores (approx. 7 points). The taste did significantly influence the preference ranking of fermented juices in this experiment. Pineapple juice came with the lowest acceptance score of taste (6.71 points). The most preferred in taste was registered for the banana with 8.29, and there was no significant different between mango (7.86 points), mixed fruit juices with ratios of 50P:25M:25B (7.86 points) and 33P:33M:33B (7.29 points)

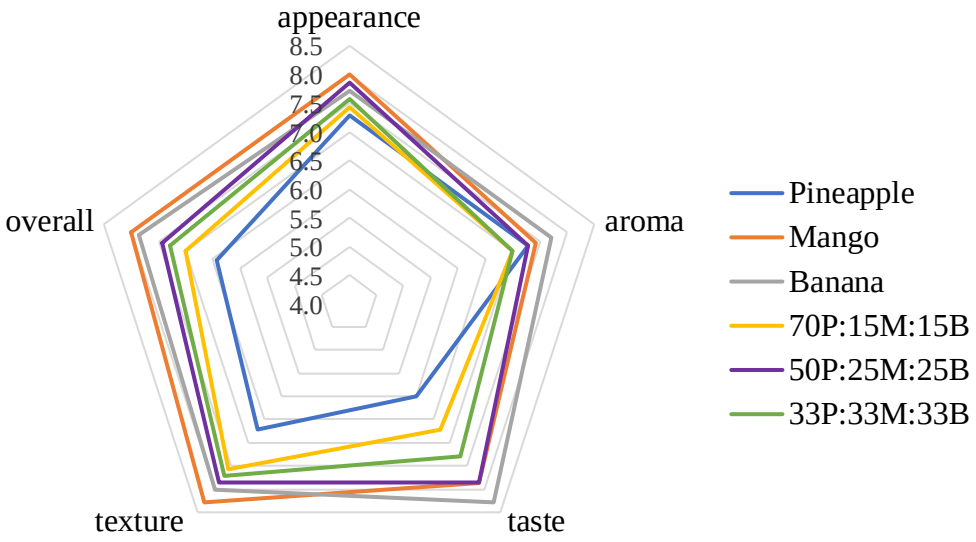


Figure 5.12. Sensory evaluation of fruit juices and mixed fruit juices fermented by mixed culture

The high acceptance of fermented banana juice may be affected by the sweet taste. Pimentel *et al.* (2015) reported that the overall acceptance of clarified apple juice was driven positively by the sweet taste, bitter aftertaste and sweet aroma. The sweetness is the most important attribute for the acceptance of apple juice (Costa *et al.*, 2013, Endrizzi *et al.*, 2015). In this study, the panellists mentioned that they prefer the sweetness in fermented mango (13.6 ^{o}Brix) and banana juice (13.8 ^{o}Brix).

Table 5.5. Solid contents of fruit juices and mixed fruit juices

Juices	°Brix
Pineapple (P)	12.2
Mango (M)	13.6
Banana (B)	13.8
70P:15M:15B	12.6
50P:25M:25B	12.8
33P:33M:33B	13.1

The data in **Table 5.5** showed that the Brix value of fermented banana juice was the highest with 13.8%, followed by fermented mango juice (13.6%) and pineapple juice came with the lowest value (12.2%). Regarding the texture, the panelists preferred significantly mango and banana juices, as well as the mixed fruit juices with ratios of 50P:25M:25B and 33P:33M:33B. Texture of fermented pineapple and mixed fruit juice with ratio of 70P:15M:15B received the lowest acceptance.

In conclusion, the fermented beverage products were well-accepted by the panelists in the range of like moderately to like very much except fermented pineapple juice with the lower preference score. Fermented banana and mango juices received the highest acceptance score, but concerning the microbial population, these media may have some limitations for the growth of bacteria. The fermented mixed fruit juice with ratio of 50P:25M:25B had the lower preference score than the fermented banana juice. Furthermore, fermentation of this mixed juice of ratio of 50P:25M:25B with the mixed culture of *Lactobacillus* and *Bifidobacterium* reached the highest cell count which is one of the most important attributes of probiotic product.

5.4. Storage study

The mixed fruit juice (Pineapple:Mango:Banana) with ratio of 50:25:25 was used for storage study. After fermentation with mixed culture of *L. casei* 01:*L. plantarum* 299v:*B. lactis* Bb12 (1:1:1) at 37°C for 16 hours, the products were stored at different temperatures including 5°C, 15°C, 25°C and 35°C until they begin to failure (pH<3.4).

5.4.1. Change of cell number

Changes of viable cell counts in fermented mixed fruit juice during storage at different temperatures (5°C, 15°C, 25°C and 35°C) are presented in **Figure 5.13**. The population of bacteria in fermented mixed fruit juice showed different trends depending on the storage temperature. A slight increase tendency at the initial stage and a significant decrease at the later stage were observed in the case of 25°C and 35°C. In detail, the bacteria kept a gradual increase in number from 9.43 log CFU/mL at beginning to approx. 9.5 log CFU/mL during three days in the case of 25°C and 1.5 days in the case of 35°C. Then, the bacteria population declined significantly to 9.36

log CFU/mL and 9.19 log CFU/mL at the end of storage, respectively. When storage at the lower temperature (5°C and 15°C), microbial population had a slight decrease at the beginning of storage period and slight increase afterwards. In the case of storage at 15°C, after 3 days with a decline tendency in population, the microbial viability started increasing and reached the number at 9.55 log CFU/mL at the end of storage (18 days). In the case of storage at 5°C, very small change of cell number was recorded. At the day 45, the number was 9.5 log CFU/mL. It could be considered that the microbial population in the products after stored at different temperatures still could fulfil the standard of probiotic products about viable cells.

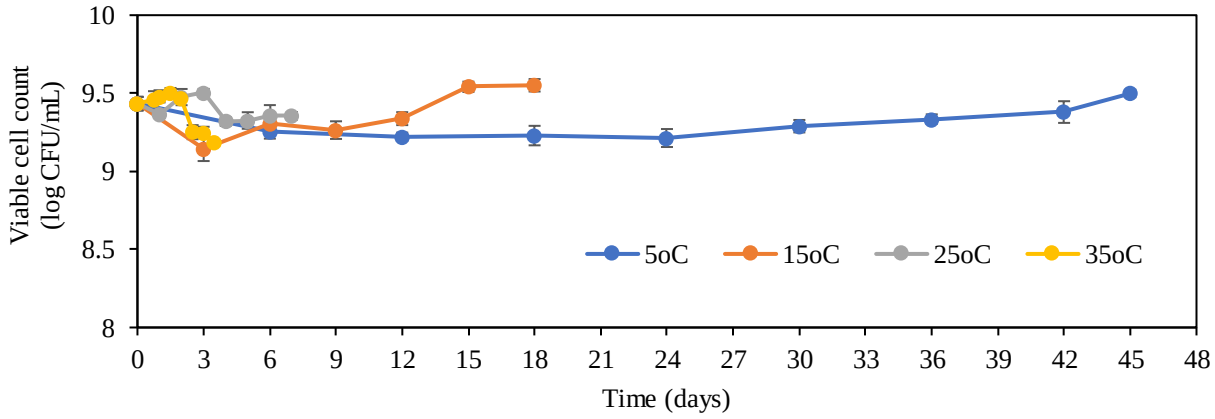


Figure 5.13. Change of microbial population in mixed fruit juice fermented by mixed culture of *L. casei* 01:*L. plantarum* 299v:*B. lactis* Bb12 (1:1:1) during storage at different temperatures

