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Effect of Fatty Acids on Hydrophobicity of the Cell Membrane of Lactobacillus Species

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Abstract

Background and Objectives: Probiotic bacteria are able to absorb fatty acids present in the culture medium and convert them into intracellular fatty acids, which may affect the physicochemical properties of probiotics. Subsequently, changing the composition of cellular fatty acids of probiotics improves the electron acceptance capacity of these microorganisms, and results in an increased adhesion to the intestinal mucus. In the present study, the effect of fatty acids on the physicochemical and adhesion properties of Lactobacillus species was investigated.

Materials and Methods: Seven fatty acids including palmitic, stearic, α-linolenic, γ-linolenic, oleic, linoleic and arachidonic acids were used for the enrichment of MRS medium. Afterwards, fatty acid content and adhesion property were measured using GC and spectrophotometer, respectively.

Results and Conclusion: The results showed that the type of microorganism and fatty acid had a significant effect ($p \leq 0.05$) on the adhesion property of probiotics. According to the results, the highest membrane fatty acid content was found for myristic and elaidic acid, and the lowest content for α-linoleic acid.

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1. Introduction

Probiotics are a mixture of one or more living microbial culture, consumed by human and animals, and their health benefits are a focus of intensive international research [1]. They are living microbial strains that, in normal doses, beneficially affect the host animal consumed by maintaining or improving their intestinal microbial balance [2]. Probiotics' adhesion to the intestines has enormous importance for microbial balance. Adhesion to cell is necessary for colonization [3].

Probiotics affect intestinal microbial flora balance and promote the immune system [4]. Their influence on improving intestinal natural micro flora has been extensively studied. Kankaanpaa et al. showed that polyunsaturated fatty acids (PUFA), in the medium influence on mucus cells. Most of bacteria can take up PUFA present in the culture medium. Marine bacteria also are able to synthesize PUFA [4], and their selection is based on their ability to adhere to the intestines [5].

Adhesive probiotic products (for example, bio-yoghurt containing probiotic bacteria) improve the health in the gastrointestinal tract. Hydrophobicity is one of the most important factors necessary in the adhesion property of probiotics. Most of hydrophobic microorganisms possess more adhesion property than the hydrophilic ones [6]. It is also known that some bacteria absorb PUFAs and others synthesize them [7].

It has been suggested that changes in the composition of probiotics' fatty acids affect the factors related to microbial adhesion through influencing lipopeptide membrane fluidity [4]. So, in the present study, the role of saturated and unsaturated

fatty acids on the physicochemical and adhesion properties of *Lactobacillus rhamnosus* cell membrane and ABY1 starter culture were studied.

2. Materials and Methods

2.1. Selection of probiotics

L. rhamnosus GG (ATCC 53103) was obtained from Razi Vaccine & Serum Research Institute, Iran, and ABY1 starter was purchased from CHR-HANSEN A/S, Denmark. The latter starter, ABY1, is a mixture of *Lactobacillus acidophilus* (LA-S) and Bifidobacterium (BB/2), *Streptococcus thermophilus* and *Lactobacillu s delbrueckii* sub sp. *Bulgaricus.*

2.2. Fatty acids

Oleic acid (18:1 n-9), linoleic acid (18:2 n-6), γ-linoleic acid (18:3 n-6), arachidonic acid (20:4 n-6), α-linolenic acid (18:3 n-3), stearic acid (18:0) and palmitic acid (16:0) were obtained from Sigma-Aldrich, Germany, and nonadecanoic acid was provided by Fluka, Switzerland.

2.3. Growth conditions

The medium culture MRS with Tween 80 was selected because it has shown to have essential growth factor for lactobacilli [8]. Bacteria were cultured in either MRS broth or MRS broth supplemented with PUFA and saturate fatty acid (SFA) at 37°C for 24 h under anaerobic conditions. To supplement the MRS broth, 5 mg ml^{-1} of the fatty acid mixture was added to the medium.
The composition of fatty acids in non-supplemented MRS growth medium was also analyzed [4]. Non-supplemented medium is widely used for lactobacillus studies. The significance of the present results necessitates the application of this medium.

