Screening of Potential Probiotic Properties of Lactobacillus and Lactococcus strains

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Abstract

The lactic acid bacteria are known to be probiotic and their important role has been known in making the biotechnological products and improving human health. Effective probiotic should be viable, safe, bile and gastric juices tolerant, able to survive throughout the human gastrointestinal tract and to colonize a specific human tract. We aimed to investigate and compare the probiotic potential of the Lactobacillus helveticus ATCC 15009 (L. helveticus), Lactobacillus plantarum ATCC 14917 (L. plantarum), Lactobacillus delbrueckii subsp. bulgaricus ATCC 11842 (L. bulgaricus), Lactococcus lactis IL1403 (L. lactis IL1403) and Lactococcus lactis IL1403 bearing pSIP plasmid (L. lactis IL403 pSIP) in different aspects that include their ability to tolerate acidic conditions, gastric acid and intestinal juice. We also evaluated their hydrophobicity and antibiotic sensitivity.

The Lactobacillus and Lactococcus species were grown at 37 ˚C and 30˚C respectively in different pH, simulated gastric acid and intestinal juice. Four of the bacteria displayed good probiotic features in low pH. Even though L. lactis IL1403 did not survive in L. pH 4.0. Among the tested organisms, cell surface hydrophobicity of L. helveticus was recorded as 79.80±0.008 at Xylene as a highest value. We also found that all bacterial strain could attain to the large intestinal area after 25 hours and are sevsitive to rifampin, chloramphenicol, gentamycin, penicillin and neomycin. Our results suggest that these strains can confer good probiotic but they need to use considering their specific features in accord with therapeutic and biotechnological purposes.

Keywords: Probiotic, *Lactobacillus, Lactococcus*, Low pH, Cell surface hydrophobicity, Antibiotic

Introduction

Probiotic was used as the term of "for life" historically, but it means to use of viable bacterial supplements currently. Probiotics are generally utilized in the nourishments and their ingredients exhibit beneficial effects on the health of the consumer[11,33].

In 2015, probiotics reached USD 33.19 billion marketing size, it reached to USD 46.55 billion today and it is estimated that its values will reach USD 64.02 billion by 2022[1]. The expansion of the market size relies upon the increase of global health awareness from consuming probiotics[1].

The lactic acid bacteria (LAB) (such as *Lactobacillus fermentum, Lactobacillus plantarum, Lactococcus lactics*) are gram positive, facultative anaerobic, catalase-negative and motile[33]. They are most known to be probiotic and their important role has been known both in making the biotechnological products such as cheese, yogurt, bread. The LAB bacteria have also important role on the food protection through the production of antimicrobial agents such as lactic acid, diacetyl, hydrogen peroxide and bacteriocins, which have potential role to keep the foods as food preservatives identified in dairy starter cultures[19]. In addition, there are important beneficial properties of probiotic bacteria to human health including improving gut microbiota balance and fighting off pathogenic bacteria, stimulating the immune system, decreasing the blood cholesterol concentrations, production of vitamins (especially vitamin B group) and anticancer and antimicrobial activity[24]. They are also used as therapeutic bacteria to handle cancer due to some limitation of traditional cancer treatments[10,14,27] and other diseases[3,15,20].

It is suggested to consume 100g/d of *Lactobacillus acidophilus, Bifidobacterium animalis ssp. lactis, Lactobacillus casei* and 6 to 7 log cfu/g or log cfu/mL of other probiotic bacteria per day[26]. Aging and using antibiotics are two fundamental factors that diminish the quantity of probiotics drastically. An efficient probiotic should be viable, safe, bile and gastric juices tolerant, able to survive throughout the human gastrointestinal tract and to colonize a specific human tract[33].