Our results are consistent with the findings of Perez and Saguir (2012) when they stored fermented orange juice with *L. plantarum* N4 at 30°C for 7 days. The growth of bacterium increased continuously till the day 3rd and reached 9.22 log CFU/mL. After this time, the number began to reduce dramatically. Similar results were shown in the study of Dahal *et al.* (2020). They observed a slight increase in *L. acidophilus* population (from 8.59 log CFU/mL to over 9 log CFU/mL) of fermented yacon juice at the beginning of storage at 37°C. At the end of the storage period (15 days), the cell number dropped significantly to 5.95 log CFU/mL. The increase in viable cell counts of bacteria at the initial of storage time could be explained that the nutrients such as sugar, vitamin, amino acid in the product have not depleted completely yet after fermentation. Therefore, bacteria can use them for their growth. Additionally, the high storage temperature (25°C-35°C) which is similar to the fermentation one can support the bacteria growth. Nematollahi *et al.* (2016) observed that there was viability lost in fermented cornelian cherry juice during the early days of cold storage at 4°C. Pereira *et al.* (2011) fermented cashew apple juice with *L. casei* then stored the product at 4°C for 42 days. They reported that there was a slight reduction in the viable cell counts, and the number was higher than 8 log CFU/mL. Costa *et al.* (2013), Dahal *et al.* (2020), Di Cagno *et al.* (2011), Valero-Cases and Frutos (2017b) also indicated the similar finding. In our study, it could be explained that the cells may be stressed by the abrupt temperature shift from a 37°C of fermentation temperature to the cold one (5°C-15°C). After that, they adapted to a cold environment and started to grow again. These tendencies are quite different compared to

the milk substrate. Al-Kadamany *et al.* (2003) conducted storage of yoghurt Labneh at different temperature (5°C, 15°C and 25°C) and found that the bacteria exhibited a lag phase initially when stored the product at 15°C and 25°C. Then a sharp drop was recorded till the end of storage time. In the case of 5°C, after reaching the highest cell number at the day 5 of storage, a gradual decrease trend was observed. Zhi *et al.* (2018) reported that the viable cell count of bacteria in yoghurt increased slightly at the initial stage of storage in four storage temperature conditions (5°C, 15°C, 25°C and 35°C), but soon, it reduced significantly. According to Al-Kadamany *et al.* (2003), Rohm *et al.* (1990) the acid accumulation during storage can cause the inhibitory effects on the growth of the cultures.

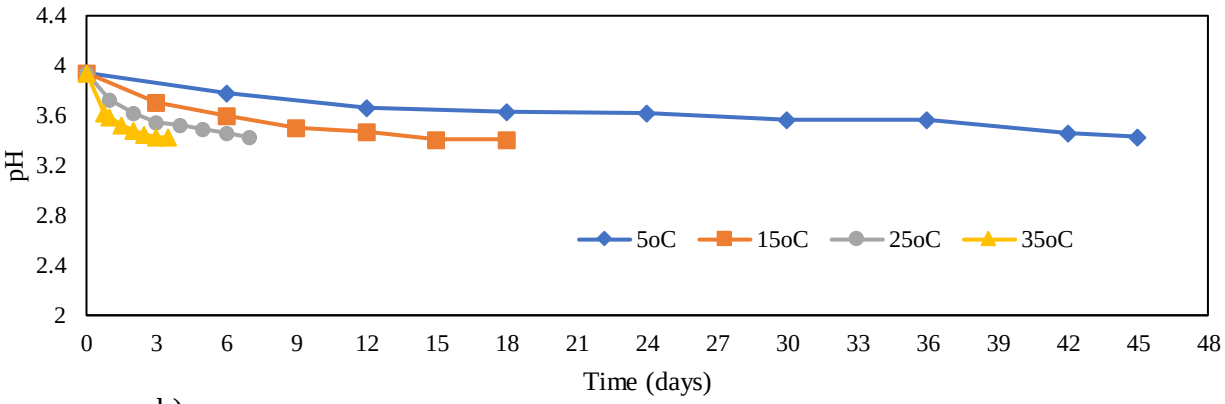
5.4.2. Change of pH, organic acid and sugar

During the storage time at different temperatures (5°C, 15°C, 25°C and 35°C), pH value of the fermented mixed fruit juice with mixed culture showed a decrease sharp (**Figure 5.14 a**) from pH 3.94 at the initial storage time to approx. pH 3.4 at the end. It was to mention that at pH<3.4 value, the product cannot be accepted sensorially. In addition, the different storage temperatures caused the difference of tendency of pH decreases. In the cases of 25°C and 35°C, the pH value declined significantly at the beginning of storage period, and then slowly. In the cases of storage at lower temperatures (5°C and 15°C), the pH values declined gradually.