2.4. Preparation of fatty acid extracts from bacterial cells

The bacteria were anaerobically grown in the MRS broth or MRS broth supplemented with PUFA and SFA and mixed. After 24 hours, the bacterial cells were harvested using a centrifuge at 1500×g and 4°C for 7 min and washed twice with phosphate-buffered saline (pH=7). Before analysis, each tube containing 100-500 mg wet cell biomass was capped and stored at 4°C. Bacterial fatty acids were extracted and analyzed by gas chromatography using Anaerl method by Microbial Identification System [9].

2.4.1. Saponification

Saponification process was implemented by addition of 1 ml of basic methanol to each tube (Basic methanol consisted of one part of 3.7 M NaOH in methanol mixed with one part of deionized distilled water). The tubes were vortexed for 5-10s. Afterwards, the samples were boiled in a water bath at 100±2°C for 5 min, cooled down slightly, and boiled again at the same condition.

2.4.2. Methylation

For Methylation of fatty acids (as sodium salts), 2 ml of methylation reagent (6.0 M HCl in Methanol [13:11, v v^{-1}]) was added to each tube. The tubes were then capped tightly and the samples were vortexed for 5-10s, heated in a water bath at 80±2°C for 10±1 min, and cooled down rapidly to the ambient temperature under tap water.

2.4.3. Extraction of methylation esters

Fatty acid methyl esters were transferred from acidic aqueous phase to organic phase by a liquid-liquid extraction method. An aliquot, 1.25 ml of the extraction solvent (hexane methyl-butyl ether; 1:1, v v^{-1}) was added to each tube. The tubes were sealed and mixed end-over-end for 10 min. The upper phase (organic) was collected. The residual free fatty acids and reagents were removed by adding 3.0 ml of 0.3 M NaOH. The firmly capped tubes were mixed end-over-end for 5 min and centrifuged (3 min at 1000 ×g) in order to clarify the interface. The upper phase (solvent) was removed and stored for GC analysis.

2.5. Gas chromatography analysis

The solvent was evaporated and the residue was dissolved in 0.5 ml of hexane and then analyzed twice by an HP 6890 auto system gas chromatograph (Agilent technologies, USA) equipped with a programmed split/split less injector and flame ionization detector controlled by Turbochrom Navigator 4. For analysis, silica capillary column (30m, 0.32 mm i.e., 0.25 µm film thickness) was used. The injection volume was 1 µl, and after 1 min, a split valve was opened. After the valve was opened (split ratio of 5:100), the flow rate of carrier gas (nitrogen) was programmed as follows: 140°C for 0.5 min, increased to 230°C at a rate of 10°C min^{-1}, and held for 7 min. The injector temperature increased from 170°C to 250°C at a rate of 20°C min^{-1}. The temperatures of detector and inlet were 250°C and 240°C, respectively. Run time was 15 min, and the peaks were identified through comparison of their retention times with those of a known internal standard mixture.

2.6. Hydrophobicity properties

Bacteria were grown in standard MRS broth or in MRS broth supplemented with free PUFA 20 µg ml^{-1}. Microbial adhesion to solvents (MATS) was studied by comparison of bacterial cell affinities for polar and non-polar solvents. A modified method (described previously) was used [10]. A pair of solvents, ethyl acetate (polar) and octane (non-polar), was used. The former is a strong electron donor. The non-polar solvent was used to estimate

the hydrophobicity properties of the lactobacilli, whereas the polar solvent was selected for estimation of the Lewis acid/base (i.e. electron donor/acceptor). We measured the affinity by comparing the polar and non polarphases. The bacteria were harvested by centrifugation at 252 ×g, 4°C for 7 min, washed twice and suspended in 0.15 M NaCl. Initially, the turbidity of microbial suspension at 600 nm was adjusted, and 1 ml sample was taken (sample A_0). An aliquot, 2.4 ml, of microbial solution was vortexed for 1 min with 0.4 ml of the solvent, and the mixture was left for 15 min to separate two phases. Another 1 ml sample was taken from the aqueous phase (sample A). The turbidity of both samples was determined at 400 nm. Bacterial cells in each solvent were expressed as percentage using the equation Eq. 1:

$$\text{Affinity } (\%) = 100 \times [1-(A/A_0)] \quad \text{Eq 1.}$$

To estimate the basic and acidic characteristics of lactobacilli, the ratio of the specific solvent pair (i.e. ethyl acetate/octane) was calculated, plotted and statistically assessed.

