HUNGARIAN UNIVERSITY OF
AGRICULTURE AND LIFE SCIENCES

MICROENCAPSULATION OF PROBIOTIC LACTIC ACID BACTERIUM *LACTIPLANTIBACILLUS PLANTARUM* 299v STRAIN AND APPLICATION POTENTIAL

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1. INTRODUCTION AND OBJECTIVES

Probiotics are living microorganisms that have health benefits on the host when they are administrated in a sufficient amount, generally more than 6 log (CFU/g) is recommended. In order to exert the functionality of the probiotics, they must be able to survive and multiply in the host. However, there are many harsh factors such as oxygen, heat, and hydrogen peroxides that can sharply affect the viability of the probiotics during the manufacturing, storage, and digestion process. In the last few decades, to respond this great challenge, intensive research, and developments are carried out worldwide, such as the selection of new strains, improvement of oxygen, acid and heat tolerance, production of some techno-functional metabolites, encapsulation. Among these developments, microencapsulation was proved to be one of the best directions.

Microencapsulation is a talented technology that can protect the probiotics by coating them with wall materials to maintain their viability and functionality. Lyophilization that sublimates the water directly from ice-phase under vacuum condition and low temperature (10 °C -20 °C) may provide a particularly good alternative solution for associating the encapsulation of probiotics. Another important part of encapsulation should be the coating materials that can be polysaccharides, proteins, lipids, and other materials, etc. Polysaccharides such as maltodextrin and resistant starch are naturally produced and GRAS (Generally Regarded as Safe) products and have been used as food additives in the food industry for a long time. Maltodextrin is a product produced by starch hydrolysis with high molecular weight, which has a good film-forming ability. Resistant starch is a small branch of starch, which has the properties to resist the hydrolysis by α-amylase and pullulanase in the upper gastrointestinal tracts (including mouth, pharynx, oesophagus, stomach, and duodenum) but it can be fermented by probiotics in the colon. Proteins such as whey protein and denatured whey protein are widely used due to their excellent physical and chemical properties. Whey proteins (WP) are considered an exceptional coating material due to their specific physical and chemical properties such us excellent emulsification, superb gelation, and exquisite fill-forming properties. Denatured whey proteins (DWP) are originated from WP, by managing with acid or heat as a denaturation approach, which can contribute to the specific properties of whey proteins, e.g., high tensile property, low oxygen permeability. Moreover, whey proteins from cheese making in the dairy industry are a main pollutant in the wastewater. However, whey proteins have a high nutritional value because they are useful sources of many valuable biological proteins, riboflavin, and minerals. The polysaccharide-protein binary complexes can be made through the Maillard reaction and get Maillard reaction products (MRPs). Several advantages of MRPs have been reported. They have excellent antioxidant and emulsifying characteristics. MRPs show great potentiality as the delivery carrier for bioactive substances. In addition, they also perform prebiotic functionality because they are resistant to digestion compared to the non-glycated proteins, which means more dietary glycoconjugates are available for the endogenous microbiota utilization in the distal colon.

Nowadays, the consumers awareness of functional foods such as probiotics, prebiotics, synbiotics

etc., is increasing significantly, due to health benefits as well as the expansion of product ranges. Most probiotic food products are dairy-based, but plant-based matrices such as fruit juice can also serve as very good carriers for the delivery of probiotic cells. Fruits are considered as fresh, nutritious, and disease-preventing foods due to their nutritional and functional properties, making fruit juices popular and preferred by consumers worldwide. Plant-based food matrices such as fruit juice, jams, vegetable juice, etc., can serve as exceptionally good alternatives of dairy products for carrying probiotics. Among those plant-based products, fruit juice is one of the most favoured forms of consumption by consumers. However, during the process fruit juice may lose on nutritional value mainly bioactive compounds. In addition, these juices also contain sufficient amount of sugars including glucose, fructose, sucrose, etc., which may lead to the over intake of carbohydrates. To compensate for the loss of nutritional compounds and facilitate the processed fruit juices still as functional food, probiotics can be fortified into it to fulfill the function. Unlike the traditional physical reduction technology of the sugar content in many soft drinks, coffee drinks, etc., the use of lactic acid bacteria to reduce the sugars in juices is an attractive method, because it does not only affect the limited taste of the juice, but it also increases the nutritional value by the production of many health beneficial intermediates such as vitamins, short chain fatty acids etc. Main advantage of this concept is multipurpose that can be realized in one food product, and these may require new types of probiotic preparation, thus topics of my Ph.D. were based on.