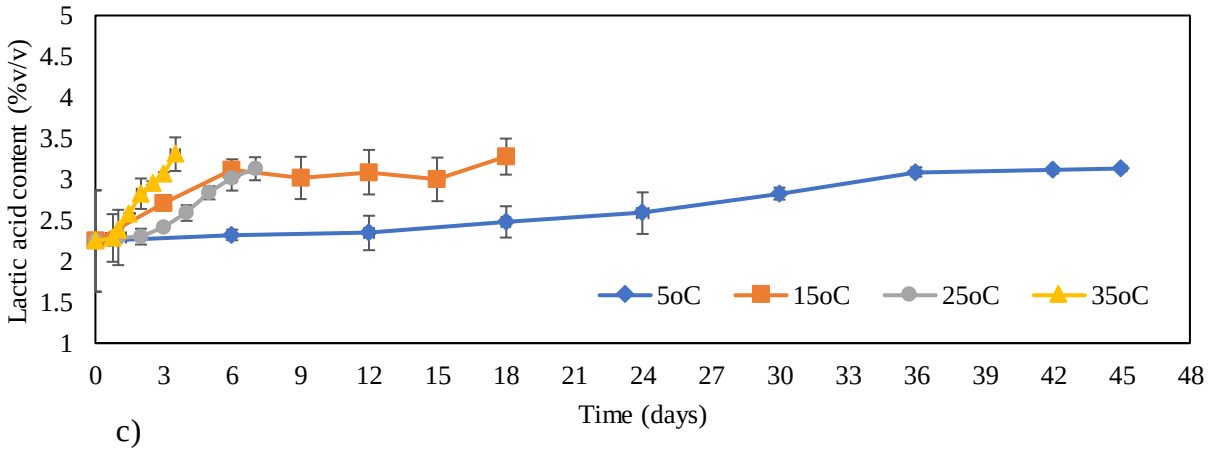
The drop of pH during storage due to the acid accumulation that is demonstrated by increasing trend in lactic acid concentration (**Figure 5.14 b)**. At the higher temperature, the rate of increase in lactic acid level was greater than that at the lower temperature. In particular, while the lactic acid concentration gradually increased by 39.11% after 45 storage days at 5°C, whereas it increased sharply by 47.11% after 3.5 days of storage at 35°C. At 15°C and 25°C, both products showed a similar increase rate in lactic acid level during first 6 days. Afterwards, the lactic acid concentration increased by 39.11% after 7 days of storage in the case of storage at 25°C, and by 44% when they were stored at 15°C for 18 days. The acetic concentration increased slowly at low temperatures (5°C and 15°C) (**Figure 5.14 c**). At higher temperatures (25°C and 35°C), a rapid increase in acetic acid level was recorded. After storage period, the acetic acid concentration of the samples reached a range of 0.07-0.13 (% v/v).

The sugar concentration (glucose and fructose) of all products decreased during storage (**Figure 5.14 d, e**). The sugars of the products stored at 25°C and 35°C had almost the same decreasing trend. Glucose and fructose contents of these products dropped significantly at the beginning, then it was followed by slower rate. At the end of storage, the concentration of glucose and fructose decreased by approx. 46% and 25%, respectively. At 5°C and 15°C, both the products displayed a gradual decrease in glucose and fructose levels at the beginning and remained almost constant at later stage. The concentration of glucose and fructose of both the products decreased in a range of 38.4%-44.4% and 17.8%-23.1%, respectively.

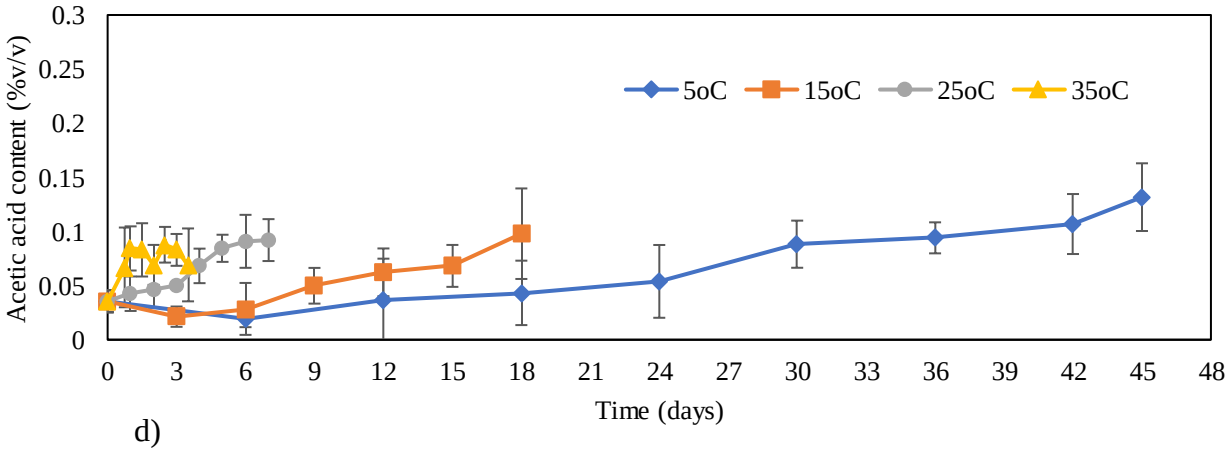
a)


pH
4.4
4
3.6
3.2
2.8
2.4
2
0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48
Time (days)
5oC
15oC
25oC
35oC

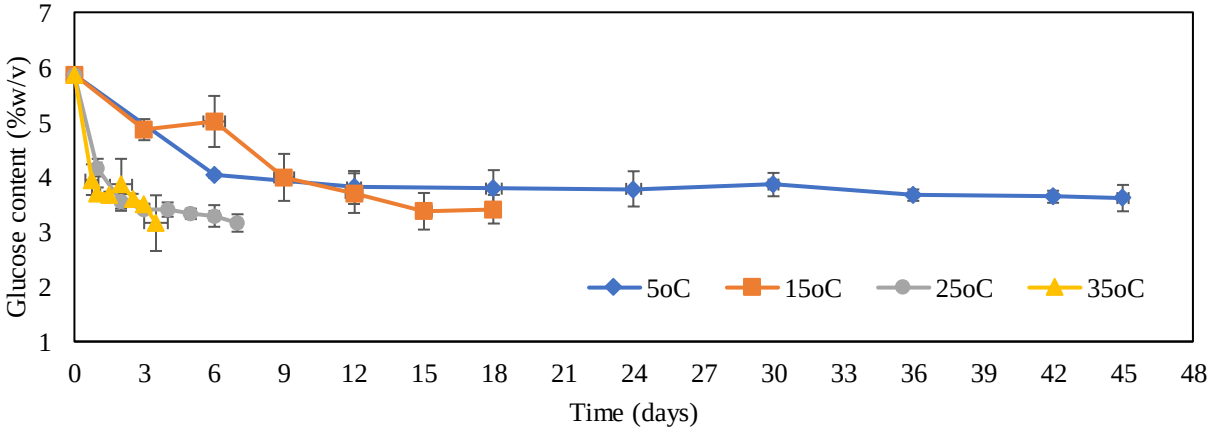
b)


Lactic acid content (%v/v)
5
4.5
4
3.5
3
2.5
2
1.5
1
0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48
Time (days)
5oC
15oC
25oC
35oC

c)


Acetic acid content (%v/v)
0.3
0.25
0.2
0.15
0.1
0.05
0
0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48
Time (days)
5oC
15oC
25oC
35oC

d)


Glucose content (%w/v)
7
6
5
4
3
2
1
0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48
Time (days)
5oC
15oC
25oC
35oC