2.7. Statistical analysis

The results were the average of three experiments and expressed as mean±SD. The analysis of variance (ANOVA) was used to calculate the difference by using SPSS software (ver. 16). The results were compared at the significance level of 0.05.

3. Results and Discussion

As shown in Tables 1 and 2, the effects of bacterial species, culture medium and their interaction on the amount of myristic acid on *L. rhamnosus* and starter bacteria walls were significant ($p \leq 0.05$). The highest amount of myristic acid was observed for the starter in the mixed culture medium (0.111 mg g^{-1} bacteria), and the lowest significant amount was found for *L. rhamnosus* in the mixed medium (0.026 mg g^{-1} bacteria).

The results showed that the effects of bacterial species, culture medium and their interaction on the amount of myristoleic acid on *L. rhamnosus* and starter bacteria walls were significant ($p \leq 0.05$).

The highest amount of myristoleic acid was found for starter *L. rhamnosus* in the saturated medium (0.560 mg g^{-1} bacteria), and the lowest significant ($p \leq 0.05$) amount was observed for *L. rhamnosus* in the mixed medium (0.017 mg g^{-1} bacteria). The results revealed that bacterial species and culture medium had significant effect on the amount of palmitic acid on *L. rhamnosus* and starter bacterial cell walls ($p \leq 0.05$); however, their interaction was not significant $p > 0.05$. The highest amount of palmitic acid was found for starter *L. rhamnosus* in the mixed culture medium (0.0501), and the lowest amount was for the starter in the unsaturated medium (0.0310 mg g^{-1} bacteria). The results suggested that bacterial species, culture medium and their interaction had insignificant effect on the variations of palmitoleic and stearic acids on the cell walls of *L. rhamnosus* and starter bacteria $p > 0.05$. The highest amount of palmitoleic acid was observed for *L. rhamnosus* in the mixed culture medium (0.075 mg g^{-1} bacteria), and the lowest amount ($p > 0.05$) was for the starter microorganisms in the mixed medium (0.045 mg g^{-1} bacteria). The highest amount of stearic acid was found for the starter in the mixed culture medium (0.03), and the lowest amount was for *L. rhamnosus* in the saturated medium (0.017 mg g^{-1} bacteria).

It was revealed that the effects of bacterial species, culture medium and their interaction on oleic acid content on *L. rhamnosus* and starter cell walls were significant ($p \leq 0.05$). Starter microorganism in the mixed culture medium showed the highest amount ($p \leq 0.05$) of oleic acid (0.571 mg g^{-1} bacteria), and the lowest amount was found for *L. rhamnosus* in the simple medium (0.014 mg g^{-1} bacteria). The data given in Table 2 indicate that the effects of bacterial species, culture medium and their interaction on the amount of elaidic acid on the cell wall of *L. rhamnosus* and starter bacteria were not significant.

The highest fatty acid content was observed for *L. rhamnosus* in the saturated medium (0.120 mg g^{-1} bacteria), and the lowest amount ($p \leq 0.05$) was found for *L. rhamnosus* in the simple culture medium (0.015 mg g^{-1} bacteria). The variations of elaidic acid showed no significant difference ($p > 0.05$) between *L. rhamnosus* and starter in different culture media. The analysis of the results revealed that the effect of none of bacterial species and culture medium and their interaction on the amount of linoleic acid absorbed onto the walls of *L. rhamnosus* and starter bacteria was significant ($p \leq 0.05$).

The highest amount ($p \leq 0.05$) of linoleic acid was found for starter microorganism in the simple medium (0.019 mg g^{-1} bacteria), and the lowest was for *L. rhamnosus* in saturated medium (0.002 mg g^{-1} bacteria). In addition, the effects of bacterial species and culture medium and their interaction on the amount of γ-linoleic acid absorbed onto the walls of *L. rhamnosus* and starter bacteria were not significant ($p \leq 0.05$). The amount of γ -linolenic acid showed no significant difference among the tested treatments ($p > 0.05$) .

