SUMMARY OF PhD THESIS

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DEVELOPMENT OF PLANT-BASED, LACTO FERMENTED DRINKS USING PROBIOTIC *LACTOBACILLUS* STRAINS

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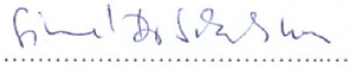

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1. INTRODUCTION

The tradition of lactic acid fermentation dates back thousands of years, and it was used by early civilizations to produce food with the primary goal of preserving raw materials and improving their digestibility. Alongside the fermentation of milk, the fermentation of vegetables using lactic acid quickly became popular, and the resulting foods remain a staple in diets on every continent to this day. However, many of these products are still produced using spontaneous fermentation, even though the use of starter cultures could ensure microbiological safety and consistent quality. The health benefits of foods created through lactic acid fermentation have been known since ancient times and have been scientifically proven since Metchnikoff's era (RASTALL et al., 2000). The *Lactobacillus* (*L.*) genus, which is responsible for lacto-fermentation, plays a particularly important role among lactic acid bacteria. Numerous members of this genus have been shown to have beneficial health effects for the consumer, making them probiotic. Due to an increase in consumer awareness and the growing trend towards a healthier lifestyle, there is now greater demand for products that not only provide nutritional value but also help to preserve consumers' health. Examples of such products include probiotic foods.

Despite the fact that the beneficial effects of probiotics have been demonstrated and published by many researchers (AVONTS et al., 2004, KUSHARYATI et al., 2011, NUALKAEKUL & CHARALAMPOPOULOS, 2011), dairy products still dominate the field of probiotic foods (HELLER, 2001, PERES et al., 2012), even though there is increasing consumer demand for non-dairy probiotic products (DE BELLIS et al., 2010). This not only limits the variety of diets for those who intend to consume probiotic products, but also prevents consumers who cannot consume dairy products for health or lifestyle reasons from enjoying the benefits of probiotic foods. Therefore, it would be important to develop a product that combines the advantages of plant-based fermented foods and probiotic microorganisms.

Vegetables and fruits can be ideal substrates for a fermented product, as they play an important role in human nutrition and already contain numerous beneficial components (minerals, vitamins, dietary fibers) while being free from allergenic compounds found in milk. Furthermore, consumers associate them closely with healthy eating (PRADO et al., 2008, RIVERA-ESPINOZA & GALLARDO-NAVARRO, 2010, PEREIRA et al., 2011). Many studies report on the use of starter cultures (HALÁSZ et al., 1999, LEROY & DE VUYST, 2004), however, few have ventured to use proven probiotic strains for fermentation or to investigate the probiotic properties of strains with good fermentation capabilities (SÁNCHEZ et al., 2012). Currently, there is no non-dairy, probiotic food product on the market that can be

incorporated into daily meals, making the development of a vegetable and/or fruit-based product with probiotic bacteria a necessary addition and an opportunity to establish a new segment in the probiotic product market. Based on this, I propose that it would be worthwhile to conduct a selection of probiotic strains on various plant-based raw materials for their suitability as starter cultures.

Aim of the research

During my research, I aimed to examine and select probiotic (and non-probiotic) strains of *Lactobacillus* that could be used to produce a novel, plant-based product that is lactic acid fermented and provides high added value through its health benefits. To achieve this:

- My goal was to investigate the fermentability and functional product development of a popular, widely consumed fruit juice with probiotic strain. Since orange juice is the most widely known and consumed fruit juice in the world, I believe it is worth starting my product development research with this juice.
- My objectives also include examining the fermentability and functional product development of juices based on several fruits and vegetables, mainly domestically grown, to select the most suitable raw materials and create a wide range of products.
- To select the appropriate strains and to determine their fermentation applicability, I will investigate the properties of *Lactobacillus* strains (proliferation) and their effects on the product (pH, titratable acidity, total soluble solids content, total polyphenol content, antioxidant and radical scavenging capacity). This is because lactic acid bacteria that proliferate in the raw materials can increase the presence of bioactive compounds through their metabolites.
- In developing a product that contains probiotic bacteria, my primary objective is to develop a method that allows for the creation and preservation of as much live flora as possible during storage, as their health effects are mostly exerted by live, active cells and only at certain concentrations. Storage tests are conducted to determine the viability of probiotic cultures and changes in the beneficial components of the product.
- In addition to using strains obtained from strain collections and starter culture distributors to create my own strain collection, I will isolate *Lactobacillus* strains from fermented dairy products to test their applicability as starter cultures and to create fermented products with better technological and functional properties.

2. MATERIALS AND METHODS

2.1. Applied plant raw materials

When selecting the applied plant raw materials (Table 1), I considered two main factors. Firstly, I aimed to choose a popular and widely consumed fruit juice, and accordingly, I selected orange juice. Secondly, I conducted fermentation experiments using domestically grown vegetables and fruits, which was made possible through collaboration with the National Agricultural Research and Innovation Center (NARIC) – Fruitculture Research Institute (FRI) and Vegetable Crop Research Department (VCRD). The selection of multiple raw materials was done to draw general conclusions since different nutritional values can have varying effects on fermentation.

The juice extraction from the washed vegetables and fruits was performed using a hand blender, followed by filtration through two different metal filters with varying pore sizes (1.0 and 0.8 mm). In order to achieve the desired consistency, some fruits required water supplementation (700 g of plums + 300 ml of water, 800 g of black chokeberries + 200 ml of water, 500 g of quinces + 500 ml of water). Commercially available orange juices did not require further treatment. However, for freshly squeezed orange juice, I used a manual juicer and examined it without filtration. Due to the natural microflora present in fresh fruits and vegetables, heat treatment became necessary in most cases. The pasteurization time and temperature in a water bath were optimized for each specific plant raw material in several instances.