Objectives

The main goal of my Ph.D. research is development and characterization of encapsulated probiotics preparation and their application in the production of probiotic apple juice. The specific objectives were the following:

- **Formulation of probiotic microcapsules**
  - Effect of coating materials
    - polysaccharides (maltodextrin, resistant starch)
    - proteins (whey protein, denatured whey protein)
    - Maillard reaction products of maltodextrin and whey protein (MRPs)
  - Effect of ratios of core-to-wall and the different wall materials
- **Characterization of probiotic microcapsules**
    - cell number and bulk density
    - morphology
    - viability change during long-term storage at different temperatures
    - tolerance to simulated gastric fluid (SGF) and simulated intestinal fluid (SIF)
- **Application of microcapsules in production of probiotic apple juice**
    - effect of production method: fermentation and fortification
    - pH changes during long-term storage at different temperatures
    - viability change during long-term storage at different temperatures

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Microorganisms

A probiotic strain *Lactiplantibacillus plantarum* 299v (Probi, Sweden) was applied.

2.1.2 Coating materials

Different types of coating materials were applied in microencapsulation process. Polysaccharides maltodextrin and resistant starch were from Ingredion (Germany), while whey protein was from Nutriversum® Ltd. (Hungary). The denatured whey protein was prepared in-house by heating 20% (w/w) whey protein solution at 90°C for 20 min. Maillard reaction products prepared by maltodextrin and whey proteins are dissolved in saline solution and the concentration was modified to 20% (w/w). The pH was adjusted to 8.0 with 4 N NaOH. The solutions were heated in a water bath at 90°C for 4 hours to form the MRPs as the coating materials. The MRPs solutions were cooled down and stored at 4°C for further practice.

2.1.3 MRS medium

MRS (Man, Rogosa, and Sharpe) medium was usually used for maintaining *Lactiplantibacillus plantarum* 299v strain. The final pH of the medium was adjusted to pH 6.8 to pH 7.0 and sterilized in the autoclave at 121°C for 15 min.

2.1.4 Simulated gastric fluid

Simulated gastrointestinal fluid (SGF) was made by dissolving the pepsin (727 U/mg) (Sigma, Germany) in the sterilized 5g/L saline solution (pH 2). The pH of the saline solution was previously adjusted to 2 by 6N HCl before sterilization.

2.1.5 Simulated intestinal fluid

Simulated intestinal fluid (SIF) was made by dissolving the bile salt (Sigma, Germany) in the sterilized K_2HPO_4 and KH_2PO_4 solution (pH 7.4). The K_2HPO_4 and KH_2PO_4 solution was made by dissolving 5.43 g K_2HPO_4 and 2.56 g KH_2PO_4 in 1L distilled/deionized water and sterilized in the autoclave at 121°C for 15 min.

2.1.4 Apple juice

Unfiltered high-quality HAZÁNK Kincsei apple juice (Lidl, Hungary) was purchased from a local supermarket. The pH of apple juice was adjusted to pH 6 by 4 N NaOH solution prior use.

2.2 Methods

2.2.1 Microencapsulation process

The *Lp. plantarum* 299v strain was grown in MRS medium at 37°C for 18 h when the cell count reached around 10^9 CFU/mL at the end of the incubation. Then the cells were collected by centrifugation at 10.000×rpm at 4°C for 20 min. After that, they were washed twice by phosphate-buffered saline solution (0.1 M and pH 7.4). The wet cell pellet was then mixed with different wall materials based on the ratios of core-to-wall, and the ratios between the wall materials (**Table 1**). In the cases of combination of maltodextrin and whey protein, the Maillard reaction was priorly

carried out by heating the solutions at 90°C for 3 h. After mixing procedure, samples were gently shaken at 150×rpm and 4°C for 1 h.

