Probiotic Biofilm: A Novel Approach to Produce a New Generation of Probiotics and Probiotic Foods

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Research

Keywords: Biofilm, Probiotic, Simulated gastrointestinal condition, Yogurt, Lactobacillus rhamnosus, Lactobacillus plantarum

Posted Date: February 19th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-212177/v1>

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Abstract

Background

Probiotic biofilm is considered a new version of an advanced natural protection method recently placed on researchers' agenda as the fourth generation of probiotics. In several studies, probiotic biofilms have been produced exclusively in the bacterial culture medium, but in this study, the biofilm of *Lactobacillus plantarum* PTCC 1745 and *Lactobacillus rhamnosus* PTCC 1637 were formed in food (milk) and evaluated for the first time.

Methods

The biofilm was produced in milk then was transferred to yogurt in whole and crushed forms to be tested in real conditions as probiotic bacteria carriers. Survival was assessed for 21 days as well as gastrointestinal conditions.

Results

Results demonstrate that the number of bacteria in biofilm did not change significantly during 21 days of refrigerated storage. In contrast, planktonic bacteria was decreased by about 2.8 log simultaneously. Another significant result is that the biofilm could appropriately protect the bacteria in the stomach and intestine simulated conditions. So, no significant reduction was observed in the number of bacteria during 120 min, but planktonic cells were destroyed after 30 min.

Conclusions

In conclusion results indicated that *L. plantarum* and *L. rhamnosus* could form a very desirable and strong biofilm in vitro and milk that can have a good protective effect on yogurt's probiotic survival process and storage. Therefore, the probiotic biofilm technique can introduce a new generation of probiotics to the food and pharmaceutical industries

1. Introduction

Probiotic biofilm is considered the fourth generation of probiotics known as the innovative probiotic encapsulation technique, which has been produced since a few years ago (Cheow & Hadinoto, 2013). Predictions suggest that this new method can revolutionize the probiotic industry (Salas-Jara et al., 2016; Speranza et al., 2020). Recently, probiotics production has increased significantly due to the growth of public knowledge about their unique properties such as detoxification, cholesterol decrease, normalization of the microbiome, the antagonistic activity towards pathogens, toxin formation suppression, maintenance of intestinal pH and permeability, and immune system stimulation (Aoudia et

al., 2016; Hu et al., 2019; Watson & Preedy, 2015). In the last few decades, efforts to produce probiotic products have led to the emergence of the first generation of probiotics. At that time, the lyophilized planktonic bacteria were used to produce probiotic dairy products such as yogurt. Later on, decreases in the probiotic population in food processing, storage, and gastrointestinal conditions led to creating the second generation in which the bacteria were coated with natural or synthetic polymers before lyophilization (de Vos et al., 2010; Salas-Jara et al., 2016). Unfortunately, this method failed to solve the digestive system's sensitivity and vulnerability (Burgain et al., 2011). Encapsulation was launched as the third generation of probiotics to solve the problems of the second generation. This generation included the bacteria entrapped by a mechanic or physicochemical processes such as extrusion, emulsification, coacervation, and spray-drying in certain polymeric materials. This generation is based on the packaging of microorganisms in nanometers to millimeters with the probiotic survival improvement goals in the gastrointestinal tract and promoting the controlled release (Burgain et al., 2011; de Vos et al., 2010). Despite the numerous studies conducted on bacterial encapsulation, the problem of survival and reaching the bacteria intact to their target site has not been completely realized. Therefore, many efforts are made to commercialize the fourth generation of probiotics and introduce them to the relevant industries by using the unique properties of biofilm (Salas-Jara et al., 2016). This generation has gone through the stages of evolution on the culture medium and laboratory scale. *L. rhamnosus* and *L. plantarum* are two species of lactic acid bacteria which is few studied for its biofilm formation capacity and probiotic properties (Ramos et al., 2012; M C Leccese Terraf et al., 2012). Nevertheless, most biofilm studies are related to intestine, the resistance profile to environmental stress, the influence of culture composition and surface on the biofilm and exopolysaccharide capacity formation, the growth temperature, and time of maturation (Chen et al., 2017; Coenye et al., 2020; Diriba et al., 2020; Gómez et al., 2016; Grossova et al., 2017; Hu et al., 2019; Jalilsood et al., 2015; Kubota et al., 2008). At the time being, to accelerate the process of commercialization and also to compete with the previous generation, it is vital to use innovative solutions to produce biofilm by an economic approach and by materials other than laboratory culture medium. However, no published research has been found on creating biofilm on food or using probiotic biofilm to produce probiotic products. Therefore, this study aims to achieve the formation of probiotic biofilm in milk through an innovative approach for the first time to improve the survival and vital activity of bacteria in probiotic products inspired by the quorum sensing of bacteria in biofilm mode.

2. Materials And Methods

2.1. Strains

In this study, *L. plantarum* PTCC 1745 and *L. rhamnosus* PTCC 1637, from Persian Type Culture Collection (code: I124), were used as probiotic strains. Lactobacillus strains are regularly grown in Man-Rogosa-Sharpe (MRS) agar medium. It was incubated under static conditions in an anaerobic jar (using Gas-Pack C) at 37°C for 48–72 h.