Table 1: Applied plant raw materials

Vegetables, fruit	**Variety or type**	**Origin**
Orange	Rauch Happy Day 100% orange juice with Vitamin C	InterSpar
	Vitafit 100% fresh orange juice	Lidl
	Greek Navelina orange	Local market
Sour cherry	Petri	NARIC – FRI (Újfehértó, Érd)
	Újfehértói Fürtös	
	Érdi Bőtermő	
Plum	Ageni	NARIC – FRI (Cegléd)
	Stanley	
Black chokeberry	Nero	NARIC – FRI (Fertőd)
	Viking	
Quince	Mezőkövesdi	NARIC – FRI (Újfehértó)
	Angersi	
	Csokonai	
Tomato	Cherrola	NARIC – VCRD
	Uno Rosso	
	Mobil	
	+ another 16 varieties	

2.2. Applied *Lactobacillus* strains

During the experiments, I examined the properties of probiotic (*L. rhamnosus* GG, *L. casei* Shirota, *L. reuteri* DSM 17938, *L. acidophilus* 150, *L. acidophilus* LA-5, *L. casei* 01) and non-probiotic *Lactobacillus* strains (*L. acidophilus* N2, *L. plantarum* 2142, *L. fermentum* DT41, *L. fermentum* D13) using microbiological methods available in our strain collection.

2.3. Examination methods

During the fermentation experiments, the *Lactobacillus* strains were previously grown and propagated in MRS broth. After two subcultures, the overnight cultures were centrifuged at 2700 g for 10 minutes, and the supernatant was discarded. The cell pellet was then resuspended in peptone physiological saline solution (0.85% w/v NaCl, 0.1% w/v peptone). The vegetable and fruit juices were inoculated with a 1% initial inoculum (1 v/v%, initial cell count in the juice was 7 log colony-forming units mL^{-1} (CFU mL^{-1})), and incubated at 30 °C for 24 hours. If storage experiments were conducted, samples placed at room temperature (24 °C) and in the refrigerator (6 °C) were also monitored for changes. During the investigations, I worked with three replicates, and the average values were calculated from the obtained results.

For assessing the cell count changes of *Lactobacillus*, the Miles and Misra method was employed, while enumeration of total viable counts was performed using spread plate technique on selective media suitable for the specific microorganism. pH measurements were carried out using a digital pH meter (Mettler-Toledo GmbH, Switzerland), and the determination of total soluble solids was conducted using a digital refractometer (Schmidt+Haensch Gmbh & Co., Germany). The titratable acidity was determined by titration with 0.1 M sodium hydroxide solution using phenolphthalein as an indicator. As lactic acid bacteria can increase the presence of bioactive compounds through their metabolic by-products, the determination of total polyphenol content was performed using the Folin-Ciocalteu reagent, and antioxidant capacity was measured using the FRAP method and DPPH radical scavenging assay.

To achieve the desired 9 log CFU mL^{-1} cell count in the fruit samples, I employed Taguchi, Plackett-Burman, and central composite design experimental methods to determine the optimal nutrient supplementation and its concentration, as well as to adjust the fermentation parameters. Statistical analysis of the measured results during lactic acid fermentation and storage experiments was performed using IBM SPSS 24 software, applying the Tukey post hoc test at a significance level of 5% ($p < 0.05$). This analysis allowed for a thorough evaluation of the obtained data and the identification of significant differences between the groups.

In addition to examining the *Lactobacillus* strains from the strain collection, I isolated lactic acid bacteria from lactic acid-fermented foods. The protocol I developed was based on MRS agar supplemented with cycloheximide (0.01% v/v). On this medium, I performed catalase testing and Gram staining on the bacterial colonies, and then observed the acid production of the presumed lactic acid bacteria on MRS agar containing bromocresol purple indicator (0.01% v/v). The identification of these preselected bacteria was carried out using *Lactobacillus*-specific PCR analysis. DNA isolation was performed using the Wizard® DNA Clean-up System (Promega, Madison, Wisconsin, USA) method, and the concentration and purity of the DNA solution were determined using the Colibri instrument (Titertek-Berthold, Berthold Detection Systems GmbH, Pforzheim, Germany). PCR reactions were conducted using the Biometra TOne (Analytik Jena AG, Jena, Germany) gradient PCR instrument. The selection of isolated lactic acid bacteria, as well as those present in our strain collection, was also based on cell viability (MTT colorimetric method) and γ-aminobutyric acid production (thin-layer chromatography method).

3. RESULTS

3.1. Orange juice

Natural forms of different types of orange juice (concentrated, non-concentrated, freshly squeezed) did not provide a suitable environment for the growth of *Lactobacillus*. This may be due to the low pH, inadequate nutrient composition for *Lactobacillus* growth, and the effects of antimicrobial components present in orange juice. However, by adding yeast extract and dextrose and adjusting the fermentation parameters, it was possible to create ideal conditions for the growth and survival of *Lactobacillus* in orange juice. To achieve the maximum cell count, the quantitative optimization of supplementary nutrients was performed using statistical methods. At a pH of 7.00, the optimal amount of dextrose was determined to be 60 g L^{-1}, considering 2 g L^{-1} of yeast extract as a nutrient supplement. In addition to optimizing the raw material, it is important to select and apply the appropriate probiotic starter culture for the fermentation of orange juice to ensure the preservation of probiotic properties over an extended period. Since the experiments indicate that the type of orange juice also influences fermentation, strain growth, and viability, it is crucial to perform starter culture selection specific to the given raw material.

Orange juice made from concentrate

In the case of orange juice made from concentrate, significant differences were observed in terms of growth and viability between different storage temperatures and selected strains. The highest cell count (9.692 log CFU mL^{-1}) during fermentation was achieved when using the *L. casei* 01 starter culture in the optimized orange juice supplemented with the appropriate nutrient quantities and adjusted pH. This viability was maintained even after 6 weeks of refrigerated storage. In the unsupplemented orange juice, the selected lactic acid bacteria achieved a comparable cell count (9.082 log CFU mL^{-1}), but a decrease of 1-2 orders of magnitude was observed by the third week. Although the desired 9 log CFU mL^{-1} cell count was achieved with most strains in the optimized orange juice, significant differences were observed among certain strains during the statistical analysis. While there was a significant difference between the two strains (*L. rhamnosus* GG and *L. casei* 01) with the highest cell counts, the results after 6 weeks of refrigerated storage (9.522 and 9.591 log CFU mL^{-1}) did not show a significant difference. Thus, in terms of strain selection combined with storage, these two strains are the most suitable for developing a product containing probiotic lactic acid bacteria. The pH of the optimized fermented juices ranged from 3.92 to 6.08, which can be considered a significant difference, and this is supported by statistical analyses as well. The highest and most extreme pH value (6.08) was obtained when using the *L. acidophilus* LA-5 starter culture, which also showed a

significant difference in total soluble solids content compared to the other strains (except *L. reuteri* DSM 17938). The titratable acidity ranged relatively widely when expressed as lactic acid (0.248 – 1.058 g 100 mL^{-1}), indicating a general significant difference among the strains.