Table 1. Experimental design for coating probiotic bacterium *Lp. plantarum* 299v

Coating materials	Ratios of core-to-wall	Wall materials	Ratios of wall materials	MD (g)	RS (g)	*Lp. plantarum* 299v (g)
Polysaccharides	1:1	MD	-	10.00	0.00	10
		MD:RS	3:1	7.50	2.50	10
		MD:RS	1:1	5.00	5.00	10
		MD:RS	1:3	2.50	7.50	10
		RS	-	0.00	10.00	10
	1:1.5	MD	-	15.00	0.00	10
		MD:RS	3:1	11.25	3.75	10
		MD:RS	1:1	7.50	7.50	10
		MD:RS	1:3	3.75	11.25	10
		RS	-	0.00	15.00	10
Proteins	1:1	WP	-	10.00	0.00	10
		WP:DWP	3:1	7.50	2.50	10
		WP:DWP	1:1	5.00	5.00	10
		WP:DWP	1:3	2.50	7.50	10
		DWP	-	0.00	10.00	10
	1:1.5	WP	-	15.00	0.00	10
		WP:DWP	3:1	11.25	3.75	10
		WP:DWP	1:1	7.50	7.50	10
		WP:DWP	1:3	3.75	11.25	10
		DWP	-	0.00	15.00	10
Polysaccharides + Proteins	1:1	MD:WP	3:1	7.50	2.50	10
		MD:WP	1:1	5.00	5.00	10
		MD:WP	1:3	2.50	7.50	10
	1:1.5	MD:WP	3:1	11.25	3.75	10
		MD:WP	1:1	7.50	7.50	10
		MD:WP	1:3	3.75	11.25	10

MD-Maltodextrin; RS-Resistant Starch; WP-Whey protein; DWP-Denatured whey protein

The suspensions were dispensed into sterilized and clean-drying glass bottles. Then, the samples were placed in the freezer (-18°C) for 24 h. The lyophilization was carried out by laboratory-scale Christ Alpha 2-4 freeze dryer (Martin Christ, Germany). The dried pressure and temperature were 0.250 mbar and 17°C, respectively. The drying process lasted for 3 days. The dried microcapsules were grounded manually under aseptic conditions, then transferred into sterilized vials, and stored at 4°C for future analysis. All preparations were conducted in duplicate.

2.2.2 Determination of viable cell numbers

The viable bacterial cells were enumerated by pour plating method using MRS agar and the serial

dilutions were made with sterile 0.85% w/v sodium chloride solution. The MRS agar plates were incubated at 37°C for 48-72 h for development of colonies. After incubation, the plates contained around 30-300 colonies were counted and expressed as CFU/g of dried samples or CFU/mL of solution. All enumerations were achieved in duplicate.

2.2.3 Determination of encapsulation yield

Encapsulation yield was determined by weighting the total amount of solid materials before and after lyophilization.

2.2.4 Determination of bulk density

Bulk density was typified by measuring the volume of 1 g microcapsule sample in a 5 mL cylinder after being tapped on a vortex for 2 min.

2.2.5 Scanning electron microscopy

Scanning electron microscopy (SEM) was used to observe the morphological structure of *Lp. plantarum* 299v microcapsules with different core-to-wall ratios and wall material formulations. The samples were transferred and stuck on a plate in the vacuum chamber and gradually decreased to 200 Pa. Observation of the samples were carried in Thermo Scientific™ Prisma™ E (Waltham, Massachusetts, USA) SEM under an accelerating voltage of 15 kV. The samples were examined under 1,000x and 14,000x magnifications.

2.2.6 Effect of storage

The viability of the microencapsulated probiotics during storage at 4°C and at 25°C was determined by enumeration on MRS agar. It was carried out every 2 weeks and the process lasted for 10 weeks. Different kinetic models were regressed based on the experimental data obtained for estimation of changes of viable cells during storage.

2.2.7 Tolerance study

Tolerance of probiotics in microcapsules during the digestion process was carried out at *in vitro* conditions. Briefly, 0.1g microcapsules of samples were added into 9.9 mL simulated gastric fluid (SGF) (0.3% pepsin, pH 2) or simulated intestinal fluid (SIF) (0.6% bile salt, pH 7.4). Samples were taken at the incubation time 0 h, 0.5 h, 1 h, 2 h, 3 h, and 0 h, 3 h, 6 h in the cases of SGF or SIF, respectively. Viable cell numbers were enumerated by the plate-counting method.

2.2.8 Application potential of microencapsulated probiotic bacteria

The probiotic capsules were applied for fermentation and fortification of apple juice. In the case of fermentation of apple juice, 0.2 g microcapsules were added into 90 mL apple juice and then incubated at 37°C for certain hours. The fermentation process was monitoring by the changing of pH, and it was completed when pH value dropped to below pH 5.0. In the case of fortification of apple juice, 0.2 g microcapsules were directly added into 90 mL apple juice.

2.3 Statistical methods

All experiments were performed in duplicates and the results were presented as means ± standard deviation. ANOVA (analysis of variance), unpaired and paired Student's t-tests with a significance level of $\alpha = 0.05$ was used to determine statistical differences among the independent variables by using SPSS AU (www.spssau.com/en).