2.2. Biofilm assay on polystyrene microplates

One milliliter of strains suspension (1.5×10^8 cfu/ml) inoculated with 9 ml of fresh MRS broth culture and dispensed per well in a 24 well microplate then incubated at 30°C for 48 h. After incubation time, the medium was removed from each well, and the plates were washed twice with sterile distilled water to remove planktonic cells attached to biofilm. Then the samples were prepared for imaging (Fig. 4) (Aoudia et al., 2016).

2.3. Biofilm assay on polystyrene jar in food medium

This method was developed by modeling the biofilm production method in an MRS broth medium (Aoudia et al., 2016) and performing several experiments by the authors to produce biofilm in the milk environment. For this purpose, the 6 oz Clear Polystyrene Straight Sided Jar (2.75 diameter × 2.76 height) with polypropylene screw closures were used. 2 ml of strains suspension (1.5×10^8 cfu/ml) inoculated with 18 ml of pasteurized fresh milk containing 3% fat and poured into each container then incubated for 48 h at 30°C. After the incubation time, the excess milk was poured, and each sample was washed twice with sterile distilled water till the planktonic cells were thoroughly removed. Then, the biofilm was used in yogurt preparation (Fig. 1 (a)).

2.4. Probiotic set yogurt production

2.4.1. Probiotic planktonic cell

According to the conventional yogurt production method, bovine milk containing 3% fat was heated up to 92°C for 12 min and cooled until it reached the incubation temperature (42–45°C). The direct starter cultures (Y100, micro milk, Italy, consists of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* spp. *Bulgaricus*) were added to milk (3.6% w/v) according to the manufacturer's instructions. The planktonic cell of *L. plantarum* and *L. rhamnosus* (1.5×10^8 cfu/ml) was inoculated. All samples were incubated at 42°C till the pH reached 4.6. Produced yogurts were stored at refrigeration temperature (4°C) for 21 days (Li et al., 2017; Yangilar & Yildiz, 2018). Figure 1 (b (3)).

2.4.2 Probiotic biofilm

According to the method mentioned in subsection 2.4.1, samples of milk inoculated with a starter were prepared, and then the biofilm (Sect. 2.2) was added to the samples in two whole (Fig. 1 (b(1))) and crushed forms (Fig. 1 (b(2))). All samples were incubated at 42°C till the pH reached 4.6. Yogurts contain biofilms were stored at refrigeration temperature (4°C) for 21 days.

2.5. Enumeration of probiotic cells in planktonic and biofilm form in yogurt

After thoroughly mixing each sample, one milliliter of each sample was diluted with 9 milliliters of peptone water 0.1% (w/v) to prepare serial dilutions. The viable probiotic bacteria were counted using surface culture technique and determined after incubating at 37°C for 72 h, on MRS agar containing 10 mg/l of vancomycin. The identification of *Lactobacillus* strains was based on colony morphology (Li et al., 2017).

2.6. Physical and chemical analysis

2.6.1. Composition Analysis

The standard method was used to determine the dry matter content, protein, ash, moisture, and nitrogen, (Wehr et al., 2004).

2.6.2. Thickness

The biofilm thickness was measured with an accuracy of 0.01 ml to perform mechanical tests in at least ten random points using the indicator clock (Meshkani et al., 2013).

2.6.3. Syneresis and pH

The samples' pH values were measured using a digital pH meter (MARTINI (ml151)). To determine syneresis, 25 g of each yogurt sample was placed on a Whatman No. 1 filter paper in a funnel. After 2 h at 4°C, the amount of extracted whey was measured, and the syneresis was expressed in percentage (Domagała, 2009).

2.7. Sensory Evaluation

A team of 15 experienced panelists was selected to evaluate yogurt samples. Each person was given 40 grams of samples stored in the refrigerator with random codes and asked to use water to rinse their mouths between evaluating both samples. Appearance, texture, taste, and overall acceptance on the 1st, 3rd, 7th, 14th, and 21st days of storage based on a five-point hedonic scale ranging from 1 (dislike extremely) to 5 (like extremely) was evaluated. (Singh & Muthukumarappan, 2008).

2.8. Evaluation of survival and digestion stability in simulated gastrointestinal conditions

The simulated gastric and intestinal methods were prepared according to the study of Clarice Gebara et al. (2013) and Madureira et al. (2011) with some modifications. Simulated gastric juice (SGJ) was obtained using potassium chloride (1.12 g/l), sodium chloride (2.0 g/l), calcium chloride (0.11 g/l), and potassium phosphate monobasic (0.4 g/l) after sterilization at 121 °C for 15 min. Then, Pepsin (0.26 g/l) was added, and the pH was adjusted (~ 2) by adding 1 N HCl. For this purpose, 50 ml of the prepared solution were added to the containers having biofilm. The same amount was mixed with 5 ml of planktonic bacterial suspension containing 1.5×10^8 cfu/ml, and it was mixed thoroughly in the control group. The containers were incubated at 37 °C in an incubator shaker for stirring regulation (90 rpm). The viability of probiotic bacteria in the biofilm and planktonic form was recorded at specific time intervals (0, 30, 60, 90, and 120 min). The simulated intestinal juice was prepared by adding 0.9 gr pancreatin and bile salts to the SGJ solution, and the pH was adjusted (~ 7) by adding 1 N NaOH. The survival of probiotic bacteria in both forms was determined at various intervals (60, 120, 180, and 240 min). (Gebara et al., 2013; Madureira et al., 2011)