Orange juice made from non-concentrate

In the case of orange juice made from non-concentrate, the strain *L. reuteri* DSM 17938 achieved the highest cell count (9.460 log CFU mL^{-1}) during fermentation, showing a significant difference compared to all other strains. Furthermore, only a half-order of magnitude decrease in cell count was observed by the 6th week of storage. Despite the numerical differences observed in cell growth, all optimized fermented samples, except for *L. acidophilus* 150, maintained a cell count above 8 log CFU mL^{-1} after 6 weeks of storage. There were no significant differences in pH among the strains, except for *L. acidophilus* 150, which exhibited an unusually high pH value. Regarding total soluble solids content, there were no significant differences among the fermented samples, while titratable acidity showed more statistical variability.

Freshly squeezed orange juice

In the case of freshly squeezed orange juice, the application of the *L. rhamnosus* GG strain resulted in a two-order-of-magnitude increase in cell count compared to the initial count during the lactic acid fermentation in the optimized orange juice. However, after 6 weeks (even in samples stored in the refrigerator), the cell count dropped below 7 log CFU mL^{-1}. Although the *L. acidophilus* LA-5 starter culture did not reach the maximum cell count within 24 hours, room temperature storage favored the growth of the lactic acid bacteria, resulting in the optimized freshly squeezed orange juice containing 8 log CFU mL^{-1} of viable cells even after 6 weeks of storage. *L. acidophilus* 150 showed only a one-order-of-magnitude increase (reaching 8 log CFU mL^{-1}) during fermentation, but its probiotic cell count was maintained during the 6-week period of storage in both room temperature and refrigerator conditions. For freshly squeezed orange juice, both *L. casei* Shirota and *L. casei* 01 proved to be excellent choices as starter cultures. During fermentation with different strains, significant differences in pH were observed, with variations of more than 1 unit (ranging from 3.86 to 5.13). Therefore, there is a significant difference in pH among all strains. There were no significant differences in total soluble solids content (ranging from 14.9% to 16.0%) among the strains, while titratable acidity showed up to threefold differences (ranging from 0.468 to 1.486 w/v%).

3.2. Sour cherry

From the preliminary experiments, it can be concluded that cherries of different varieties (Petri, Újfehértói fürtös, Érdi bőtermő) in their natural form did not meet the requirements for *Lactobacillus* growth. This could be attributed to the acidic pH, inadequate nutrient composition for *Lactobacillus* proliferation, and the negative effects of phenolic compounds present in sour cherry juice. Additionally, the high initial microbial count in cherries posed a problem, necessitating pasteurization of the juices after processing. The time and temperature of pasteurization were optimized using a central composite design, and treating the sour cherry juice at 60 °C for 15 minutes proved to be the most effective, reducing the microbial count present in the raw material. To achieve the desired 9 log CFU mL^{-1} *Lactobacillus* cell count, the fermentation parameters were also optimized using statistical methods. As a result, at a pH of 5.80, the optimal amount of yeast extract supplementation was determined to be 3 g L^{-1} when diluting the sour cherry juice with water in a 6 : 4 (v/v) ratio.

Újfehértói fürtös

Despite achieving the desired 9 log CFU mL^{-1} cell count with all the strains used during strain selection, there were statistically significant differences observed in the cell counts of certain *Lactobacillus* strains. Among the Újfehértói fürtös sour cherry variety, *L. acidophilus* LA-5 exhibited the highest cell count (9.425 log CFU mL^{-1}), which showed significant differences only compared to the *L. plantarum* 2142 and *L. acidophilus* N2 strains. The pH ranged from 4.21 to 4.56 after 24 hours of fermentation, but this minimal difference is not considered significant for fermentation and product development purposes. Although the overall difference in total soluble solids content was only 0.5%, significant variations were observed among certain strains. Typically, the *L. casei* 01 and *L. acidophilus* N2 strains showed significant differences from the other strains, resulting in the smallest decrease in total soluble solids content when applied as starter cultures. The titratable acidity showed significant increases, with more than a twofold difference observed among the strains (0.4444 – 0.9548 w/v%). At the end of fermentation, substantial differences were measured in polyphenol content among certain strains, which were also confirmed by statistical analysis, although it is not a predominant characteristic. However, it is worth noting that while *L. casei* 01 and *L. acidophilus* N2 showed a decrease in polyphenols, *L. acidophilus* 150 resulted in nearly a 30% increase compared to the initial sample. Although the use of certain *Lactobacillus* strains led to a decrease in antioxidant capacity, *L. acidophilus* 150, *L. acidophilus* LA-5, *L. casei* Shirota, and *L. fermentum* DT41 strains showed an increase compared to the initial sour cherry juice, while fermentation with *L. rhamnosus* GG strain preserved the initial antioxidant activity of

Újfehértói fürtös cherries. However, there were no statistically significant differences in this regard among the strains. The free radical scavenging capacity showed an increase of up to 40% after 24 hours of fermentation with all the applied *Lactobacillus* strains compared to the 0-hour sample.

Petri

In the Petri sour cherry variety, the desired 9 log CFU mL^{-1} cell count was also achieved with all the *Lactobacillus* strains used as starter cultures. However, the significance of strain selection for this variety is demonstrated by the fact that *L. acidophilus* 150 resulted in the highest cell count (9.598 log CFU mL^{-1}), which showed significant differences compared to the other strains, except for *L. acidophilus* LA-5. There were no significant differences in pH for this variety (4.22 – 4.43). The minimal differences observed in total soluble solids content are not characteristic of being statistically significant, except for *L. rhamnosus* GG, which showed such differences compared to certain strains, resulting in the lowest total soluble solids content. The titratable acidity ranged from 0.4384 to 0.7567 w/v%, which not only showed significant differences statistically, for example, between *L. rhamnosus* GG and *L. acidophilus* 150. In general, an increase in total polyphenol content, as well as in antioxidant and radical scavenging capacity, was observed in the Petri variety. However, *L. reuteri* DSM 17938 and *L. plantarum* 2142 resulted in a slight decrease of less than 10% in total polyphenol content, showing significant differences from the other strains. The antioxidant capacity also generally showed an increase, with minimal differences among the strains that were not considered statistically significant.