3. RESULTS AND DISCUSSION

My Ph.D. work focused on the encapsulation of the probiotic *Lp. plantarum* 299v strain. Investigation of the effect of different types and ratios of different coating materials such as polysaccharides (maltodextrin and resistant starch), proteins (whey protein and denatured whey protein), Maillard reaction products as well as ratios of core-to-wall on the protection of probiotics was aimed. Additionally, the application potentials of probiotic microcapsules were also aimed to explore.

- **Formulation of probiotic microcapsules**

Yield is an essential parameter during the manufacturing, packaging, and storage process of probiotic microcapsules. The yields of the microencapsulation process were determined. In terms of yield, the best results were 66.74%, 68.02%, and 66.76%, respectively corresponding to polysaccharides (ratio of core-to-wall 1:1.5 and MD), proteins (ratio of core-to-wall 1:1.5 and DWP), and MRPs (ratio of core-to-wall 1:1.5 and MD:WP 3:1).

- **Characterization of probiotic microcapsules**

The cell number of living microorganisms is an indicator for all the probiotic products, it is recommended by FAO/WHO with a minimal number of 6 log (CFU/g). Hence, analyzing the viability change of the microencapsulated probiotics focuses on protecting ability of coating materials, temperature, etc., during storage process can not only check the quality of the microcapsules but also predict the shelf-life of the microcapsules.

Probiotic microcapsules were successfully produced by encapsulation with different coating materials, ratios of core-to-wall, and ratios of wall materials. The highest cell numbers were observed in the microcapsules coated with polysaccharides (ratio of core-to-wall 1:1.5 and MD:RS 3:1), proteins (ratio of core-to-wall 1:1 and WP:DWP 3:1), and MRPs (ratio of core-to-wall 1:1 and MD:WP 1:1) were 11.93 log (CFU/g), 11.29 log (CFU/g) and 13.75 log (CFU/g), respectively. Scanning electron microscopy was performed to study the morphological properties of the microcapsules. The rod-shaped *Lp. plantarum* 299v cells were all homogenously microencapsulated and coated. Additionally, in the case of polysaccharides with a ratio of core-to-wall 1:1.5, the surface of the microcapsules was smoother, and had a more uniform structure compared to the samples with a ratio of core-to-wall 1:1. This observation was not noted in the microcapsules coated with proteins nor MRPs.

- **Evaluation of physiological properties of the probiotic microcapsules**

The viability loss of the probiotics during storage is mainly owing to the membrane lipid oxidation, thus oxygen content and temperature are the crucial factors that affect probiotic viability. Hence, the microencapsulation process of probiotics with different ratios of core-to-wall and ratios of wall materials have effect on the stability of microencapsulated *Lp. plantarum* 299v during 4°C and 25°C storage.

Different models obtained by regression analysis of experimental data were applied for monitoring

the viability change of the probiotics. The viability of bacterial cells in microcapsules stored at 4°C had significantly lower loss compared to those stored at 25°C. However, no significant difference in viability loss was observed between the samples with a ratio of core-to-wall 1:1 and 1:1.5 even stored at 4°C or at 25°C. The comparison of the highest viability after 8 weeks of storage of three types of coating materials is listed in **Table 2**.

Table 2. Summary table of the highest viability, viability reduction and final cell number of probiotics coated by three kind of coating materials (storage for 8 weeks) at 4°C and 25°C

	parameters	polysaccharides	proteins	MRPs
4°C	ratio of core-to-wall	1:1.5	1:1	1:1
	ratio of wall materials	MD:RS 3:1	WP	MD:WP 1:1
	cell number changes	-0.81 log (CFU/g)	-0.14 log (CFU/g)	-1.16log (CFU/g)
	highest number	**11.12 log (CFU/g)**	**10.96 log (CFU/g)**	**12.59 log (CFU/g)**
25°C	ratio of core-to-wall	1:1	1:1	1:1
	ratio of wall materials	MD:RS 1:3	WP:DWP 3:1	MD:WP 1:1
	cell number changes	-0.91 log (CFU/g)	-1.40 log (CFU/g)	-0.64 log (CFU/g)
	highest number	**9.90 log (CFU/g)**	**9.90 log (CFU/g)**	**13.11 log (CFU/g)**

MD: maltodextrin; RS: resistant starch; WP: whey protein; DWP: denatured whey protein