2.9. Biofilms microstructure

Biofilm was fixed in 2.5% glutardialdehyde solution in 10 Mm sodium cacodylate buffer for 24 h at 4°C to study the microstructure of biofilm by scanning electron microscope. It was then washed three times for 15 min in 10 mM sodium cacodylate buffer by gentle mixing at room temperature and dehydrated in a graded ethanol series (50, 70, 80, 90, 95, and 100%). The samples were air-dried, placed on SEM stub, coated with gold/palladium by Sputter Coater Device Model SC7620 (England), and investigated by a LEO1450VP scanning electron microscope (Germany) with the resolution of 2.5 nm and the maximum voltage of 35 kV. Images were taken in different magnifications at a voltage of 20 kV (Kubota et al., 2008).

2.10. Statistical Analysis

The experiment was performed according to a completely randomized factorial design with three replications. Analysis of variance (ANOVA) was performed using Minitab software (Minitab Release 19, Minitab Inc., and the USA). The Tukey method was used at a 5% significance level to compare the significant differences in treatment means.

3. Results And Discussion

3.1. Viability of Lactobacillus strain in biofilm and planktonic form during storage time

As shown in Tables 1 and 2, Lactobacillus strain biofilm's survival in whole and crushed form was compared with planktonic.

Table 1
Viability of *L. rhamnosus* (log cfu/ml) during storage for 21 days at 4°C

Treatment	1	3	7	14	21
Planktonic	7.95 ± 0.49 _a	6.9 ± 0.70 _b ^c	5.8 ± 0.50 _c ^b	5.60 ± 0.26 _c ^b	5.15 ± 0.07 _d ^b
Crushed biofilm	8.00 ± 0.56 _a ^a	7.94 ± 0.48 _a ^b	8.17 ± 0.56 _a ^a	8.20 ± 0.94 _a ^a	8.4 ± 0.51 _a ^a
Whole biofilm	8.34 ± 0.16 _a ^a	8.50 ± 0.42 _a ^a	8.52 ± 0.73 _a ^a	9.15 ± 0.21 _a ^a	8.3 ± 0.00 _a ^b

Table 2
Viability of *L. plantarum* (log cfu/ml) during storage for 21 days at 4°C

Treatment	1	3	7	14	21
Planktonic	$7.8 \pm 0.45_a^a$	$5.4 \pm 0.28_b^b$	$5.3 \pm 0.14_b^b$	$5.15 \pm 0.07_b^b$	$4.95 \pm 0.07_c^b$
Crushed biofilm	$7.80 \pm 0.45_a^a$	$8.25 \pm 0.07_a^a$	$8.42 \pm 0.14_a^a$	$8.36 \pm 0.14_a^a$	$8.00 \pm 0.00_a^a$
Whole biofilm	$8.50 \pm 0.28_a^a$	$8.82 \pm 0.46_a^a$	$9.30 \pm 0.57_a^a$	$8.56 \pm 0.79_a^a$	$8.8 \pm 0.14_a^a$

The results show that the biofilm protected the initial number of bacteria, and there is no significant reduction in their population ($p\text{-value} > 0.05$). However, in planktonic mode, it has decreased by an average of 3.5 log. It should be considered that the crushed and whole biofilm was acted with a similar protective effect, and there is no significant difference between them ($p\text{-value} > 0.05$). It indicates that if the biofilm structure is well-formed, each biofilm piece will act like the original complete structure and repair itself. It is of utmost importance to apply this idea in the industry and probiotic products because it allows using probiotic biofilms in crushed form and various formulations in products with uniform texture such as stirred yogurt. However, more studies are required to understand how the repair mechanism and stability work in crushed biofilm pieces.

Examination of the bacterial survival process during storage for three weeks shows that the bacteria in the biofilm conditions, as predicted, reduced their metabolism to basal metabolism and adapted well to the new environment (yogurt) with pH and Eh different from the environment in which they were formed (milk). Although there is no significant increase in the number of bacteria during storage, the biofilm could protect probiotic bacteria well in the new environment, but in similar conditions, a considerable reduction (2.83 log) of planktonic bacteria was observed. Due to the biofilm structure of bacteria (Dufour et al., 2010; Hobley et al., 2015; Hou et al., 2019), they can continue their logarithmic growth and multiplication in the yogurt environment without being affected by the new environment parameters. This good feature can ensure probiotics' survival in the minimum standard value of 10^6 in probiotic products. On the other hand, both bacteria's survival is similar in this respect, indicating a general behavior among the biofilm formation bacteria of *Lactobacillus* species (M C Leccese Terraf et al., 2012). The findings of recent studies on survival with new methods (Table 3) show that the reduction of bacteria was noticeable, and reduction was observed in different conditions from 1 to 7 log. However, biofilm saber-rattling compared to other common techniques (Table 3) can be described as a 3.1 logarithmic increase from the planktonic state, which shows the power of the biofilm (Afzaal et al., 2019; González-Ferrero et al., 2018; Holkem et al., 2017; Huq et al., 2017; Liao et al., 2019; Pop et al., 2017; Sohail et al., 2011). It can potentially revolutionize the probiotic industry since the philosophy of biofilm formation is resistant and increases survival in difficult and new conditions (Hobley et al., 2015; O'Connell et al., 2006; Okuda et al., 2018).