Érdi bőtermő

After strain selection, I conducted an 8-week storage experiment in a refrigerator (6°C). For the lactic acid fermentation of the Érdi bőtermő sour cherry variety, I used the *L. acidophilus* LA-5 strain, applying the fermentation environmental parameters determined during optimization. During lactic acid fermentation, I was able to achieve a cell count of nearly 9 log CFU mL^{-1}. Subsequently, I examined the properties of the strain and its effects on the raw material in three types of packaging: glass and plastic bottles, as well as Tetra Pak carton packages. These samples were stored in the refrigerator for 8 weeks. Even after the 8-week period, the cell count did not drastically decrease in any of the storage types. Although there were no significant differences in the cell count between the storage types on a weekly basis, it can be generally observed that the glass bottle consistently had the highest cell count. Regarding pH and titratable acidity, there were no significant differences among the storage types, while the results of total soluble solids content in the fourth week showed significant differences. Up until

the 6th week, there were no significant differences in total polyphenol content among the storage types, but the samples stored in Tetra Pak cartons contained less polyphenols compared to glass and plastic bottles. Regarding the increase in antioxidant capacity due to fermentation, the samples stored in Tetra Pak packaging showed a greater reduction by the 8th week compared to those in glass or plastic bottles, although no significant differences were observed in the measured values. The fermented sour cherry juice preserved its radical scavenging capacity best when stored in glass bottles.

3.3. Plum

Based on the preliminary experiments, it can be concluded that the different varieties of plums (Ageni, Stanley) in their natural form do not provide a suitable environment for *Lactobacillus* growth. However, to reduce the existing natural microflora, it is necessary to pasteurize the plum juices (60 °C, 15 minutes). In order to maximize the *Lactobacillus* cell count, the optimal values of supplementary nutrients and parameter settings were determined using a central composite design. Thus, at a pH of 6.50, the ideal quantity of yeast extract is 6 g L^{-1}, when the plum juice is supplemented with water in a ratio of 5.5 : 4.5 (v/v).

Ageni

For the Ageni plum variety, *L. plantarum* 2142 and *L. fermentum* DT41 achieved a cell count of 9 log CFU mL^{-1} (9.215 and 9.174 log CFU mL^{-1}), which are statistically similar but significantly different from the other strains. Since these two strains had relatively higher initial cell counts, to ensure proper comparability, growth rates were calculated. According to the growth rate (based on logarithmic cell count), *L. acidophilus* 150, *L. casei* Shirota, and *L. acidophilus* N2 exhibited at least a 20% increase. The pH ranged from 3.68 to 3.99 after 24 hours of fermentation, and the total soluble solids content decreased from the initial 10.8% to 9.9 – 10.3%. Certain strains showed variations in titratable acidity compared to others: while *L. casei* 01 exhibited lower acidity, *L. rhamnosus* GG resulted in higher acid production in Ageni plum juice. There were no significant differences among the applied *Lactobacillus* strains in terms of bioactive compounds such as total polyphenol content, antioxidant capacity, and radical scavenging capacity. However, only three strains (*L. casei* Shirota, *L. plantarum* 2142, and *L. fermentum* DT41) showed an increase in total polyphenol content during fermentation. Except for *L. casei* 01 and *L. acidophilus* N2, the antioxidant capacity and radical scavenging capacity showed an increase compared to the 0-hour sample.

Stanley

For the Stanley plum variety, three strains, *L. plantarum* 2142, *L. acidophilus* N2, and *L. fermentum* DT41, achieved a cell count of 9 log CFU mL^{-1} (9.091, 9.100, and 9.075 log CFU mL^{-1}) in the plum juice with the set parameters. In terms of growth rate, *L. reuteri* DSM 17938 exhibited the highest level of growth on the raw material, approaching a cell count of 9 log CFU mL^{-1} (8.908 CFU mL^{-1}). The initial pH of 6.52 decreased to a range of 3.68 to 4.10, while the total soluble solids content ranged from 6.3% to 6.9%, but these differences were not significant among the strains. However, there were significant differences in titratable acidity, particularly with *L. acidophilus* 150 showing significantly lower acid production in Stanley plum compared to *L. reuteri* DSM 17938, *L. casei* 01, *L. acidophilus* N2, and *L. rhamnosus* GG. During the measurement of total polyphenol content, a decrease was observed for all strains, with the most significant decrease observed in the *L. rhamnosus* GG strain. There were no significant differences in antioxidant capacity among the applied *Lactobacillus* strains, but in terms of DPPH assay-based radical scavenging capacity, different strains could result in larger differences, even more than twofold (for example, *L. rhamnosus* GG exhibited significantly lower radical scavenging capacity in plum juice compared to all strains except *L. acidophilus* 150).

3.4. Black chokeberry

The native form of Nero and Viking black chokeberries is not ideal for the growth of *Lactobacillus*. However, by adding peptone and adjusting the fermentation parameters, the necessary conditions for *Lactobacillus* growth were successfully established in pasteurized (60°C, 15 minutes) black chokeberry juice. The optimization of fermentation parameters and supplementary nutrients was carried out using statistical methods, resulting in the requirement of 5.62 g L^{-1} of peptone at a pH of 4.50. When diluting the black chokeberry juice with water, a ratio of 8 : 2 (v/v) is recommended.

Nero

In the case of Nero black chokeberries, certain strains showed an increase of approximately one order of magnitude from the initial 7 log CFU mL^{-1} cell count (e.g., *L. reuteri* DSM 17938 and *L. acidophilus* N2), while other strains (*L. rhamnosus* GG, *L. acidophilus* LA-5, *L. casei* Shirota, *L. casei* 01) exhibited a decrease. Significant differences were observed primarily between strains that reached cell counts above 8 log CFU mL^{-1} and those below 7 log CFU mL^{-1}. The strain with the highest cell count, *L. reuteri* DSM 17938 (8.587 log CFU mL^{-1}), showed a significant difference compared to all strains except *L. acidophilus* N2 and *L. fermentum*

DT41. The growth rate was also highest for *L. reuteri* DSM 17938 (15.22%). Adjusting the initial pH to 4.50 resulted in only a slight decrease during growth (4.27 – 4.49). The total soluble solids content minimally decreased from 15.9% to a range of 15.2 – 15.7%, and the titratable acidity increased in certain strains (e.g., *L. casei* Shirota) while decreasing in others (e.g., *L. acidophilus* N2). The total polyphenol content increased with all applied *Lactobacillus* strains, and statistically significant differences were observed only between the extreme values produced by *L. reuteri* DSM 17938 and *L. casei* 01. The antioxidant capacity showed an increase in all cases compared to the initial value, except for *L. casei* 150. Although the measurement of free radical scavenging capacity resulted in both decreases and increases depending on the strain, no significant differences were observed among the *Lactobacillus* strains.