The highest viability of probiotics coated by polysaccharides, proteins, and MRPs are 11.12 log (CFU/g), 10.96 log (CFU/g), and 12.59 log (CFU/g) when stored at 4°C. The highest viability of probiotics coated by polysaccharides, proteins, and MRPs are 9.90 log (CFU/g), 9.90 log (CFU/g), and 13.11 log (CFU/g) when stored at 25°C. In the case of storage at 4°C, the best combinations of coating materials for the protection of probiotics were polysaccharides with a ratio of core-to-wall 1:1.5 and MD:RS 3:1, proteins with a ratio of core-to-wall 1:1 and WP, and MRPs with a ratio of core-to-wall 1:1 and MD:WP 1:1. These samples had viability reduction of 0.81 log (CFU/g), 0.14 log (CFU/g), and 1.16 log (CFU/g), respectively. In the case of storage at 25°C, the best combinations of coating materials for the protection of probiotics were polysaccharides with a ratio of core-to-wall 1:1 and MD:RS 1:3, proteins with a ratio of core-to-wall 1:1 and WP:DWP 3:1, and MRPs with a ratio of core-to-wall 1:1 and MD:WP 1:1. These samples had viability loss of 0.91 log (CFU/g), 1.40 log (CFU/g), and 0.64 log (CFU/g), respectively, during storage at 25°C for 8 weeks.

Low pH and high bile salt content indicate the harsh environment that probiotics may suffer during the digestion process. Consequently, the sustainability of the living characteristics during the digestion process is the other paramount property of probiotics incorporated with coating materials, which can effectively consolidate them into functional foods. Microencapsulating effect on probiotic survival ability manifests a conspicuous increase in viable cell number, ensures more viable cells pass through the digestion process with low pH and high bile salt conditions safely, minimizes the viability loss of probiotic product, together with safeguarding complete releasing

microencapsulated probiotic bacteria into the intestine in quantities large enough for further colonization.

The tolerance of different probiotic microcapsules to SGF and SIF was evaluated using an *in vitro* simulated gastrointestinal testing system. The comparison of the highest viability of probiotics coated by three kinds of coating materials after the SGF and SIF test is listed in **Table 3**.

Table 3. Summary table of the highest viability, viability reduction and final cell number of probiotics coated by three kind of coating materials after SGF and SIF test

parameters		polysaccharides	proteins	MRPs
SGF	ratio of core-to-wall	1:1	1:1.5	1:1.5
	ratio of wall materials	MD:RS 1:1	WP:DWP 3:1	MD:WP 1:3
	cell number changes	-0.65 log (CFU/g)	-0.12 log (CFU/g)	-0.28 log (CFU/g)
	cell number after SGF	**9.04 log (CFU/g)**	**10.23 log (CFU/g)**	**10.79 log (CFU/g)**
SIF	ratio of core-to-wall	1:1.5	1:1	1:1.5
	ratio of wall materials	MD:RS 3:1	WP	MD:WP 1:3
	cell number changes	-0.11 log (CFU/g)	-0.35 log (CFU/g)	-0.52 log (CFU/g)
	cell number after SIF	**9.51 log (CFU/g)**	**8.92 log (CFU/g)**	**11.02 log (CFU/g)**

MD: maltodextrin; RS: resistant starch; WP: whey protein; DWP: denatured whey protein

The highest viability of probiotics after the SGF test coated by polysaccharides, proteins, and MRPs are 9.04 log (CFU/g), 10.23 log (CFU/g), and 10.79 log (CFU/g), respectively. The highest viability of probiotics after the SIF test coated by polysaccharides, proteins, and MRPs are 9.51 log (CFU/g), 8.92 log (CFU/g), and 11.02 log (CFU/g), respectively. The samples coated with polysaccharides in the ratio of core-to-wall 1:1 and MD:RS 1:1, with proteins in the ratio of core-to-wall 1:1.5 and WP:DWP 3:1, and with MRPs in the ratio of core-to-wall 1:1.5 and MD:WP 1:3 had the viability reduction of 0.65 log (CFU/g), 0.12 log (CFU/g), and 0.28 log (CFU/g), respectively after treatment in the simulated gastric fluid for 3 hours. Moreover, after placement of microcapsules in the simulated intestinal fluid for 6 hours, viability reduction of 0.11 log (CFU/g), 0.35 log (CFU/g), and 0.52 log (CFU/g) of the viability of viable cells was determined in the cases of coating with polysaccharides in the ratio of core-to-wall 1:1 and MD:RS 3:1, with proteins in the ratio of core-to-wall 1:1 and WP, or with MRPs in the ratio of core-to-wall 1:1.5 and MD:WP 1:3, respectively.