Table 3
Comparison of the capability of some techniques in the survival of probiotics

Strain	Technique	Time		Survival reduction (log cfu/g)(log cfu/ml)		References
		Storage (day)	Gut (min)	Storage	Gut	
<i>L. rhamnosus</i> GG	Encapsulation	-	120	-	1.7	Asma Sohail. et al. (2013)
<i>L. acidophilus</i>			120		1.27	
Cell free of <i>L. rhamnosus</i> GG			20		undetectable levels	
Cell free of <i>L. acidophilus</i>			90		5.45	
<i>Lactobacillus rhamnosus</i> ATCC 9595	Nanocomposite	42	120	1.73	1.45	Huq et al. (2017)
Cell free of <i>L. rhamnosus</i>		42	30	4.36	below the detection level	
<i>Lactobacillus plantarum</i> CECT 220	Microparticle	-	480		1	González- Ferrero et al. (2018)
			480		4	
Cell free of <i>Lactobacillus plantarum</i>			480		1.6	
			480		4	
<i>Lactobacillus casei</i> CECT 475						
Cell free of <i>Lactobacillus casei</i>						
Bifidobacterium BB-12	Micro capsulation	120	180	6.74	0.5	Tasch Holkem et al. (2017)
Cell free			90	7.3	3	
<i>Lactobacillus fermentum</i>	Encapsulation	-	240	-	1	Liao et al. (2018)
Cell-free			240		7.65	
<i>Lactobacillus casei</i>	Encapsulation	-	120	-	1	Pop et al. (2017)
			120		1.5	

Strain	Technique	Time		Survival reduction (log cfu/g)(log cfu/ml)		References
		Storage (day)	Gut (min)	Storage	Gut	
L. acidophilus	Sodium alginate and carrageenan	120	120	1	4.28	Afzaal et al. (2019)
Cell-free				4	8.1	

3.2. Physical and chemical properties of biofilm

3.2.1. Composition analysis

The physical and chemical properties of *L. plantarum* and *L. rhamnosus* biofilms are presented in Table 4. Biofilm of probiotic strains has more protein and polysaccharides and less water than biofilm of pathogenic bacteria, which may be due to the nature of probiotics and their growth medium. (Donlan, 2002; Dufour et al., 2010). According to previous research, more protein and polysaccharides will have a greater protective effect (Limoli et al., 2015; Salas-Jara et al., 2016; Vu et al., 2009). The results show no significant difference (p-value > 0.05) between the two probiotic strains in terms of the number of compounds, and their structure is very similar in terms of constituents.

Table 4
Physicochemical properties of *Lactobacillus* strains biofilm produced in milk

Treatment	Ash (%)	Moisture (%)	Nitrogen (%)	Protein (%)	Dry matter (%)	Polysaccharide	Thickness (mm)
<i>L. rhamnosus</i>	0.31 ± 0.12 ^a	85 ± 0.21 ^a	1.18 ± 0.13 ^a	7.62 ± 0.11 ^a	15 ± 0.13 ^a	7.06 ± 0.15 ^a	0.025 ± 0.27 ^a
<i>L. plantarum</i>	0.26 ± 0.15 ^a	83 ± 0.24 ^a	1.17 ± 0.17 ^a	7.47 ± 0.14 ^a	17 ± 0.16 ^a	9.26 ± 0.17 ^a	0.020 ± 0.31 ^a

3.2.2. Thickness

The results of the present study showed that there is no significant difference in the thickness of biofilm produced by the two strains. However, some previous studies indicated that the power of biofilm

production depends on the type of strain and intraspecific differences in biofilm production have been reported (Aoudia et al., 2016; Ramírez et al., 2015)

3.2.3 Syneresis and pH

The syneresis and pH measurement results in yogurt samples containing planktonic cell, whole biofilm, and crushed biofilm for both probiotic strains of *L. plantarum*, *L. rhamnosus*, and control samples are presented in Tables 5 and 6.

Table 5
Physicochemical characteristics of probiotic yogurt with *L. plantarum*

Analysis	Storage time (days)	Treatment			
		Whole biofilm	Crushed biofilm	Planktonic	Control
pH	1	4.36 _a ^c	4.37 _a ^c	4.43 _b ^b	4.50 _a ^a
	3	4.30 _a ^c	4.35 _a ^c	4.42 _b ^b	4.48 _a ^a
	7	4.27 _b ^c	4.30 _b ^c	4.38 _c ^b	4.42 _b ^a
	14	4.20 _c ^d	4.28 _b ^c	4.4 _b ^b	4.41 _b ^a
	21	4.18 _b ^c	4.20 _b ^c	4.48 _a ^b	4.40 _b ^a
Syneresis (%)	1	24.44 _c ^c	26.76 _c ^{bc}	28.20 _d ^{ab}	30 ^a
	3	30.62 _a ^b	32.40 _a ^{ab}	33.12 _a ^a	35.60 _b ^a
	7	31.02 _a ^b	33.46 _a ^{ab}	33.96 _b ^{ab}	36.42 _a ^a
	14	26.62 _b ^c	28.20 _{bc} ^{bc}	29.68 _d ^b	36.81 _a ^a
	21	26.70 _b ^c	30.98 _{ab} ^b	32.20 _c ^b	37.20 _a ^a
Index letters and Power letters indicate the comparison of the averages in the columns and rows, respectively. ($p \leq 0.05$)					