Viking

In the case of Viking black chokeberry juices, significant differences were observed in the growth of the strains. Only the cell count of *L. rhamnosus* GG decreased, while the other strains showed an increase of approximately one order of magnitude. As a result, *L. rhamnosus* GG significantly differed from all strains except *L. acidophilus* LA-5, but significant differences in cell count were detected among most applied strains at a significance level of $p = 0.05$. The highest cell count in Viking black chokeberry juices was achieved by *L. fermentum* DT41 (8.876 log CFU/ml, 20.93% growth rate), while the highest growth rate was observed with the probiotic strain *L. reuteri* DSM 17938 (8.812 log CFU mL^{-1}, 21.32%). However, the cell counts of these two strains did not show a significant difference. The pH difference of up to 0.5 decimal places, measured after fermentation and ranging from 4.10 to 4.62, can be considered more significant, with *L. fermentum* DT41 resulting in significantly lower pH compared to all other strains. In the case of Viking black chokeberries, certain strains showed significant differences in total soluble solids content and titratable acidity, but these differences were negligible from a fermentation perspective. The total polyphenol content decreased with all strains compared to the initial value, except for *L. acidophilus* LA-5, which showed a non-significant increase. The results of antioxidant and free radical scavenging capacity varied, with some strains showing an increase (e.g., *L. rhamnosus* GG) and others showing a decrease (e.g., *L. casei* Shirota) during fermentation, but significant differences were not prominent.

3.5. Quince

Similar to other fruits, such as Angersi, Csokonai, and Mezőkövesdi varieties, raw quince did not provide a suitable environment for the growth of lactic acid bacteria. However, by adjusting the fermentation parameters, it was possible to create the ideal conditions for *Lactobacillus*

growth in quince juice as well. To achieve the desired cell count, I optimized these parameters, determining that at a pH of 6.00, the optimal amount of peptone is 5 g L^{-1} when quince juice is supplemented with water in an 8 : 2 (v/v) ratio. To reduce or eliminate the microbial load present in quince, I applied pasteurization using parameters optimized for tomato juice (65°C for 60 minutes).

Angersi

In the case of Angersi quince juice, the highest cell count (8.865 log CFU mL^{-1}) and growth rate (19.79%) during fermentation were achieved with the *L. reuteri* DSM 17938 strain, which showed a significant difference compared to all other strains except for *L. acidophilus* N2 (8.776 log CFU mL^{-1}). However, for some strains, even with the determination and adjustment of optimal conditions, quince juice was not an ideal substrate for their growth, as evidenced by a decrease in cell count of *L. rhamnosus* GG, *L. acidophilus* LA-5, and *L. casei* 01 over 24 hours. Significant differences in pH were observed among the strains. For those strains where a decrease in cell count was observed, the pH decreased by only a few tenths of a point (5.68 – 5.78), while for other *Lactobacillus* strains, the pH decreased to as low as 4.07 (*L. acidophilus* N2). The results of total soluble solids were effectively divided into two groups: there was little change in *L. rhamnosus* GG, *L. acidophilus* LA-5, and *L. casei* 01, which remained at 6.1% from the initial 6.3%, while for other strains, it decreased to 5.5 – 5.8%. Significant differences were also observed in the titratable acidity.

Csokonai

A 24-hour fermentation period resulted in the highest cell count (8.824 log CFU mL^{-1}) in Csokonai quince juice with the *L. plantarum* 2142 strain, while the highest growth rate (16.55%) was achieved with *L. reuteri* DSM 17938. However, the cell count of *L. plantarum* 2142 was not significantly different from that of *L. acidophilus* 150, *L. casei* Shirota, *L. acidophilus* N2, and *L. fermentum* DT41, as all of these strains approached the desired concentration of 9 log CFU mL^{-1}. On the other hand, *L. casei* 01 showed a significant decrease in cell count, dropping by two orders of magnitude in Csokonai quince juice (5.824 log CFU mL^{-1}). The cell count of *L. rhamnosus* GG and *L. acidophilus* LA-5 was also lower compared to the initial sample. The initial pH set for fermentation ranged from 4.03 to 5.72 after the 24-hour fermentation period, which represents a wide pH range. However, the strain with the highest decrease in total soluble solids did not correspond to the strain with the lowest pH. In fact, when using the *L. fermentum* DT41 strain, the total soluble solids decreased from 6.5% to 5.9%. Significant differences were also observed in the titratable acidity, as non-growing strains

showed minimal lactic acid production compared to the initial amount, distinguishing them statistically from the other strains.

3.6. Tomato

Cherrola, Mobil, Uno Rosso, and the other 16 varieties of tomatoes were generally found to be suitable raw materials for producing lactic acid-fermented products with probiotic *Lactobacillus* starter cultures. Apart from reducing the natural microflora through heat treatment (60 minutes at 65°C), no additional treatment or supplementation was required for the proliferation of lactic acid bacteria. There were significant differences in cell counts among the strains, but all *Lactobacillus* strains reached the ideal concentration of 9 log CFU mL^{-1}. Despite the varying behavior of different strains in different tomato varieties, *L. acidophilus* N2 achieved the highest cell count in both Mobil, Uno Rosso, and Cherrola tomato juices, with probiotic *L. casei* Shirota coming in second place. Based on cell counts, it is evident that *Lactobacillus* strains generally proliferate more effectively in Uno Rosso, reaching higher cell numbers compared to Cherrola or Mobil varieties. The pH of all samples dropped below the important microbiological safety value of 4 (ranging from 3.43 to 3.94). The Cherrola variety had the highest total soluble solids, which was also noticeable in the sweeter taste of the raw tomato juice. This higher total soluble solids content may counterbalance the higher acidity and lower pH in terms of taste. The raw Cherrola tomato juice had the highest titratable acidity, which typically resulted in high acidity levels in the fermented juices, although among the fermented tomato juices, Uno Rosso generally had the highest titratable acidity. Even after four weeks of storage in the refrigerator, numerous *Lactobacillus* strains showed significant survival rates and high cell counts. However, there were substantial differences among strains in this regard as well. In extreme cases, the cell count dropped below 5 log CFU mL^{-1} by the fourth week (Uno Rosso, *L. acidophilus* N2), while in other cases, it maintained almost the initial concentration of 9 log CFU mL^{-1} (Uno Rosso, *L. casei* 01). However, depending on the strain and tomato variety, the cell count generally ranged from 6 to 8 log CFU mL^{-1} after four weeks of storage at 6°C. The best survival results were obtained in Cherrola tomato juice (0.86 to 0.94), where, for each strain, 85% of the cells remained viable after the four-week storage period.