- **Application of microcapsules in production of probiotic apple juice**

The stability of microencapsulated cells is quite different from diverse food matrices, even for the same kind of food matrices, the properties will change after the fermentation process. Hence, the stability of the microencapsulated cells in food matrices is related not only to the characteristics of the food matrices but also has a linkage with the coating materials.

Table 4. Summary table of the highest viability, viability reduction and final cell number of probiotics coated by three kind of coating materials to produce fortified and fermented apple juice store at 4°C and 25°C after storage for 8 weeks

parameters			polysaccharides	proteins	MRPs
4°C	fermented	ratio of core-to-wall	1:1	1:1	1:1.5
		ratio of wall materials	MD	WP:DWP 1:1	MD:WP 1:1
		highest cell number	8.26 log (CFU/ml)	8.97 log (CFU/ml)	9.27 log (CFU/ml)
	fortified	ratio of core-to-wall	1:1	1:1	1:1.5
		ratio of wall materials	MD	DWP	MD:WP 1:3
		highest cell number	8.43 log (CFU/ml)	9.24 log (CFU/ml)	9.44 log (CFU/ml)
25°C	fermented	ratio of core-to-wall	1:1.5	1:1	1:1.5
		ratio of wall materials	RS	WP:DWP 1:1	MD:WP 3:1
		highest cell number	7.39 log (CFU/ml)	8.44 log (CFU/ml)	5.10 log (CFU/ml)
	fortified	ratio of core-to-wall	1:1	1:1	1:1
		ratio of wall materials	MD:RS 1:1	WP:DWP 1:3	MD:WP 1:1
		highest cell number	7.67 log (CFU/ml)	8.47 log (CFU/ml)	6.04 log (CFU/ml)

The production of fortified apple juice was successfully performed using probiotic microcapsules coated with polysaccharides, proteins, and MRPs. Application of microcapsules coated by polysaccharides in apple juice, the fortification method resulted in higher viability compared to fermentation. The comparison of the highest viability of probiotics coated by three kinds of coating materials to produce fortified and fermented apple juice after storage for 8 weeks at 4°C and 25°C are listed in **Table 4**. When stored at 4°C, the highest cell number of fortified apple juice produced by probiotics coated by polysaccharides, proteins, and MRPs are 8.43 log (CFU/g), 9.24 log (CFU/g), and 9.44 log (CFU/g), respectively. While the juice produced by the fermented method is 8.43 log (CFU/g), 9.24 log (CFU/g), and 9.44 log (CFU/g) when stored at 4 °C. Similar results were found for those stored at 25°C. The highest viability of fortified apple juice produced by probiotics coated by polysaccharides, proteins, and MRPs was 7.67 log (CFU/g), 8.47 log (CFU/g), and 6.04 log (CFU/g), respectively. The highest viability of fermented apple juice produced by probiotics coated by polysaccharides, proteins, and MRPs was 7.39 log (CFU/g), 8.44 log (CFU/g), and 5.10 log (CFU/g), respectively. In addition, the highest viability of probiotic apple juice with the same coating materials for microcapsules was also found to be higher when stored at 4°C compared to 25°C.

Table 5. Summary table of characteristics of microcapsules

Parameters		Polysaccharides	Proteins	MRPs
samples	ratios of core-to-wall	1:1	1:1	1:1.5
	ratios of wall materials	MD:RS 1:1	WP	MD:WP 3:1
viability changes (8 weeks)	4°C/25°C if different	4 °C better	4 °C better	4 °C better
	4°C	-1.23 log (CFU/g)	-0.20 log (CFU/g)	-0.43 log (CFU/g)
	25°C	-2.14 log (CFU/g)	-1.17 log (CFU/g)	-1.81 log (CFU/g)
viability changes in juices (8 weeks)	fortified/fermented if different	fortified better	fortified better	fortified better
	4°C	0.15 log (CFU/ml)	0.16 log (CFU/ml)	-0.30 log (CFU/ml)
	25°C	-0.93 log (CFU/ml)	0.94 log (CFU/ml)	0.78 log (CFU/ml)
viability changes during simulated digestion process	SGF	-0.65 log (CFU/g)	-0.33 log (CFU/g)	-1.30 log (CFU/g)
	SIF	-0.94 log (CFU/g)	-0.35 log (CFU/g)	0.27 log (CFU/g)
viability after microencapsulation		11.19 log (CFU/g)	11.09 log (CFU/g)	12.39 log (CFU/g)
final viability		8.53 log (CFU/g)	11.21 log (CFU/g)	11.71 log (CFU/g)

MD: maltodextrin; RS: resistant starch; WP: whey protein; DWP: denatured whey protein

In summary, these results (**Table 5**) provided crucial information for the development of microcapsules systems with effective protection ability and potential application.