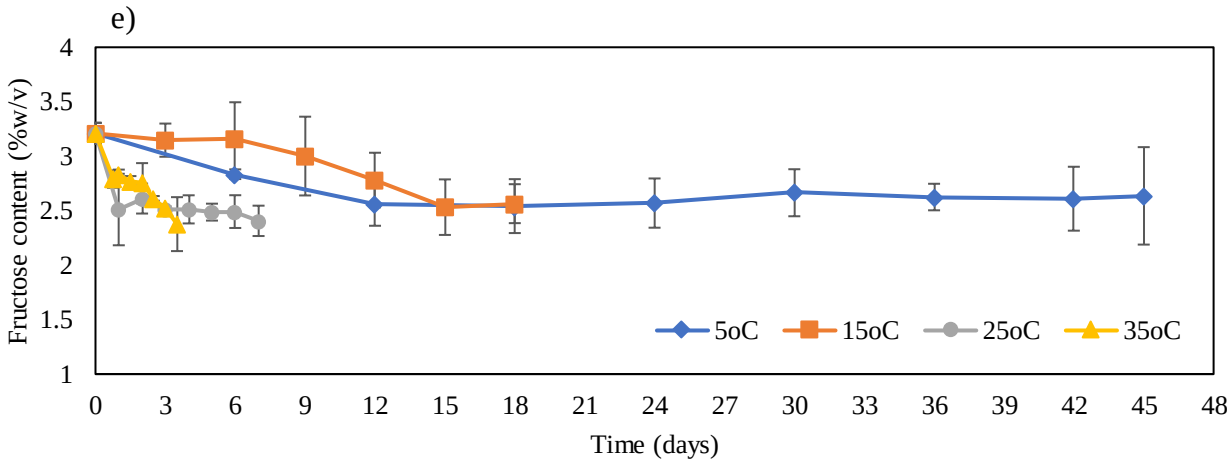


Figure 5.14. Change of pH value a), lactic acid concentration b), acetic acid concentration c), glucose concentration d), and fructose concentration e) of mixed fruit juice fermented by mixed culture of *L. casei* 01:*L. plantarum* 299v:*B. lactis* Bb12 (1:1:1) during storage at different temperatures

These findings indicated that the storage temperature had a significant influence on changes of pH, organic acid and sugar content of product. Higher temperature has been linked to speed up the increase rate in organic acid concentration and reduction rate in sugar concentration. The effects of storage temperature on changes of pH and acidity level in fermented food were observed in the study of Al-Kadamany *et al*. (2003) when they stored Labneh yoghurt at 5^{o}C, 15^{o}C and 25^{o}C for 21, 11 and 5 (days), respectively. The lactic acid increased with rates of change increasing in parallel with storage temperature, while the pH dropped. The concentration of sugars decreased by 46.15% for fructose, 88.89% for lactose and 96.43% for glucose during storage of yoghurt at different temperatures (5^{o}C, 10^{o}C, 15^{o}C, 20^{o}C) (Mataragas *et al*. (2011). These results confirmed the hypothesis that the bacteria continued utilization of sugars for their growth during storage.

5.4.3. Change of TPC and FRAP

Storage condition affected significantly on TPC and FRAP of fermented mixed fruit juices (**Figure 5.15**). Before storage, the concentration of TPC compounds and FRAP capacity of fermented mixed fruit juice were 0.42 µg/mL gallic acid and 3.49 mM $FeSO_4$, respectively. After storage, TPC value decreased by 8.06%-10.4% and FRAP dropped by 11.17%-34.05% varied on storage temperature conditions. Fermented fruit juice stored at 25^{o}C and 35^{o}C had the lowest decrease in these parameters (11.17% and 8.06%, respectively). There was no significant difference in the TPC value of 15^{o}C, 25^{o}C and 35^{o}C. However, the antioxidant activity of the fermented mixed juice stored at 15^{o}C was significantly lower than those at 25^{o}C and 35^{o}C. The highest lost in TPC and FRAP values were recorded in the case of 5^{o}C (10.4% and 34.05%, respectively.

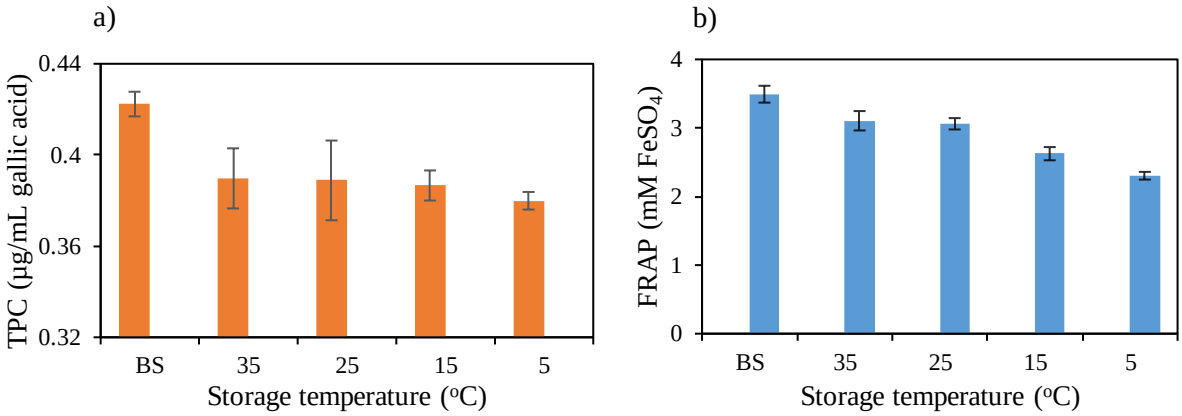


Figure 5.15. Changes of total phenolic content (a) and antioxidant activity (b) of mixed fruit juice with ratio of 50P:25M:25B fermented by mixed culture of *L. casei* 01:*L. plantarum* 299v:*B. lactis* Bb12 (1:1:1) before (BS) and after storage at 5°C for 45 days, 15°C for 18 days, 25°C for 7 days and 35°C for 3.5 days

The decreases of TPC and FRAP during storage were observed in many studies. Nematollahi *et al.* (2016) reported a significantly lower value of the antioxidant activity and phenolic content in fermented cornelian cherry juice after 28 days of cold storage. Di Cagno *et al.* (2011) confirmed this tendency in the fermentation of red and green smoothies with LAB. The decrease of these parameters can be explained that the activity of bacteria during storage may cause the reduction of these parameters (Di Cagno *et al.*, 2011, Nematollahi *et al.*, 2016). For instance, the metabolism of *L. plantarum* may breakdown some phenolic compounds and/or other strictly related chemical compounds such as oleuropein, hydroxycinnamic acid derivates (p-coumaric and ferulic acid), caffeic, gallic and protocatechuic acids (Di Cagno *et al.*, 2011). Furthermore, Nematollahi *et al.* (2016) added that the presence of dissolved oxygen in products causes the oxidation phenolic compounds. Silalahi *et al.* (2018) reported the same statement that the rising storage time reduced the antioxidant activity of yoghurt drinks. However, they found out that the product had higher antioxidant activity when stored at cold storage temperature (4°C-10°C) compared to the storage at room temperature. These authors stored samples for two weeks at both temperatures (refrigerator and room temperature), while we stored our samples at different temperatures during different timeline.