Table 6
Physicochemical characteristics of probiotic yogurt with *L. rhamnosus*

Analysis	Storage time (days)	Treatment			
		Whole biofilm	Crushed biofilm	Planktonic	Control
pH	1	4.32 _a ^c	4.34 _a ^c	4.41 _a ^b	4.5 _a ^a
	3	4.28 _a ^c	4.32 _a ^c	4.41 _a ^b	4.48 _a ^a
	7	4.20 _{ab} ^c	4.28 _b ^c	4.36 _b ^b	4.42 _b ^a
	14	4.18 _c ^c	4.20 _b ^c	4.38 _b ^b	4.41 _b ^a
	21	4.10 _b ^b	4.16 _b ^b	4.40 _a ^a	4.40 _b ^a
Syneresis (%)	1	22.20 _c ^b	23 _c ^b	28.6 _e ^a	30 _b ^a
	3	20.22 _d ^c	24.86 _{bc} ^{bc}	30.08 _d ^{ab}	35.60 _a ^a
	7	22.04 _c ^c	29.40 _{ab} ^b	31 _c ^b	36.42 _a ^a
	14	23.46 _b ^c	25.04 _{bc} ^c	32.20 _b ^b	36.81 _a ^a
	21	29.7 _a ^b	31.58 _a ^b	35.14 _a ^a	37.20 _a ^a

The pH of yogurt containing whole biofilm and crushed biofilm had a direct relationship with bacteria's survival. According to Tables 5 and 6, yogurt samples containing crushed biofilm and whole biofilm have a lower pH than yogurt samples containing the planktonic form of probiotics. It could be related to biofilm's protective effect on probiotics (Koohestani et al., 2018). Since biofilm can lead to the survival of most probiotics (Kokare et al., 2009), the production of lactic acid is higher, and the final pH of the product is lower compared to yogurt samples containing the planktonic form of probiotics due to the presence of more probiotic's bacterial population. Besides, samples containing the whole biofilm had a lower pH than samples containing crushed biofilm, which may be because the bacterial population is better preserved in the whole biofilm than in crushed biofilm.

Syneresis is defined as the extracted water of yogurt that accumulates on yogurt's surface and is visible. This parameter affects the appearance quality of the yogurt and general product acceptance. In the dairy industry, stabilizers such as pectin and gum are used to reduce or prevent this phenomenon, or the protein content is increased (Lee & Lucey, 2010). In the present study, the percentage of syneresis was low in the yogurt samples containing whole and crushed biofilm compared to the planktonic form due to the biofilm's three-dimensional structure. Leccese et al. (2016) evaluated the biofilm matrix formed by *L.*

rhamnosus CRL 1332, demonstrating that the biofilm matrix contains large amounts of polysaccharides, carbohydrates, and proteins (María Cecilia Leccese Terraf et al., 2016). These natural compounds produced by probiotic bacteria in the biofilm network can play the same role as industrial stabilizers. Due to their hydrophilic groups, they can absorb yogurt water and reduce industrial stabilizers' consumption. The biofilm integrated structure is an important factor in holding water capacity in the biofilm structure (Kokare et al., 2009; Salas-Jara et al., 2016). In Figs. 4 (a, c) and (b, d), the channels in the biofilm structure created by water can have an effective role in maintaining and absorbing water by this structure while providing the nutritional requirements of microorganisms. So, this is another structural advantage of the fourth-generation probiotic compared to its first-generation counterpart.

3.3. Sensory evaluation

The results indicated that yogurt containing biofilm was significantly better in color and odor. The acidity of the product could affect its pleasant odor. On the other hand, the more solids exist in the product, the clearer and whiter the color will be due to light scattering (Walstra et al., 2005). Therefore, the color of the product was whiter due to polysaccharide and protein compounds in biofilm samples.

The texture of yogurt is considered one of the most significant parameters. As shown in Table 7 of the results, yogurt that has biofilm contains internal force with many bonds due to biofilm's special and unique structure. So, in the survey done by consumers in terms of texture and cohesion, it was more acceptable than control samples ($p < 0.05$). On the other hand, extracellular polysaccharides in the biofilm structure have led to a perfect mouth feeling.

The present study results demonstrated that yogurt samples containing biofilm taste significantly better than control samples, and this property was maintained during storage ($p < 0.05$). Since the biofilm matrix contains components such as proteins and polysaccharides of about 6 to 7%, these compounds can undoubtedly affect other tissue properties of the product (Mousavi et al., 2019). Also, since biofilm had a protective effect on probiotics' bacterial population, due to the higher bacterial population and more lactic acid production, the product had a more acidic and more pleasant taste. The samples containing biofilm presented significantly ($p < 0.05$) higher values in all cases compared to control and planktonic samples (without biofilm). This finding indicated that the new yogurt has a high industrial potential since the sensory evaluation showed higher overall acceptability scores while they mentioned no defect. Comparison of the results with other techniques such as nano capsulation and microencapsulation (Afzaal et al., 2019; Iravani et al., 2015; Yao et al., 2020) showed that the use of biofilm did not cause adverse sensory changes but can also be used as a technique to improve the organoleptic properties in yogurt and similar products.