I have fermented an additional 15 tomato varieties (numbered 452, 455, 458, 461, 463, 464, 465, 467, 469, 470, 472, 473, 475, 477, 479) using the two best strains selected based on growth rate (*L. acidophilus* N2 and *L. casei* Shirota). Comparing these results with the results from Mobil, Uno Rosso, and Cherrola, I observed the highest cell counts in the same five varieties (Uno Rosso, Mobil, Cherrola, 479, 477) for both strains. Among the tomato varieties

I examined, Uno Rosso exhibited exceptionally high cell counts, indicating that Uno Rosso is a suitable choice for the lactic acid fermentation of tomato juice.

3.7. Isolation of *Lactobacillus* from fermented vegetables

Isolation of *Lactobacillus* from fermented vegetables (olives, sauerkraut, pickles, kimchi) was carried out based on the protocol I established. These fermented vegetables were either homemade or commercially prepared, presumably not heat-treated, making them potential sources of *Lactobacillus*. I pre-selected lactic acid bacteria on cycloheximide MRS agar, as the *Lactobacillus* selective medium supplemented with cycloheximide has inhibitory effects against various moulds, yeasts, and phytopathogenic fungi. The potential *Lactobacillus* strains grown on the medium were subjected to a catalase test, which indicates the presence of the catalase enzyme that breaks down hydrogen peroxide. *Lactobacillus* strains belonging to the *Lactobacillus* genus react negatively to the test in the absence of catalase, hence I further examined the bacteria if no bubbling was observed. *Lactobacillus* is a Gram-positive bacterium, and during the Gram staining, it stains purple. Bacterial cultures giving a negative catalase test and Gram-positive staining were examined for acid production on bromocresol purple MRS agar. The change in color from purple to yellow on bromocresol purple indicator-containing MRS agar indicates acid production by the bacteria. Finally, the species pre-selected in this way were confirmed to belong to the *Lactobacillus* genus through PCR analysis.

I confirmed the applicability of the selected *Lactobacillus* strains (designated as 476) for the lactic acid fermentation of tomatoes through investigation. From the initial cell count of 7 log CFU mL^{-1}, nearly all of the isolated strains reached or approached the desired cell count of 9 log CFU mL^{-1}. This can be considered a satisfactory result, as the cell proliferation of *Lactobacillus* strains from the strain collection yielded very similar results (strain collection: 8.857 – 9.315 log CFU mL^{-1}, isolated: 8.176 – 9.364 log CFU mL^{-1}). While there are statistical differences observed among certain strains, they do not segregate into distinct groups based on their *Lactobacillus* origin. Therefore, it would be worthwhile to further investigate these strains for their potential probiotic properties.

3.8. Conclusions

Based on the combined heatmap diagram (Table 2), it can be seen that there is no specific *Lactobacillus* strain that consistently performed better in all tested plant materials. However, it can be generally said that only in the sour cherry juice did we manage to achieve a very high cell count, exceeding 9 log CFU mL^{-1}, with all strains tested, even after creating the necessary environment for the growth of lactic acid bacteria. Nonetheless, during the fermentation of each

raw material, I was able to select at least one *Lactobacillus* strain that approached this cell concentration.

Table 2: Visualization of the live cell count dynamics of *Lactobacillus* strains in the form of a heatmap, following 24 hours of fermentation on specific plant material. The rows of the heatmap correspond to different vegetable or fruit juices, while the columns represent the bacterial strains.

		L. rhamnosus GG	*L. acidophilus* LA-5	*L. acidophilus* 150	*L. casei* Shirota	*L. casei* 01	*L. reuteri* DSM 17938	*L. plantarum* 2142	*L. acidophilus* N2	*L. fermentuum* DT41
Orange	Concentrate	9.221	n.d.	8.612	9.086	9.692	7.779	n.d.	n.d.	n.d.
	Non-concentrate	8.305	9.345	7.326	9.262	9.219	9.460	n.d.	n.d.	n.d.
	Freshly squeezed	9.233	6.889	8.319	9.461	9.477	n.d.	n.d.	n.d.	n.d.
Sour cherry	Újfehértói f.	9.354	9.425	9.377	9.307	9.301	9.341	9.138	9.167	9.363
	Petri	9.127	9.538	9.598	9.333	9.282	9.349	9.233	9.094	9.216
Plum	Ageni	8.824	n.d.	8.178	8.942	8.917	8.282	9.215	8.950	9.174
	Stanley	8.873	n.d.	8.705	8.921	8.802	8.908	9.091	9.100	9.075
Black chokeberry	Nero	6.942	6.699	8.084	7.211	6.699	8.587	8.363	8.410	8.452
	Viking	6.884	7.789	8.391	8.536	8.728	8.812	8.635	8.641	8.876
Quince	Angersi	6.138	6.273	8.704	8.579	6.211	8.865	8.540	8.776	8.590
	Csokonai	6.211	6.176	8.622	8.560	5.824	8.561	8.824	8.661	8.689
Tomato	Mobil	7.993	9.000	8.987	9.079	8.762	7.176	8.926	9.251	8.895
	Uno Rosso	8.938	8.945	9.295	9.372	9.219	8.200	8.896	9.514	9.195
	Cherrola	9.130	n.d.	8.950	9.154	n.d.	n.d.	8.832	9.177	n.d.