The highest amount ($p \leq 0.05$) of γ-linoleic acid was observed for *L. rhamnosus* in the simple culture medium (0.034 mg g^{-1} bacteria), and the lowest was for starter microorganism in the saturated medium (0.014 mg g^{-1} bacteria). The bacterial species and culture medium had no significant effect on the amount of α-linoleic acid absorbed onto the walls of *L. rhamnosus* and starter bacteria ($p \leq 0.05$); however, their interactive effect was significant.

Table 1. Amount of fatty acids (%) in membrane of *L. rhamnosus* and starter.

Tests	*L. rhamnosus*			
	Simple[1]	Unsaturarted[2]	Saturated[3]	Mix[4]
Myristic acid 14:0	0.046±0.001^{c}	0.038±0.002^{b}	0.064±0.004^{e}	0.026±0.004^{a}
Myristoleic acid 14:1	0.021±0.0028^{a}	0.022±0.001^{a}	0.056±0.002^{c}	0.017±0.004^{a}
Palmitic acid 16:0	0.039±0.003^{bc}	0.032±0.006^{a}	0.041±0.006^{c}	0.050±0.007^{d}
Palmitoleic acid 16:1	0.060± 0.002^{a}	0.045± 0.004^{a}	0.070±0.081^{a}	0.075±0.002^{a}
Stearic acid 18:0	0.020±0.006^{ab}	0.018±0.006^{ab}	0.017±0.004^{ab}	0.014±0.004^{a}
Cis oleic acid 18:1	0.014±0.004^{a}	0.034±0.025^{c}	0.015±0.006^{ab}	0.020±0.004^{abc}
Trance Elaidic acid 18:1	0.015±0.000^{a}	0.075±0.086^{a}	0.120±0.138^{a}	0.023±0.000^{a}
Linoleic acid 18:2	0.003±0.001^{ab}	0.004±0.000^{abc}	0.002±0.000^{a}	0.006±0.001^{bc}
γ-linolenic acid 18:3	0.034± 0.041^{a}	0.015±0.004^{a}	0.017± 0.003^{a}	0.016±0.004^{a}
α -linolenic acid 18:3	0.002±0.000^{abc}	0.002±0.000^{a}	0.003±0.000^{bc}	0.002±0.000^{ab}
Arachidonicacid 20:4	0.005± 0.000^{ab}	0.005±0.001^{ab}	0.013±0.007^{bc}	0.027±0.013^{d}
Sum	0.263±0.065	0.293±0.140	0.420±0.257	0.280±0.045
Percent	9.82	10.92	15.69	10.43

1. MRS without fatty acid
2. MRS with saturated fatty acids (palmetic acid and stearic acid)
3. MRS with polyunsaturated fatty acids (oleic acid, linoleic acid, α-linolenic acid, γ- linolenic acid and arachidonic acid)
4. MRS with saturated fatty acids and polyunsaturated fatty acids (palmetic acid, stearic acid, oleic acid, linoleic acid, α-linolenic acid, γ- linolenic acid, and arachidonic acid)
5. Ratio of bacteria in polar solvent to non polar solvent
6. Amount of bacteria base on mgr in per gram of bacteria
7. ABY1 starter is a mix of *lactobacillus acidophilus* LA-5, and *bifidobacterium* BB/12 and *stereptoccocus thermiphillus* and *lactobacillus delberocii sub.bulgaricus*
8. Different letters ($^{a\text{-}e}$) denote significant differences within the columns ($p \leq 0.05$).