Table 7
Sensory evaluation of probiotic yogurt made by planktonic and biofilm forms of probiotic

Sensory parameter	Treatment	Storage time (days)				
		0	3	7	14	21
Appearance	Whole biofilm. <i>LP</i>	3.00 ± 0.93 _a ^{ab}	2.73 ± 0.70 _a ^b	3.00 ± 0.93 _a ^{ab}	3.53 ± 0.64 _a ^a	3.07 ± 1.10 _a ^{ab}
	Crushed biofilm. <i>L.P</i>	3.00 ± 0.76 _a ^a	2.40 ± 1.06 _{ab} ^a	2.53 ± 1.13 _a ^a	2.40 ± 1.24 _c ^a	2.33 ± 0.98 _b ^a
	<i>L. P</i> (planktonic)	2.73 ± 0.96 _{ab} ^a	2.60 ± 1.06 _{ab} ^a	2.60 ± 1.12 _a ^a	2.47 ± 0.92 _{bc} ^a	2.33 ± 1.05 _b ^a
	Whole biofilm. <i>L.rh.</i>	3.13 ± 0.74 _a ^a	3.07 ± 0.80 _a ^a	3.00 ± 1.07 _a ^a	3.13 ± 0.74 _{ab} ^a	3.40 ± 0.74 _a ^a
	Crushed biofilm <i>L. rh</i>	3.40 ± 0.74 _a ^a	2.93 ± 0.96 _a ^a	2.87 ± 0.74 _a ^a	3.13 ± 1.06 _{ab} ^a	3.20 ± 0.86 _a ^a
	<i>I. rh.</i> (planktonic)	2.27 ± 1.22 _{bc} ^a	2.60 ± 0.91 _{ab} ^a	2.27 ± 1.16 _{ab} ^a	2.87 ± 0.92 _{abc} ^a	2.33 ± 0.62 _b ^a
	Control	1.93 ± 0.03 _c ^a	1.93 ± 1.03 _b ^a	1.73 ± 0.59 _b ^a	1.60 ± 0.51 _d ^a	1.00 ± 0.00 _c ^b
Texture	Whole biofilm <i>LP</i>	3.38 ± 0.73 _a ^a	2.90 ± 1.00 _a ^a	3.00 ± 0.80 _{ab} ^a	3.33 ± 0.60 _a ^a	3.22 ± 0.86 _a ^a
	Crushed biofilm. <i>L.P</i>	2.85 ± 0.90 _a ^a	3.02 ± 0.95 _a ^a	2.25 ± 1.01 _c ^a	2.94 ± 1.02 _a ^a	2.94 ± 0.72 _a ^a
	<i>L. P</i> (planktonic)	2.81 ± 0.84 _a ^a	2.84 ± 1.10 _a ^a	2.61 ± 1.13 _{bc} ^a	3.06 ± 0.85 _a ^a	2.94 ± 0.87 _a ^a
	Whole biofilm <i>L.rh.</i>	3.10 ± 0.81 _a ^a	2.91 ± 1.01 _a ^a	3.34 ± 0.94 _a ^a	3.42 ± 0.76 _a ^a	3.22 ± 0.77 _a ^a
	Crushed biofilm <i>L. rh</i>	3.09 ± 1.07 _a ^a	3.04 ± 1.22 _a ^a	3.21 ± 0.82 _{ab} ^a	3.24 ± 0.81 _a ^a	3.08 ± 0.81 _a ^a
	<i>I. rh.</i> (planktonic)	1.89 ± 1.20 _b ^c	2.29 ± 1.12 _{ab} ^{bc}	2.07 ± 1.10 _c ^{bc}	3.24 ± 0.81 _a ^a	2.74 ± 0.81 _a ^{ab}
	Control	1.86 ± 1.23 _b ^a	1.74 ± 1.05 _b ^a	1.01 ± 0.01 _d ^b	1.01 ± 0.01 _b ^b	1.01 ± 0.01 _b ^b