5.4.4. Estimation of shelf-life

Among the environmental conditions having a major effect on the growth kinetics of microorganism, pH would be valuable for the understanding of what happens on the product during its storage (Kwaw *et al.*, 2018). Generally, a decline in a pH value could lead to conclude the activity of bacteria involving the production of organic acids. But, an extremely low pH generally causes bacteria inhibition (Cabello-Olmo *et al.*, 2020). CoSeteng *et al.* (1989) stated that pH levels also strongly influence acceptability of fruit beverages, thus pH measurement is widely used as a rapid, accurate measure of the acidity of fluids of all sorts (Webster, 2003). In my study, the shelf-

life prediction model of fermented fruit juice products was established based on the change of pH values. The pH 3.4 was considered as minimum value to evaluate the sensorial acceptance of product.

The declines of pH at different temperatures were regressed using several models: zero-order, first-order, second-order and third-order. The highest coefficients of determination (R^2) of each temperature cases were selected and used for choosing the suitable order of reaction. The regression equations obtained from the change of pH are listed in **Table 5.6**.

Table 5.6. Regression equations of pH against time under different storage temperatures using different models

Temperature	Models	Regression equations	Regression coefficient
35°C	0 order	pH = -0.1258t + 3.781	R^2 = 0.7597
	1st order	Ln(pH) = -0.0346t + 1.3296	R^2 = 0.7763
	2nd order	$\frac{1}{pH}$ = 0.0095t + 0.2647	R^2 = 0.7927
	3rd order	$\frac{1}{pH^2}$ = 0.0053t + 0.0701	R^2 = 0.8086
25°C	0 order	pH = -0.0631t + 3.8137	R^2 = 0.8355
	1st order	Ln(pH) = -0.0173t + 1.3386	R^2 = 0.8499
	2nd order	$\frac{1}{pH}$ = 0.0047t + 0.2622	R^2 = 0.8639
	3rd order	$\frac{1}{pH^2}$ = 0.0026t + 0.0688	R^2 = 0.8773
15°C	0 order	pH = -0.0277t + 3.8279	R^2 = 0.8633
	1st order	Ln(pH) = -0.0076t + 1.3424	R^2 = 0.8761
	2nd order	$\frac{1}{pH}$ = 0.0021t + 0.2612	R^2 = 0.8882
	3rd order	$\frac{1}{pH^2}$ = 0.0012t + 0.0682	R^2 = 0.8996
5°C	0 order	pH = -0.0093t + 3.8504	R^2 = 0.8954
	1st order	Ln(pH) = -0.0026t + 1.3487	R^2 = 0.904
	2nd order	$\frac{1}{pH}$ = 0.0007t + 0.2594	R^2 = 0.9115
	3rd order	$\frac{1}{pH^2}$ = 0.0004t + 0.0672	R^2 = 0.9178

These regressions showed that in zero- and first-order models, the slope of equations decreased against the increase of storage temperature while in the second- and the third-order, the slope increase with the rising temperature. Based on the coefficient of determination (R^2), the third order model with the highest R^2 value (over 0.8) was as good as describing the pH kinetic, thus it was used to establish the Arrhenius equation. The reaction rate constant of the Arrhenius equation was calculated using linear regression analysis between Ln(k) and 1/T (**Figure 5.16, Eq. 5.1**).

$$\text{Ln(k)} = -7317.2 \times \frac{1}{T} + 17.881 \quad \textbf{(Eq. 5.1)}$$

The equation had a very good determination coefficient (R^2=0.9941) which indicating the reliability and accuracy of the model for predicting the shelf-life of the product in the range of

tested temperatures (5°C-35°C). Based on the **Eq. 4.6** and **Eq. 5.1**, the activation energy (E_a) required for initiating the change of pH was calculated and its value was 15.42 kJ/mol.

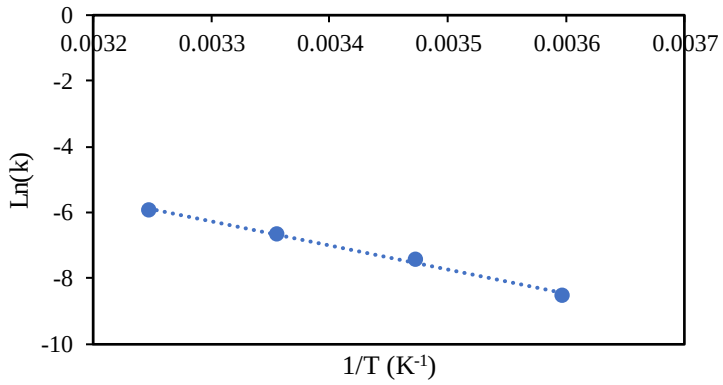


Figure 5.16. Linear regression curve of temperature against pH change of sample based on the Arrhenius model

Based on the order of reaction and Arrhenius equation (**Eq. 5.1**), the shelf-life kinetic model of change in pH value was described as **Eq. 5.2**.

$$\frac{1}{pH^2} = 1.1658 \times 10^8 \times e^{\frac{-7317.2}{T}} \times t + \frac{1}{pH_o^2} \quad \textbf{(Eq. 5.2)}$$

where, pH is the expected pH value at the end of storage period, T is the storage temperature (K), t is the storage time (days) and pH_o is the initial pH value of fermented juice before storage

The root mean square error (RMSE) was calculated to evaluate the fit of the model using sets of data from experimental tests and model prediction of the product's shelf-life stored at 30°C. The initial pH of fermented fruit juice is pH_o = 4.09, and the final pH was pH 3.4. The shelf-life of the sample was predicted as t = 7.05 days (obtained from **Eq. 5.2**). The predicted and experimental data were compared in **Figure 5.17,** and a strong correlation was observed. The RMSE value closes to zero (0.1272), which reflects the ability of the model to predict the data accurately.

Based on the results, the shelf life of the fermented juices was calculated with the initial parameters including pH of juice after fermentation (3.94) and the ending pH (3.4). At 5°C, by using the **Eq. 5.2**, the shelf-life prediction of fermented juice was 51.1 days. Similarly, the shelf lives of the fermented juices stored at 15°C, 25°C and 35°C were estimated to be 20.5 days, 8.7 days and 3.9 days, respectively.