Table 1. continued

Tests	Starter			
	Simple[1]	Unsaturated[2]	Saturated[3]	Mix[4]
Myristic acid 14:0	0.076±0.001^{e}	0.035±0.002^{b}	0.093±0.001^{f}	0.111±0.002^{h}
Myristoleic acid 14:1	0.022± 0.004^{a}	0.0190± 0.001^{a}	0.039±0.001^{b}	0.021±0.002^{a}
Palmitic acid 16:0	0.033±0.002^{ab}	0.031±0.005^{a}	0.035±0.006^{abc}	0.042±0.004^{a}
Palmitoleic acid 16:1	0.067±0.001^{a}	0.058±0.0014^{a}	0.063±0.002^{a}	0.045±0.002^{a}
Stearic acid 18:0	0.021±0.011^{ab}	0.017± 0.014^{ab}	0.020±0.011^{ab}	0.031±0.010^{a}
Cis oleic acid 18:1	0.048± 0.007^{d}	0.030± 0.006^{bc}	0.025±0.003^{abc}	0.057±0.005^{d}
Trance Elaidic acid 18:1	0.025±0.004^{a}	0.081±0.001^{a}	0.081±0.004^{a}	0.030± 0.004^{a}
Linoleic acid 18:2	0.019±0.005^{e}	0.018±0.004^{e}	0.013±0.001^{d}	0.007±0.002^{c}
γ-linolenic acid 18:3	0.016 ±0.001^{a}	0.019±0.005^{a}	0.014±0.002^{a}	0.014±0.003^{a}
α -linolenic acid 18:3	0.002±0.000^{ab}	0.003±0.000^{c}	0.002±0.000^{abc}	0.0026±0.000^{abc}
Arachidonic acid 20:4	0.006±0.002^{ab}	0.002±0.0021^{a}	0.015±0.008^{c}	0.005±0.001^{ab}
Sum	0.337±0.042	0.315±0.044	0.403±0.043	0.368±0.040
Percent	12.56	11.76	15.05	13.71

Table 2. Significant probability (p-values) of the impact of independent variables on the amount of fatty acids (%) in the membrane of *L. rhamnosus* and starter.

Tests	p-value		
	Bacteria type	Medium type	Bacteria type*Medium type
Myristic acid 14:0	0.000*	0.000*	0.000*
Myristoleic acid 14:1	0.031*	0.000*	0.004*
Palmitic acid 16:0	0.006*	0.000*	0.568
Palmitoleic acid 16:1	0.773	0.891	0.734
Stearic acid 18:0	0.111	0.680	0.166
Cis oleic acid 18:1	0.000*	0.005*	0.000*
Trance Elaidic acid 18:1	0.884	0.219	0.920
Linoleic acid 18:2	0.000*	0.001*	0.000*
γ-linolenic acid 18:3	0.354	0.462	0.428
α -linolenic acid 18:3	0.439	0.171	0.012*
Arachidonic acid 20:4	0.012*	0.000*	0.001*

* Significant level was adopted on $p \leq 0.05$.

Table 3. Effect of various media on the bacterial cell surface properties (membrane polarity).

Bacteria type	Solution	Octane Mean± SE	Ethylacetate Mean± SE	Mean difference Mean± SE
L	Simple	0.186±0.071	0.105±0.015	0.081±0.07^{ns}
L	Unsaturate	0.468±0.063	0.188±0.015	0.28±0.05**
L	Saturate	0.258±0.071	0.174±0.012	0.08±0.08^{ns}
L	Mix	0.231±0.069	0.091±0.027	0.14±0.09^{ns}
S	Simple	0.535±0.149	0.094±0.047	0.44±0.16*
S	Unsaturate	0.170±0.012	0.020±0.004	0.15±0.12^{ns}
S	Saturate	0.453±0.008	0.119±0.017	0.33±0.01**
S	Mix	0.209±0.017	0.335±0.018	-0.13±0.01**

ns = non significant, *=significant in 0.05 level, **=significant at 0.01 level
L= *L. rhamnosus*, *S*=ABY1 starter culture