Sensory parameter	Treatment	Storage time (days)				
		0	3	7	14	21
Taste	Whole biofilm <i>LP</i>	3.33 ± 0.82 _a ^{ab}	3.13 ± 0.83 _a ^b	3.13 ± 0.74 _{ab} ^{ab}	3.20 ± 0.68 _a ^a	3.13 ± 0.84 _a ^{ab}
	Crushed biofilm <i>LP</i>	3.00 ± 0.66 _a ^{ab}	2.80 ± 0.86 _{ab} ^a	2.47 ± 0.99 _{bc} ^{ab}	2.40 ± 0.74 _b ^{ab}	2.07 ± 0.59 _b ^b
	<i>L. P</i> (planktonic)	3.00 ± 1.00 _{ab} ^a	3.07 ± 0.88 _{ab} ^a	2.60 ± 0.83 _{abc} ^a	2.80 ± 0.78 _{ab} ^a	2.60 ± 0.91 _{ab} ^a
	Whole biofilm <i>L.rh.</i>	2.93 ± 0.88 _{ab} ^a	2.87 ± 0.83 _{ab} ^a	3.20 ± 0.94 _a ^a	3.33 ± 0.62 _a ^a	3.07 ± 0.59 _a ^a
	Crushed biofilm <i>L. rh</i>	3.00 ± 1.00 _{ab} ^a	3.07 ± 1.16 _{ab} ^a	3.13 ± 0.83 _{ab} ^a	3.33 ± 0.82 _a ^a	3.00 ± 0.76 _a ^a
	<i>I. rh.</i> (planktonic)	2.07 ± 1.22 _c ^b	2.40 ± 0.83 _{bc} ^{ab}	2.13 ± 1.06 _c ^{ab}	2.93 ± 0.80 _{ab} ^a	2.27 ± 1.16 _b ^{ab}
	Control	2.27 ± 1.22 _{bc} ^a	2.07 ± 1.05 _c ^a	1.47 ± 0.52 _d ^b	1.33 ± 0.49 _c ^b	1.00 ± 0.00 _c ^b
Overall acceptance	Whole biofilm <i>LP</i>	3.40 ± 0.74 _a ^a	2.93 ± 1.03 _a ^a	3.07 ± 0.88 _{ab} ^a	3.40 ± 0.63 _a ^a	3.27 ± 0.88 _a ^a
	Crushed biofilm <i>LP</i>	2.87 ± 0.92 _a ^a	3.00 ± 0.93 _a ^a	2.27 ± 1.03 _c ^a	2.93 ± 1.03 _a ^a	2.93 ± 0.70 _a ^a
	<i>L. P</i> (planktonic)	2.80 ± 0.86 _a ^a	2.87 ± 1.13 _a ^a	2.60 ± 1.12 _{bc} ^a	3.07 ± 0.88 _a ^a	2.93 ± 0.88 _a ^a
	Whole biofilm <i>L.rh.</i>	3.13 ± 0.83 _a ^a	2.93 ± 1.03 _a ^a	3.33 ± 0.98 _a ^a	3.40 ± 0.74 _a ^a	3.20 ± 0.76 _a ^a
	Crushed biofilm <i>L. rh</i>	3.07 ± 1.10 _a ^a	3.00 ± 1.20 _a ^a	3.20 ± 0.86 _{ab} ^a	3.27 ± 0.80 _a ^a	3.07 ± 0.80 _a ^a
	<i>I. rh.</i> (planktonic)	1.87 ± 1.30 _b ^c	2.27 ± 1.10 _{ab} ^{bc}	2.07 ± 1.10 _c ^{bc}	3.27 ± 0.80 _a ^a	2.73 ± 0.80 _a ^{ab}
	Control	1.87 ± 1.25 _b ^a	1.73 ± 1.03 _b ^a	1.00 ± 0.00 _d ^b	1.00 ± 0.00 _b ^b	1.00 ± 0.00 _b ^b

3.4. Survival and digestion stability in simulated gastrointestinal conditions

Two samples were used to evaluate probiotic bacteria survival in the simulated gastrointestinal conditions, namely probiotic biofilms and planktonic cells. Tables 8 and 9 show the viability of *L. rhamnosus* and *L. plantarum* in free cell and biofilm form after 120 min of exposure to the simulated gastric (pH 2.0) and 240 min of exposure to the simulated intestinal juice (pH 7.0), respectively. The free cell viability of *L. rhamnosus* and *L. plantarum* bacteria was decreased to 4.03 and 4 log CFU/ml after 30 min, respectively, and undetectable levels were declined after 60 minutes (Table 8). In the present study, the survivability of *L. rhamnosus* and *L. plantarum* were greatly enhanced by biofilm technique in the high acid condition (pH 2.0) to the extent that only 0.5 and 1.1 log CFU/ml reduction were observed in 120 min and the survival was at 8.10 and 7.70 log CFU/ml, respectively. No other probiotics could survive in any other treatments after 120 min ($p < 0.05$) at that time. After the gastrointestinal digestion assay, it can be concluded that the biofilm technique positively influenced the viability of the probiotic cells.

Table 8

The viability of Lactobacillus strain (log CFU/ml) during the exposure to the simulated gastric condition

Treatment	Time (min)				
	0	30	60	90	120
<i>L. plantarum</i> biofilm	8.60 _a ^a	7.65 ± 0.03 _b ^b	7.70 ± 0.5 _a ^b	7.70 ± 0.14 _b ^b	7.70 ± 0.70 _a ^b
<i>L. plantarum</i> planktonic	8.60 _a ^a	4 _d ^b	0 _b ^c	0 _c ^c	0 _b ^c
<i>L. rhamnosus</i> biofilm	8.60 _a ^a	8.40 _a ^a	8.30 ± 0.30 _a ^a	8.20 ± 0.28 _a ^a	8.10 ± 0.07 _a ^a
<i>L. rhamnosus</i> planktonic	8.60 _a ^a	4.03 _c ^b	0 _b ^c	0 _c ^c	0 _b ^c

As shown in Table 3, even in relatively advanced protection techniques such as nanocomposites and microencapsulation, a 1 to 7 logarithmic reduction is observed in the number of bacteria. A comparison between the results of the new biofilm technique with previous studies of third-generation techniques (encapsulation) is presented in Table 3 and reveals that biofilm, as a unique natural method, has had an amazing performance in increasing the survival of probiotics (Afzaal et al., 2019; González-Ferrero et al., 2018; Holkem et al., 2017; Huq et al., 2017; Liao et al., 2019; Pop et al., 2017; Sohail et al., 2011). The unique feature of this new method is its naturalness, which is an inherent property of bacteria. In the report of Sohail et al. (2013), probiotics encapsulation in alginate gel microbeads could protect probiotics in highly acidic environments but compare to the biofilm technique, and there was a greater reduction in the survival (Sohail et al., 2011). Also, Huq et al. (2017) reported a reduction in the survival rate of the probiotic bacterium *L. rhamnosus* in the alginate-based nanocomposite state when passing through the simulated gastric environment after 120 minutes of 1.45 log (Huq et al., 2017). In this study, free cells

were undetectable after 30 minutes. Compared to the present study results, biofilm efficiency was higher in the survival of *L. rhamnosus*. Other comparative studies are presented in Table 3.