4. CONCLUSIONS AND RECOMMENDATIONS

In my Ph.D. research, microencapsulation with three types of coating materials in different ratios of core-to-wall and ratios of wall materials as well as industrial application potential were studied for the development of microcapsules as delivery systems with good protection for probiotic *Lp. plantarum* 299v strain. Three types of microcapsules coated with polysaccharides, proteins, and MRPs were developed successfully. Yield and efficiency of the encapsulation process as well as cell number and bulk density of the microcapsules were influenced by the types of coating materials, the ratios of core-to-wall, and the ratios of wall materials used. Among the investigated coating materials, the MRPs were the bests, because microcapsules coated with them resulted in significantly higher resistance to SGF and SIF than with the two other ones. Additionally, the viability of probiotic cells in microcapsules during storage was dependent on the nature of coating materials, the ratios of core-to-wall, the ratios of different wall materials, the storage temperature, and the storage time. The probiotic microcapsules were ready to apply in the fortification of apple juice, but the developed probiotic drink should be stored at 4°C temperature. Overall, this study provided valuable insights into the development of effective probiotic delivery systems through microencapsulation, and the newly developed microcapsules have high application potential in the fortification of foods.

There are several directions that could be pursued in future research:

(1) Optimization of microencapsulation process with MRPs as coating materials
(2) Study of control release properties of probiotic microcapsules
(3) Evaluation of the administration efficiency of probiotics
(4) Assession of the viability of probiotic microcapsules in the *in vivo* systems.

5. NOVEL CONTRIBUTIONS

1. Probiotic microcapsules were produced by encapsulation with different coating materials, ratios of core-to-wall, and ratios of different wall materials. The highest viabilities in the cases of polysaccharides (ratio of core-to-wall 1:1.5 and MD:RS 3:1), proteins (ratio of core-to-wall 1:1 and WP:DWP 3:1), and MRPs (ratio of core-to-wall 1:1 and MD:WP 1:1) were 11.93 log (CFU/g), 11.29 log (CFU/g) and 13.75 log (CFU/g), respectively.
2. The particle state and the surface morphology of probiotic microcapsules are different depending on the nature of different coating materials, ratios of core-to-wall, or ratios of wall materials. The cells of *Lp. plantarum* 299v strain were homogenously encapsulated and covered in all microcapsules.
3. Different models were developed and used for monitoring the changes in the viability of probiotic cells during storage at different temperatures. The probiotic microcapsules coated with MRPs in the ratio of core-to-wall 1:1 and MD:WP 1:1 showed the highest cell counts of the probiotic microcapsules stored at 4°C and 25°C after 8 weeks of storage with 12.59

log (CFU/g) and 13.11 log (CFU/g), respectively.

4. The highest cell number of probiotic cells was obtained to the tolerance of SGF and SIF tests in the case of the microcapsules coated with MRPs with a ratio of core-to-wall 1:1.5 and MD:WP 1:3 with 10.79 log (CFU/g) and 11.02 log (CFU/g), respectively.
5. The application of probiotic microcapsules in apple juice was achieved successfully through fortification and fermentation methods. The highest cell number of fortified apple juice in the cases of polysaccharides (core-to-wall 1:1 and MD), proteins (ratio of core-to-wall 1:1 and DWP), and MRPs (ratio of core-to-wall 1:1.5 and MD:WP 1:3) that stored at 4°C after 8 weeks storage were 8.43 log (CFU/g), 9.24 log (CFU/g) and 9.44 log (CFU/g), respectively. The fortified apple juice should be stored at 4°C. The microcapsules coated with MRPs with the mentioned conditions with the fortification method and stored at 4°C have the highest cell number in the application of apple juice.