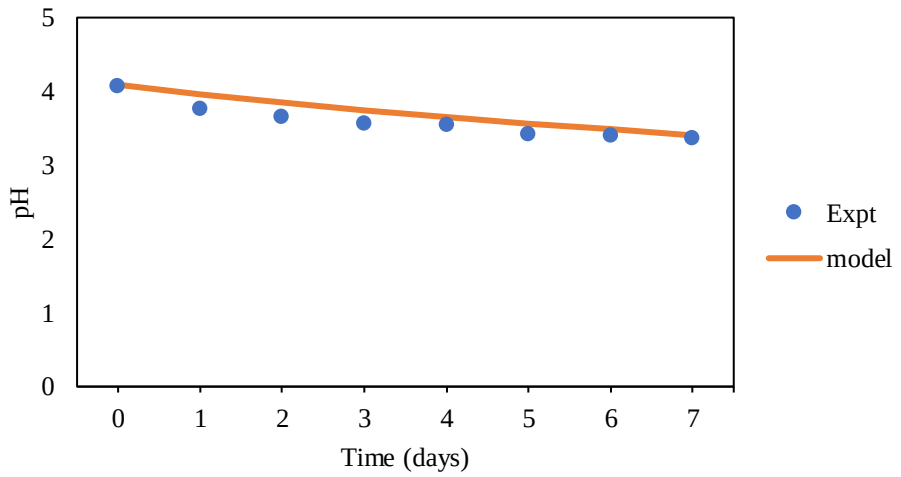


Figure 5.17. Plot of experimental vs. predicted values in a regression model

Bambara groundnut probiotic beverage fermented with *L. bulgaricus* alone and mixture of *L. bulgaricus* and *L. plantarum* had shelf-life estimation for 28 days at 5^{o}C and 2 days at 25^{o}C for both products. At 15^{o}C, the shelf life of the product fermented with monoculture was 18 days and with mixed culture was 10 days (Murevanhema and Jideani, 2020). The estimated shelf-lives of their products were shorter than our products. Murevanhema and Jideani (2020) also calculated the activation energies for pH changes of the fermented Bambara groundnut probiotic beverage. In the range of storage temperature of 5^{o}C-25^{o}C, the E_a value of the sample inoculated with individual strain was 12.8 kJ/mol. An activation energy of 16.9 kJ/mol was estimated for the sample with mixed culture. These results are consistent with our finding that the E_a value for pH changes of juice fermented using mixed culture of *L. casei* 01:*L. plantarum* 299v:*B. lactis* Bb12 was 15.42 kJ/mol. Aini *et al.* (2021) estimated shelf life of corn yoghurt (the ingredients were 50% sweet corn extract, 15% red sweet potato extract, 10% mung bean extract, 15% v/v sugar, and 10% w/v skim milk powder) at different storage temperatures. They reported that the shelf-life estimation of the products stored at 5^{o}C, 10^{o}C, 15^{o}C, and 20^{o}C were 41 days, 40 days, 39 days and 38 days, respectively. The activation energy of 97.1 kJ/mol (significantly higher than our result) was needed for pH change of this product which was stored at a range of temperature of 25^{o}C-35^{o}C (Aini *et al.*, 2021). It can be concluded that the pH of the fermented juice in our study was higher sensitive to temperature than that of corn yoghurt. The difference in activation energy could be explained by the different strains which were used for fermentation (Murevanhema and Jideani, 2020).

6. CONCLUSIONS AND RECOMMENDATIONS

In this study, pineapple, mango and banana juices were demonstrated to be good media for the growth of probiotic bacteria without any nutrient supplementation. Pineapple juice was observed as the most suitable medium compared to other substrates. All investigated *Lactobacillus* strains (*Lactobacillus acidophilus* 150, *Lactobacillus casei* 01 and *Lactobacillus plantarum* 299v), and *Bifidobacterium* strains (*Bifidobacterium lactis* Bb12 and *Bifidobacterium longum* DSM16603) could grow and maintain their viability (over 8 log CFU/mL) in the tropical juices during fermentation and storage. The *L. casei* 01, *L. plantarum* 299v and *B. lactis* Bb12 strains were promising cultures for production of probiotic tropical fruit drinks. During fermentation and storage period, the probiotics utilized sugars (glucose and fructose) of the juices for their growth and produced lactic acid as the main product. Slight decreases in total phenolic content and antioxidant activity of the juices during both fermentation and storage processes were observed. The incubation in simulated gastro-intestinal conditions did not affect significantly the viability of these strains. The combination of the three strains was successfully applied for fermentation of single and mixed juices of pineapple, mango and banana. The products were well accepted by consumers except the fermented pineapple juice. The shelf life of product was estimated with the fermented mixture of pineapple, mango and banana with ratio of 50:25:25 and mixed culture of probiotic *L. casei* 01*:L. plantarum* 299v*:B. lactis* Bb12 (1:1:1). The stability of the product was affected significantly by storage temperature. The shelf life of the fermented probiotic juice drink product is expected to be 51.1 days at 5°C.

This study provided promising results for the development of new non-dairy probiotic food products. Some directions for further research can be proposed as follows:

- Complementary studies on the impact of the fermentation process on the formation of aroma compounds and bioactive compounds produced by probiotic bacteria.
- Experimental results showed that pineapple juice was the most suitable substrate for all the investigated probiotic strains. This is likely due to the presence of some components in the juice which may confer positive effects on the growth of bacteria. Deeper research is recommended to discover such components.
- Scaling up, optimisation as well as comprehensive sensorial evaluation are needed for adaptation of these results in production of commercialised probiotic fruit juices.

7. NOVEL CONTRIBUTIONS

1. Pineapple, mango and banana juices are as good as media for growth of three probiotic *Lactobacillus* strains (*Lactobacillus acidophilus* 150, *Lactobacillus casei* 01 and *Lactobacillus plantarum* 299v), and two probiotic *Bifidobacterium* strains (*Bifidobacterium lactis* Bb12 and *Bifidobacterium longum* DSM16603) without any supplement of nutrients. The *L. casei* 01, *L. plantarum* 299v and *B. lactis* Bb12 strains were promising cultures for production of probiotic tropical fruit drinks.
2. The survival of *Lactobacillus* and *Bifidobacterium* strains in all fermented fruit juices after exposure to pepsin and bile salt solution was over 80%. Storage condition did not affect significantly on the survival of these bacteria except in the case of *B. longum* DSM16603 in mango juice.
3. The fermented beverage products including mango, banana juices and mixed juices of pineapple, mango and banana with ratio of 70P:15M:15B, 50P:25M:25B and 33P:33M:33B were well-accepted by the panelists in the range of like moderately to like very much.
4. The mixed culture demonstrated a good viability after stored at different temperatures (5°C, 15°C, 25°C and 35°C). The rate of acid increase (lactic and acetic) and sugar decrease (glucose and fructose) linked with the increase of the storage temperatures. Slight decrease in total phenolic contents and antioxidant activity values during fermentation and storage were observed.
5. The third order model and Arrhenius equation were adequate for the prediction of shelf lives of the fermented fruit juices based on the changes of pH during storage. The shelf lives of probiotic drinks were estimated to be 51.1 days, 20.5 days, 8.7 days and 3.9 days when stored at 5°C, 15°C, 25°C and 35°C, respectively.