Table 9

Viability of Lactobacillus strain (log CFU/ml) during exposure to the simulated intestine condition

Treatment	Time (min)			
	60	120	180	240
<i>L. plantarum</i> biofilm	$6.30 \pm 0.30_a^a$	6.30_a^a	$6.35 \pm 0.40_a^a$	$6.25 \pm 0.20_a^a$
<i>L. plantarum</i> planktonic	0_b	0_b	0_b	0_b
<i>L. rhamnosus</i> biofilm	$6.55 \pm 0.30_a^a$	$6.50 \pm 0.50_a^a$	$6.60 \pm 0.20_a^a$	$6.50 \pm 0.07_a^a$
<i>L.rhamnosus</i> planktonic	0_b	0_b	0_b	0_b

Survival studies in the simulated intestinal environment demonstrated interesting results. As shown in Table 9, the examined probiotics were decreased slightly during going from the stomach to the intestine. However, they were increased in the following hours of digestion. Finally, after 4 h of incubation, the final reduction germane to the primary cells for *L. rhamnosus* and *L. plantarum* was 0.59 and 1.05 log, respectively. González-Ferrero et al. (2018) stated that the reduction rate of *L. plantarum* and *L. casei* in the encapsulated state after incubation time was 1 and 1.4 log, respectively, which was in line with the results of the present study (González-Ferrero et al., 2018). However, in this study, *L. rhamnosus* biofilm showed a higher survival rate comparing to *L. plantarum*. Numerous studies in this field have suggested that the strength of biofilm formation depends on the strains. (Aoudia et al., 2016; Ramírez et al., 2015) Liao et al. (2019) reported that *L. fermentum* reduced about 1 log in the encapsulated state after 240 min of the incubation time (Liao et al., 2019). Pop et al. (2017) reported the viability of *L. casei*, which was reduced about 1 log after 120 min of the incubation time (Pop et al., 2017).

In comparison with the results of other studies, the current study results show the unique capability of the biofilm technique in maintaining the survival of probiotics. Therefore, these findings demonstrated biofilm's uniqueness for the survival of probiotics both in simulated stomach and intestine conditions and during 21 days of storage. This technique can be an inexpensive and straightforward method compared to third-generation techniques. It is the most efficient technique leading to the survival of these beneficial bacteria.

3.5. Biofilms microstructure

The microstructure of biofilm in MRS broth and milk medium in two different strains (*L. rhamnosus* and *L. plantarum*) was analyzed microscopically. In the image of *L. rhamnosus* biofilm that has been grown in milk, a complex three-dimensional structure and a diffuse extracellular material are observed due to

aggregation of bacterial cells (Fig. 4b). In contrast, in the MRS broth culture medium, the biofilm shows less density (Fig. 4a). Also, the *L. plantarum* form stronger biofilm in milk was compared with MRS broth. (Fig. 4c and d). All of the biofilm structure studies have been done on a culture medium (Irfan et al., 2017; Jones & Versalovic, 2009; Kubota et al., 2009), and so far, the formation of biofilm in food medium has not been studied. So, further studies are required for better comparisons.

4. Conclusion

The present study results show that biofilm can be grown well in food media and used to prepare probiotic products. As predicted, nature-inspired biofilm, as a natural and cost-effective method, can greatly improve probiotics' challenges, including survival and delivery. On the other hand, as it is a creative and straightforward method to produce biofilm, it can be welcomed by researchers and make a change in related industries.

Declarations

Acknowledgements:

The authors express their gratitude. The authors would like to thank the Faculty of Veterinary Medicine at Ferdowsi University of Mashhad for providing raw material and for financial support and providing the facilities that make this project possible.

Author Contributions:

Z.R. A. S and S.Kh. contributed equally to this study. A. S. Created the original idea. Z.R. and S.Kh. expanded the idea. Z.R. carried out the experiments, and A. S and S. Kh directed the project. All authors analyzed and interpreted the data and contributed to the writing of the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflicts of interest.

Funding

None.

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Figures

(a)

(b)

Figure 1

(a). Biofilm formation with milk by probiotic bacteria in PS containers (polystyrene) (b). Yogurt preparation by a biofilm, (1): yogurt with the whole biofilm, (2): yogurt with crushed biofilm, (3): yogurt containing planktonic cells, and (4): conventional yogurt.

Figure 2

Viability of *L. plantarum* in yogurt

Figure 3

Figure 4

Scanning electron microscopy images of biofilm-forming *L. rhamnosus* and *L. plantarum* in MRS agar (a, c) at 2500× and biofilm-forming *L. rhamnosus* and *L. plantarum* in milk (b, d) at 2500×