Table 3 shows the membrane polarity of *L. rhamnosus* and starter bacteria using octane (non-polar) and ethyl acetate (polar) as solvents. For simple culture medium with *L. rhamnosus*, octane resulted in higher (0.186) and ethyl acetate resulted in lower (0.105) polarity, showing no significant difference $p>0.05$. For unsaturated medium with *L. rhamnosus*, octane caused higher (0.468) and ethyl acetate caused lower ($p\leq0.05$) (0.188) polarity. For unsaturated medium using *L. rhamnosus*, octane caused higher (0.468) and ethyl acetate caused lower (0.258) polarity, indicating significant difference ($p\leq0.05$). Mixed medium using *L. rhamnosus*, showed higher amount of polarity (0.231) with octane and lower polarity (0.091) with ethyl acetate. For simple medium using starter microorganisms, octane resulted in higher (0.535) and ethyl acetate gave lower (0.93) polarity, showing a significant difference ($p\leq0.05$). The saturated medium using starter bacteria had higher 0.170) polarity with octane and lower (0.020) with ethyl acetate. In the unsaturated medium using starter bacteria, octane resulted in higher (0.453) and ethyl acetate resulted in lower (0.119) polarity, showing significant difference ($p\leq0.05$). Mixed medium using starter bacteria had higher (0.209) polarity with octan and lower (0.335) with ethyl acetate, being significantly different ($p\leq0.05$). For comparison of octane in different culture media, the highest polarity was found for the starter (0.535) in the simple medium, and the lowest was for the starter in the saturated medium (0.170), showing a significant difference ($p\leq0.05$).

One of the main criteria for selection of potential probiotics is the capability of microorganisms to adhere to the intestines [11]. Adhesion to the intestinal mucosa is considered an important characteristic for colonization through preventing washout [7], especially in the small intestine where flow rates are relatively high [12]. It is also suggested that mucosa enhances the ability to stimulate the immune system [5,13]. O'Halloran et al. found a direct relation between the ability of probiotic strains adhering to the intestines and the serum antibody titers in the patients treated with probiotic bacteria [13]. It has been also observed that the adhesion of Lactobacilli to damaged mucosa stimulates healing of the tissue [14,15].

In this study, *L. rhamnosus* and starter bacteria both were cultured in simple and enriched media with saturated and unsaturated fatty acids and their mixed medium; then the amount of fatty acids in the bacterial cell walls was measured. According to the results, the highest amount was found for elaidic acid (9*t*-18:1) by *L. rhamnosus* grown in the medium enriched with saturated fatty acids.

It has been previously reported that the highest amount of fatty acids was observed for 18:1 without its isomers such as oleic and vaccenic acids [16-19]. High availability of oleic acid (e.g. Twin 80 in MRS) led to an increased level of dihydrosterculic acid [20]. The results of the present study showed that the total amount of fatty acids in the cell wall of *L. rhamnosus* grown in saturated culture medium was higher than in other samples (0.42). These variations are due to the difference between the microorganisms and the composition of enriched media. The tested bacterial strains could adhere to different intestinal surfaces, and the enrichment of the cultures with free PUFA could influence the adhesion property of the bacteria [4,21]. Furthermore, the previous research showed that all Lactobacilli grown in the medium enriched with free PUFA could properly absorb the fatty acids into their cell wall. Additionally, PUFA-dependent differences were observed in other cellular fatty acids (including SFA, mono-unsaturated fatty acids and PUFA), indicating that fatty acid conversion reaction [19,20] could balance the applied bacterial stress [6].

Probiotic bacteria possess hydrophobic properties. Table 1 shows the membrane polarity by octane and ethyl acetate as solvents. According to the results, the highest membrane polarity and adhesion to the intestinal membrane was observed for the starter grown in the simple medium and measured by non polarsolvent octane. The reason may be the type of organism and the composition of fatty acid in the bacterial cell wall.

Changes in the composition of probiotic fatty acids might be among the factors affecting the adhesion, and membrane fluidity, as well as lipopep-

tid interferences. Being consistent with the results obtained by other researched, hydrophobicity decreases in unsaturated medium [7]. Furthermore, Lactobacilli showed more affinity for non polar solvents such as octane than for polar ones like ethyl acetate.

4. Conclusions

The results showed that enrichment of probiotics culture medium with fatty acids (saturated and unsaturated) had significant ($p \leq 0.05$) effect on the probiotics' adhesion to the intestinal wall.

Fatty acids in culture medium could be absorbed into the probiotics cell wall and increase the microorganisms' adhesion to the intestinal wall. It is suggested that fatty acids are more effective when they are assimilated. The results further revealed that the type of probiotic microorganism and culture medium had significant ($p \leq 0.05$) effect on fatty acids adsorption into the bacterial cell wall.

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6. Conflict of interest

The authors declare that there is no conflict of interest.

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