8. SUMMARY

The research “Production of probiotic tropical fruit juices by fermentation with probiotic bacteria” aimed to develop a new non-dairy probiotic food product which is suitable for consumers, especially for such groups who do not tolerate lactose or who are allergic to milk proteins or are vegetarian.

Fermentation of three kinds of tropical fruit juice including pineapple, mango and banana with individual probiotic bacteria strains of *Lactobacillus acidophilus* 150, *Lactobacillus casei* 01, *Lactobacillus plantarum* 299v, *Bifidobacterium lactis* Bb12 and *Bifidobacterium longum* DSM16603 were investigated. Additionally, changes of some properties in the beverage during storage were monitored. After 16 h of fermentation for *Lactobacillus* and 24 h of fermentation for *Bifidobacterium* strains, the pH of fermented fruit juices dropped from the initial pH 6.4 to a range of pH 3.8-3.96 in pineapple juice, pH 4.05-4.5 in mango juice and pH 4.12-4.34 in banana juice. All tested strains exhibited good growth properties on the juices without supplementation of any nutrient compounds. The viable cell counts of most bacteria reached approx. 10 log CFU/mL in pineapple juice and 9 log CFU/mL in mango and banana juice. *L. acidophilus* 150 showed the lowest growth in fruit juices. The population of *L. acidophilus* 150 reached a range of 8.22-8.63 (log CFU/mL) in three kinds of juice. Sugar concentration of juices decreased during fermentation. In the case of *Lactobacillus* strains, glucose was observed as the main carbohydrate source of their metabolism. Meanwhile *Bifidobacterium* utilized both glucose and fructose as the main carbohydrate source. The bacteria consumed more sugar quantity in pineapple juice than in other juices. Lactic acid was registered as a major product of sugar metabolism of both *Lactobacillus* and *Bifidobacterium* during fermentation. After fermentation, the highest concentration of lactic acid was recorded in fermented pineapple juice in both *Lactobacillus* and *Bifidobacterium* strains. The values were determined to be in the range of 1.7-2.01 (% v/v) and 2.7-3.06 (% v/v), respectively. Concentration of acetic acid of the fermented fruit juices was very low. The quantities of this acid were in a range of 0.04-0.21 (% v/v) depending on the strains and juices. Regarding total phenolic content and antioxidant activity, these parameters decreased slightly during fermentation. In the juices inoculated with *Lactobacillus* strains, TPC content decreased approx. 1.4%-14.2%, while in the case of the juices fermented with *Bifidobacterium*, TPC concentration decreased more significantly (8.28%-22.54%). The antioxidant activity of samples with *Lactobacillus* strains dropped approx. 1.6%-13.9%. For *Bifidobacterium* strains, FRAP values of juices reduced by 10.89%-23.82%. The survival of *Lactobacillus* and *Bifidobacterium* strains in all fermented fruit juices after exposure to simulated gastro-intestinal conditions (0.3% pepsin and 0.6% bile salts solution) was over 80%. During 4-week storage at 4^{o}C, the parameters continued changing except cell number. In detail, the pH value of fermented juices slightly decreased in both cases of *Lactobacillus* and *Bifidobacterium* strains. Most bacteria remained stable in population and remained over 8 log CFU/mL. The sugar concentration of fermented juices continued decreasing in both cases of *Lactobacillus* and *Bifidobacterium* strains. For organic acid, a significant increase in lactic acid quantity was observed in the case of *Lactobacillus*. The lactic

acid concentration of fermented juices inoculated with *Lactobacillus* strains after storage was in a range of 2.01-2.85 (% v/v) in pineapple juice, 1.23-1.75 (% v/v) in mango juice and 1.59-1.93 (% v/v) in banana juice. For *Bifidobacterium*, lactic acid concentration of fermented pineapple juice continued increasing during storage and reached 2.97-3.44 (% v/v) at the end while the quantity of this acid remained unchanged in mango and banana juice. A significant decrease of TPC and FRAP of fermented juices was observed after storage. Storage condition did not affect significantly on the survival of these bacteria through the test except *B. longum* DSM16603 in mango juice. A 70.69% and 56.42% of survival of this strain through pepsin and bile salts solution, respectively, were observed. These results indicated that pineapple is the most suitable substrate for the growth of probiotic bacteria. *Lactobacillus casei* 01, *Lactobacillus plantarum* 299v and *Bifidobacterium lactis* Bb12 is suggested to be used for development probiotic tropical juice drink.

In order to improve the quality of products, fermentation of individual fruit juices (pineapple -P, mango -M and banana -B) and mixture of these juices with ratio of 70P:15M:15B, 50P:25M:25B and 33P:33M:33B using mixed culture of *L. casei* 01*: L. plantarum* 299v*: B. lactis* Bb12 (1:1:1) were conducted at 37^{o}C for 16 hours. Then, the fermented samples were stored at 4^{o}C for 4 weeks. After fermentation, the pH values of pineapple, mango and banana decreased significantly from pH 6.4 to pH 4.25, pH 4.33 and pH 4.49, respectively. Meanwhile, lower pH values were observed in mixed fruit juices (approx. pH 4). The microbial viability in most media obtained in a range of 9.18-9.44 (log CFU/mL), except in banana juice with a slighter viable cell count of 8.92 log CFU/mL. Fermented pineapple and mixed juices contained high amount of lactic acid (in a range of 2.03% v/v-2.68% v/v). Fermented mango and banana juice had the lower quantity of lactic acid concentration (0.56% v/v and 0.98% v/v, respectively). Acetic acid was found with a small amount in all fermented juices and mixed juices. The sugar (glucose and fructose) in pineapple juice and mixed fruit juices were utilized more rapidly than in mango and banana juice during fermentation. A slight decrease of TPC and FRAP was observed. TPC dropped by 11%-15.4%. Mixed culture was not significantly affected by artificial gastric fluid and bile salt through the model. The survival of bacteria in both *Lactobacillus* and *Bifidobacterium* strains were over 90%. After storage, these parameters continued their trend. The pH of mixed fruit juices after storage was approx. pH 3.5. Fermented pineapple, mango and banana after storage presented a pH value of 3.51, 3.64 and 3.76, respectively. The population of bacteria in fermented juices still remained over 9 log CFU/mL. The mixed culture continued metabolizing sugars and produced acid. The concentration of lactic acid rose dramatically during 4 weeks of storage. The mixed fruit juice with ratio of 70P:15M:15B and pineapple juice obtained the highest lactic acid concentration (approx. 4.2% v/v). Fermented mango and banana juice had the lowest acid quantity (1.67% v/v-1.82% v/v). The concentration of TPC and FRAP kept reducing slightly. After four weeks of storage at 4^{o}C, the survival of these strains still remained over 90%. The results indicated that the bacteria would not enhance the sensitivity to gastric fluid and bile salt of each other when growing in the media simultaneously.