6. PUBLICATIONS

Journal articles

1. **Sun, W.**; Nguyen, Q.D.; Sipiczki, G.; Ziane, S.R.; Hristovski, K.; Friedrich, L.; Visy, A.; Hitka, G.; Gere, A.; Bujna, E. Microencapsulation of *Lactobacillus plantarum* 299v Strain with Whey Proteins by Lyophilization and Its Application in Production of Probiotic Apple Juices. *Appl. Sci.* 2023, *13*, 318. https://doi.org/10.3390/app13010318
2. Pham, T.M.; **Sun, W.**; Bujna, E.; Hoschke, Á.; Friedrich, L.; Nguyen, Q.D. Optimization of Fermentation Conditions for Production of Hungarian Sour Cherry Spirit Using Response Surface Methodology. *Fermentation* 2021, *7*, 209. https://doi.org/10.3390/fermentation7040209
3. **Sun, W.**; Nguyen, Q.D.; Süli B. K.; Alarawi F.; Szécsi A.; Gupta V. K.; Friedrich L.; Gera A.; Bujna E. (2023). Microencapsulation and Application of Probiotic Bacteria *Lactiplantibacillus plantarum* 299v Strain. *Microorganisms*. (Under reviewing process)

Poster presentation

International conferences:

1. **Sun, W.**; Bujna, E.; Nguyen, Q.D. (2021). Stability of apple juice fermented and fortified by microencapsulated *Lactobacillus plantarum* 299v during storage. *The 4th International Conference on Biosystems and Food Engineering*. Hungarian University of Agriculture and Life Sciences, Budapest, 4th June. http://www.biosysfoodeng.hu/
2. Pham, T.M.; **Sun, W.**; Bujna, E.; Hoschke, Á.; Nguyen, Q.D. (2021). Application of response surface methodology for fermentation optimization of cherry by *saccharomyces cerevisiae*. *The 4th International Conference on Biosystems and Food Engineering*. Hungarian University of Agriculture and Life Sciences, Budapest, 4th June. http://www.biosysfoodeng.hu/
3. **Sun, W.**; Bujna, E.; Nguyen, Q.D. (2021). The effect of whey protein and denatured whey

protein on microencapsulation of *Lactobacillus plantarum* 299v by lyophilization. *The 6th Central European Forum for Microbiology [CEFORM]*. Aranyhomok Hotel, Kecskemét, 13th October. http://ceform2021.eventcloud.hu

4. Nguyen, Q.D.; **Sun, W.**; Bujna, E.; Ta, L.P.; Nguyen, T.B.; Rezessy-Szabó, J. (2021). *"The Belt and Road" China-Europe International Academic Forum on Science and Technology Development and the First International Food Nutrition, Health and Flavor Innovation Forum*. Beijing Yulong International Hotel, Beijing, 12th October. https://spxy.btbu.edu.cn/xytz/fc0774537c8a4f48b51b1a098ef4fd77.htm
5. **Sun, W.**; Bujna, E.; Nguyen, Q.D. (2022). Viability of protein microencapsulated *Lactobacillus plantarum* 299v during *in vitro* digestion process. *The 4th Food Conference*. MATE Buda campus, Budapest,10-11th of June. http://www.foodconf.hu/
6. **Sun, W.**; Bujna, E.; Nguyen, Q.D. (2022). Microencapsulation of *Lactobacillus plantarum* 299v with Maillard reaction products of maltodextrin and whey proteins by lyophilization. *The 9th Asia Pacific Probiotics Symposium*. Online, 20th of December. https://apifp2015.wixsite.com/apifp

National conferences:

1. **Sun, W.**; Bujna, E.; Nguyen, Q.D. (2020).The effect of prebiotics on microencapsulation of *Lactobacillus plantarum* 299v by lyophilization. *IV. SZIEntific Meeting for Young Researchers conference*. Szent István University, Budapest,7th December. http://itt.budaicampus.szie.hu/
2. Pham, T.M.; **Sun, W.**; Bujna, E.; Hoschke, Á.; Nguyen, Q.D. (2021). Application of response surface methodology for fermentation optimization of pear by *Saccharomyces cerevisiae*. *Chemical Engineering Day'21*. University of Pannonia, Veszprém, 21st April. https://mkn.uni-pannon.hu/index.php/en/program-2021.html
3. **Sun, W.**; Bujna, E.; Nguyen, Q.D. (2021). The effect of whey protein and denatured whey protein on viability loss of lyophilized probiotic under different storage temperatures. *Lippay János – Ormos Imre – Vas Károly (LOV) Scientific Conference*. Hungarian University of Agriculture and Life Sciences, Budapest, 29th November. https://lov.uni-mate.hu