> 9 log CFU mL^{-1} = 9 log CFU mL^{-1} = 8 log CFU mL^{-1} = 7 log CFU mL^{-1} < 7 log CFU mL^{-1}. n.d. = no data

The importance of strain selection for specific raw materials is evident by the outstanding performance of all nine tested *Lactobacillus* strains derived from the strain collection on at least one specific raw material (only *L. rhamnosus* GG and *L. casei* Shirota strains did not produce the highest cell counts in any juice). In several cases (Petri and Újfehértói sour cherry varieties, Angersi quince variety), the highest cell counts were achieved with probiotic strains, contradicting the general assumption that probiotic strains do not possess as favorable technological properties as non-probiotic lactic acid bacteria. Currently, commercial strains are largely selected based on their technological properties, thereby excluding numerous strains that could be promising for maintaining consumer health (LACROIX & YILDIRIM, 2007).

Lactobacillus strains are capable of metabolizing simple sugars found in fruit juices, thereby increasing the acidity of the product (SENGUN et al., 2019). Upon reaching an

adequate cell count, the pH is consistently lowered to a level sufficient for microbiological safety. The increase in bioactive compounds can contribute to a product of higher added value, but their decrease is not necessarily a drawback as probiotic microorganisms are capable of rapidly consuming phenolic compounds present in fruits, thereby enhancing their own survival (OZCAN et al., 2015). Based on my obtained results, I was also interested in determining which raw material showed the most favorable overall changes in bioactive compounds due to fermentation (Table 3). If growth occurred compared to the initial (0 h) value, it was assigned a score of 1. If there was no change, a score of 0 was given, and in case of a decrease, a score of -1 was assigned to the strain on the respective fruit. The maximum score corresponded to the total points the fruit variety would have received if all applied strains had resulted in growth in all three measured bioactive compounds. Thus, I calculated a percentage aggregate value. Accordingly, among the three fruits, sour cherry proved to be the most suitable raw material in terms of overall changes in bioactive compounds during lactic acid fermentation, as significant growth was observed in the bioactive compounds of both varieties. In the case of black chokeberry, the Nero variety also obtained a high aggregate score, while for the Viking variety, I observed a decrease on average. Similarly, with plums, there was minimal growth in the case of the Ageni variety, whereas the Stanley variety exhibited more decreases than increases in the quantity of bioactive compounds.

My results support the statement made by RANADHEERA et al. (2010) that the growth and viability of probiotic bacteria are influenced by both the microbial strain and the composition of the fruit juice (acidity, carbohydrate content, nitrogen source). In addition to storage temperature, the survival of lactic acid bacteria depended on the type and variety of the raw material, as there were several orders of magnitude difference, for example, in the cell count of *L. acidophilus* LA-5 during the 4th week of storage in orange juice concentrate (0.166 log CFU mL^{-1} decrease) and Uno Rosso tomato juice (3.526 log CFU mL^{-1} decrease). During storage following fermentation with *L. plantarum* in fermented fruit juices (orange, grapefruit, blackcurrant, pineapple, pomegranate, blueberry, and lemon), significant differences (0.02 – 8.02 log CFU mL^{-1} decrease) in cell count were observed at the 6th week depending on the type of raw material (NUALKAEKUL & CHARALAMPOPOULOS, 2011).

Table 3: The changes in bioactive compounds due to fermentation compared to the initial value (growth 1, decrease -1, no change 0) and their overall percentage values in the respective plant raw materials.

			L. rhamnosus GG	*L. acidophilus* LA-5	*L. acidophilus* 150	*L. casei* Shirota	*L. casei* 01	*L. reuteri* DSM 17938	*L. plantarum* 2142	*L. acidophilus N2* 2142	*L. fermentuum* DT41	Overall
Sour cherry	Újfehértói fürtös	Polyphenol	1	1	1	1	-1	1	1	-1	1	51.85%
		FRAP	0	1	1	1	-1	-1	-1	-1	1	
		DPPH	1	1	1	1	1	1	1	1	1	
	Petri	Polyphenol	1	1	1	1	1	-1	-1	1	1	77.78%
		FRAP	1	1	1	1	1	1	1	1	1	
		DPPH	1	1	1	1	1	1	1	-1	1	
Plum	Ageni	Polyphenol	-1	n.d.	-1	1	-1	-1	1	-1	1	20.83%
		FRAP	1	n.d.	1	1	0	1	1	-1	1	
		DPPH	-1	n.d.	1	1	-1	1	1	-1	1	
	Stanley	Polyphenol	-1	n.d.	-1	-1	-1	-1	-1	-1	-1	-45.83%
		FRAP	-1	n.d.	-1	-1	1	-1	-1	-1	-1	
		DPPH	-1	n.d.	-1	1	1	1	1	0	1	
Black chokeberry	Nero	Polyphenol	1	1	1	1	1	1	1	1	1	70.37%
		FRAP	1	1	-1	1	1	1	1	1	1	
		DPPH	-1	1	1	1	-1	1	1	-1	1	
	Viking	Polyphenol	-1	1	-1	-1	-1	-1	-1	-1	-1	-25.93%
		FRAP	1	1	1	-1	-1	1	1	1	1	
		DPPH	1	1	-1	-1	-1	-1	-1	-1	-1	

n.d. = no data

Based on my investigations, it can be concluded that by using appropriate lactic acid bacteria and creating the necessary conditions for their growth, it is possible to develop a lactic acid fermented product that contains the desired live cell count of 9 log CFU mL^{-1}. This allows for the creation of a product that contributes to health preservation, can be incorporated into daily meals, is compatible with a vegan diet, and does not require individuals with milk protein allergies or lactose intolerance to miss out on the health benefits offered by probiotic foods.

4. RECOMMENDATIONS

I have successfully selected *Lactobacillus* strains for all examined plant-based raw materials, resulting in a more durable product with improved sensory and physiological properties compared to the perishable and lower-value vegetables and fruits. Through the process of biopreservation, I have created a product containing probiotic strains without the need for

artificial additives, making it suitable for everyday consumption. These naturally acidic fruit and vegetable juices, produced using a preservative-free method, retain their sensory characteristics while maintaining their nutritional properties without significant changes. In fact, in some cases, the quantity of bioactive compounds in these juices increases. These products, along with the optimized production technology involving fermentation and storage parameters, not only offer probiotics but also contribute to the expanding range of vegetable and fruit products available to consumers.