Sensory analysis was carried out by seven panelists (3 females and 4 males), ranging in age from 25 to 45. The appearance, aroma, taste, texture and overall attributes of the fermented fruit

juice formulations were chosen for acceptance testing using a 9-points hedonic scale (9-like extremely and 1-dislike extremely). The fermented beverage products were well-accepted by the consumer in the range of like moderately to like very much except fermented pineapple juice with the lower preference score. Fermented banana and mango juice receive the highest acceptance score, and there was no significant difference between them and the fermented mixed fruit juice with ratio 50P:25M:25B. Based on the sensory evaluation and the viable cell counts, the mixed juice of ratio of 50P:25M:25B is the most suitable substrate for development of produce probiotic fruit drink using the mixed culture of *Lactobacillus* and *Bifidobacterium.*

With the aim to develop a prediction model to estimate the shelf-life of a probiotic fruit drink product, the mixed fruit juice (pineapple: mango: banana) with ratio of 50:25:25 was fermented by the mixed starter of *L. casei* 01*:L. plantarum* 299v*:B. lactis* Bb12 (1:1:1) at 37^{o}C for 16 hours. Then, the fermented juices were stored at different temperatures including 5^{o}C, 15^{o}C, 25^{o}C and 35^{o}C until their ending pH reached around pH 3.4 which sensorially cannot be accepted. The initial pH value (pH 3.94) dropped to approx. pH 3.4 after 45 days in the case of 5^{o}C, 18 days for 15^{o}C, 7 days for 25^{o}C and 3.5 days for 35^{o}C. The microbial population of juices remained over 9 log CFU/mL after the storage period. In addition, the rate of acid increase (lactic and acetic) and sugar decrease (glucose and fructose) linked with the increase of the storage temperatures. Complex test procedure was conducted to estimate the shelf life of fermented fruit juice products based on the rate laws and Arrhenius equation. Based on the coefficient of determination (R^2), the third order model with the highest R^2 value (> 0.8) was in accordance with the pH kinetic. Arrhenius prediction shelf-life model was obtained with high R^2 value (>0.99). The shelf-life prediction model of fermented juice was calculated: $\frac{1}{pH^2} = 1.1658 \times 10^8 \times e^{\frac{-7317.2}{T}} \times t + \frac{1}{pH_o^2}$, where, pH is the expected pH value at the end of storage period, T is the storage temperature (K), t is the storage time (days) and pH_o is the initial pH value of fermented juice before storage. The fit of model was determined by correlation between the modelled data and empirical data collected from the fermented juice stored at 30^{o}C. The root mean square error (RMSE) were calculated with 0.1272. Finally, based on the results, the shelf life of the fermented juices was calculated with the initial parameters including pH of juice after fermentation (pH 3.94) and the ending pH (pH 3.4). The shelf-lives of fermented juices stored at 5^{o}C 15^{o}C, 25^{o}C and 35^{o}C were predicted to be 51.1 days 20.5 days, 8.7 days and 3.9 days, respectively.

Summarizing, my results are very promising and may serve good bases for development of probiotic tropical drinks.

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LIST OF TABLES

LIST OF FIGURES

PUBLICATIONS

Journal articles

1. **NGUYEN, B. T.,** BUJNA, E., FEKETE, N., TRAN, T. M. A., REZESSY-SZABÓ, J., PRASAD, R., NGUYEN, D. Q. (2019): Probiotic beverage from pineapple juice fermented with *Lactobacillus* and *Bifidobacterium* strains. *Frontiers in Nutrition,* 6(54):7 (IF: 6.576)
2. TRAN, T. M. A., **NGUYEN, B. T.,** NGUYEN, D.V., BUJNA, E., DAM, S. M., NGUYEN, D. Q. (2020) Changes in bitterness, antioxidant activity and total phenolic content of grapefruit juice fermented by *Lactobacillus* and *Bifidobacterium* strains. *Acta Alimentaria*, 49(1):103-110 (IF: 0.5)
3. **NGUYEN, B. T.,** BUJNA, E., WHEIZE, S., SZÉCSI, A., REZESSY-SZABÓ, J., PHAM, M. T., HITKA, G., FRIEDRICH, L., USMANI, Z., SHARMA, M., K. GUPTA, K. V., NGUYEN, D. Q. (2021): Storage stability of probiotic mango juice fermented with Lactobacillus and Bifidobacterium. *Food Chemistry* (**under reviewing process**)
4. PHAM, M. T, VARJÚ, R., BUJNA, E., HOSCHKE, Á., CSERNUS, O., **NGUYEN, B. T.**, GUPTA, K. V., NGUYEN, D. Q. (2021): Chemical and volatile composition of fermented apple juice with different commercial yeast strains of Saccharomyces cerevisiae. *International Journal of Food Microbiology* (**under reviewing process**)

Poster presentations

1. **NGUYEN, B. T.,** BUJNA, E., NGUYEN, D. Q. (2021): Estimation of shelf-life of probiotic fruit juice by using physicochemical change during storage. *The 4th International Conference on Biosystems and Food Engineering.*
2. **NGUYEN, B. T.,** BUJNA, E., NGUYEN, D. Q. (2020): Suitability of some tropical fruit juices for the production of probiotic product fermented by Bifidobacterium lactis Bb12. *Ifjú Tehetségek Találkozója*.
3. **NGUYEN, B. T.,** BUJNA, E., TRAN, T. M. A., NGUYEN, D. Q. (2019): Effect of fermentation of mango juice by some lactic acid bacteria on the antioxidant activity and phenolic compound. *18th International Congress of The Hungarian Society for Microbiology.*
4. **NGUYEN, B. T.,** BUJNA, E., TRAN, T. M. A., NGUYEN, D. Q. (2018): Storage stability of pineapple juice fermented by probiotic bacteria Lactobacillus sp. *3rd International Conference on Food Science and Technology*.
5. **NGUYEN, B. T.,** BUJNA, E., TRAN, T. M. A., NGUYEN, D. Q. (2018): Fermentation of pineapple juice by some probiotic *Lactobacillus sp. Fiatal Biotechnologusok Országos Konferenciája (FIBOK)*.

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