In the production of fermented foods, the use of multiple species (mixed cultures) can be employed to ensure product flavor or safety (ZHOU et al., 2020). The application of various *Lactobacillus* strains as a mixed culture is not a novel concept, particularly in enhancing substrate conversion efficiency in lactic acid production (CUI et al., 2011). Microorganisms can have a positive influence on each other, resulting in higher cell counts, organic acid production, and scavenging capacity compared to pure cultures (BAGHER HASHEMI & JAFARPOUR, 2020). This approach could potentially enhance the viability of less prolific probiotic *Lactobacillus* strains by synergistic effects exerted by a well-fermenting non-probiotic strain or even bacteria from a different genus. This could lead not only to an increase in cell count but also in the quantity of metabolic products and bioactive compounds in the fermented product.

Compared to other substrates, fruits have significantly lower levels of free amino acids (RUIZ RODRÍGUEZ et al., 2020). This can be a limiting factor for the growth of lactic acid bacteria, as observed in the fruit juices I examined, where the lactobacilli required supplementation with protein due to their high demand for organic nitrogen sources. Therefore, instead of yeast extract supplementation, the use of inactive yeast could be recommended, as it improves the assimilation of nitrogen compounds and provides nutrients for the bacteria (ŠUKLJE et al., 2016). Additionally, it would be advisable to investigate the fermentation of fruit juices mixed with a higher protein-containing plant material, and experiments are currently underway in this regard (such as the combination of black chokeberry juice with soy milk). This approach could potentially lead to the creation of products that do not require added additives. Instead of using inulin, which was examined in orange juice, the addition of natural, plant-based prebiotic components could further enhance the growth and survival of *Lactobacillus* on raw materials. Therefore, it would be worth conducting fermentation experiments with fruit and vegetable juices mixed with Jerusalem artichoke or artichoke, for example (LAVERMICOCCA et al., 2016). I also conducted experiments on the fermentation of green peas, which provided a suitable environment for the growth of lactic acid bacteria, similar to tomato juice. However,

based on the organoleptic properties observed, I would recommend using green peas as a supplementary raw material for further experiments.

The *Lactobacillus* strains isolated from lactic acid fermented vegetables exhibited similar good fermentation properties to the strains from the culture collection. Therefore, it would be worthwhile to further investigate these strains in terms of their resistance to gastric and bile acids and their potential probiotic properties, as only a few studies have focused on exploring the probiotic potential of strains with good fermentation properties. Additionally, in the future, it is recommended to isolate *Lactobacillus* strains directly from raw vegetables and fruits, which could be used as starter cultures for lactic acid fermentation. Numerous studies have highlighted the advantages of these naturally occurring microorganisms present in plant-based raw materials for the production of fermented products with improved technological and functional properties (VERÓN et al., 2017, VERÓN et al., 2019).

5. NEW SCIENTIFIC RESULTS

1. I determined the optimal nutrient supplementation, pH values, and juice dilution ratios required for the lactic acid fermentation of different fruit juices. For orange juice, at a pH of 7.00, the optimal amount of dextrose supplementation is 60 g L^{-1}, along with 2 g L^{-1} of yeast extract as a nutrient supplement. For sour cherry juice, the optimal initial values are a pH of 5.80, 3 g L^{-1} of added yeast extract, and a dilution ratio of 6 : 4 (v/v) with water. For plum juice, with a pH set at 6.50, the ideal amount of yeast extract is 6 g L^{-1}, and the plum juice should be diluted with water in a 5.5 : 4.5 (v/v) ratio. For black chokeberry juice, the pH needs to be adjusted to 4.50, and supplementation of 5.62 g L^{-1} of peptone is required when diluting the black chokeberry juice in an 8 : 2 (v/v) ratio with water. For quince juice fermentation, at a pH of 6.00, the optimal amount of peptone supplementation is 5 g L^{-1}, and the quince juice should be diluted with water in an 8 : 2 (v/v) ratio.

2. I successfully selected *Lactobacillus* strains on all tested raw materials that have achieved or approached the desired 9 log CFU mL^{-1} cell count. The importance of strain selection is evident as not all raw materials yielded the highest cell count with the same strain, and each strain behaved differently in different fruit and vegetable juices. It is crucial to perform strain selection specifically for each raw material and its variety when choosing a starter culture. For non-concentrated orange juice, the application of *L. reuteri* DSM 17938 resulted in the highest cell count, while for concentrated and freshly squeezed orange juice, *L. casei* 01 was the most effective starter culture. For the Újfehértói fürtös sour cherry, *L. acidophilus* LA-5 performed

best, while for the Petri variety, *L. acidophilus* 150 was the most successful. The Ageni plum showed the highest cell count with *L. plantarum* 2142, whereas the Stanley variety responded best to *L. acidophilus* N2. As for the Angersi quince, *L. reuteri* DSM 17938 yielded the highest cell count, and for the Csokonai variety, *L. plantarum* 2142 was the most effective strain under the specified parameters. Among the tomato varieties I examined, the Uno Rosso variety is recommended for lactic acid fermentation, with *L. acidophilus* N2 as the most successful starter culture in terms of cell count.

3. I have demonstrated through the results of sour cherry fermentation that there is no significant difference in cell counts between probiotic strains (*L. rhamnosus* GG, *L. casei* Shirota, *L. reuteri* DSM 17938, *L. acidophilus* 150, *L. acidophilus* LA-5, *L. casei* 01) and non-probiotic strains (*L. acidophilus* N2, *L. plantarum* 2142, *L. fermentum* DT41) after fermentation. Furthermore, it has been proven that the highest cell count is achieved by a probiotic strain in both sour cherry varieties. Similar results were obtained with tomato juice, where one probiotic strain stood out in terms of growth and survival.

4. During my research, I developed lacto-fermented plant-based products that contain a high number of probiotic culture propagated in the respective raw material. These products, when stored in the refrigerator, maintained the recommended cell count even after 8 weeks.

5. Based on the protocol I established (cycloheximide MRS agar, catalase test, Gram staining, bromocresol purple MRS agar), I isolated strains belonging to the *Lactobacillus* genus from non-heat-treated, commercially available, and homemade fermented vegetables such as kimchi, olives, sauerkraut, and pickles. These strains have been confirmed to exhibit good fermentation properties.

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PUBLICATIONS

Journal articles published

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2. **Perjéssy, J.**, Hegyi, F., Nagy-Gasztonyi, M., Zalán, Zs. (2021). Effect of the lactic acid fermentation by probiotic strains on the sour cherry juice and its bioactive compounds. Food Science and Technology International. (Q2 – IF.: 2,023) https://doi.org/10.1177/10820132211018044
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