So, investigating the probiotic potential of the organism will improve our knowledge to assist promoting human health, food content and keep up the characteristic parity of intestinal microflora during anti-microbial medications. In our research, we determined the probiotic properties of *Lactobacillus helveticus* ATCC 15009 *(L. helveticus), Lactobacillus plantarum* ATCC 14917 *(L. plantarum), Lactobacillus delbrueckii subsp. bulgaricus* ATCC 11842 *(L. bulgaricus), Lactococcus lactis IL1403 (L. lactis IL1403), Lactococcus lactis IL1403 bearing* pSIP *plasmid (L. lactis IL403 pSIP).*

Material and Methods

Three species of LAB *[Lactobacillus helveticus* ATCC 15009 *(L. helveticus), Lactobacillus plantarum* ATCC 14917 *(L. plantarum), Lactobacillus delbrueckii subsp. bulgaricus* ATCC 11842 *(L. bulgaricus)]* were obtained

from Departments of Food Engineering at the Inonu University. *L. lactis IL1403* and *L. lactis IL1403* pSIP were requested from Departments of Biology at Ankara University and Departments of Chemistry, Biotechnology and Food Science, at Norwegian University respectively. M17 culture medium (sigma), MRS culture medium, Agar (sigma). Pepsin (sigma), trypsin (sigma), antibiotic discs (sigma) were used in our experiments. The others used chemicals were of analytical grade.

Survival of Bacterial Strains in Low pH: *Lactobacillus* species were grown in 10 ml sterile MRS broth (1% peptone, 1% meat extract, 0.5% yeast extract, 2% glucose, 0.2% K_2HPO_4, 0.5% sodium acetate, 0.2% tri-ammonium citrate, 0.02% $MgSO_4 \cdot 7H_2O$, 0.005% $MnSO_4 \cdot 4H_2O$, pH 6.3) with shaking at 110 rpm at 37 °C overnight[18,32].

The *L. lactis IL 1403* was grown according to study of Karlskas et al[16] in 10 ml sterile M17 culture medium (pH 6.9) including 0.5% glucose concentration without shaking at 30°C during overnight. The *L. lactis IL 1403* pSIP was cultivated in 10 ml sterile M17 culture medium (pH 6.9) including 0.5% glucose concentration and erythromycin (10 µg/ml) antibiotics without shaking at 30°C during overnight.

Two milliliters of the all bacterial species were centrifuged at 14000 rpm at +4°C for 2 minutes. The pellets were added in MRS broth fresh medium (pH 6.3, pH 4.0, pH 3.0, pH 2.0) and M17 broth fresh medium (pH 6.9, pH 4.0, pH 3.0, pH 2.0) and M17 broth fresh medium with erythromycin (pH 6.9, pH 4.0, pH 3.0, pH 2.0) respectively. Bacterial survival rate was examined at different pH ranging from 6.9 to 2. Their growing absorbance values were recorded versus time. Growth was measured as OD value. Cell growth rates were presented as growth curves with standard deviation. All experiments were conducted in triplicate.

Tolerance to Simulated Gastric Acid Juice: The bacterial species were examined in simulated gastric juice with minor modifications[13]. We prepared fresh stimulated gastric juice including 3 g/l pepsin in 1^x PBS buffer at pH 2.0 and the solution was sterilized by 0.20 nm filter. All bacterial strains were cultivated overnight under the optimum conditions as mentioned previously. 2 ml of each bacterial culture was centrifuged at 14000 rpm at +4°C for 2 minutes. The pellets were added into 10 ml of 1^x PBS pH 2 and serial dilutions in sterile saline (0.9%) were prepared. They were held in optimum condition for 5 hours and 100 µl the bacterial samples were inoculated by MRS and M17 agar for growth overnight. Bacterial colonies were counted and their survival percentage in the artificial gastric juice after 5 hours was calculated by the following formula:

Survival rate % = ((N0- N1) / N0) x100

where (N1 = Total bacterial number after 5 hours in stimulated gastric juice and N0= Total bacterial number at first time in stimulated gastric juice.

Tolerance to Simulated Intestinal Juice: To investigate the tolerance to intestinal juice, fresh artificial intestinal juice including 1 g/l trypsin in 1x PBS buffer at pH 8.0 was prepared and sterilized by 0.20 nm filter.[23] The bacterial samples in simulated gastric acid juice were collected via centrifugation at 4000 rpm at +4°C for 5 minutes. The supernatant was removed and the pellet was dissolved in 9 ml fresh simulated intestinal juice at pH 8.0. All bacterial samples were kept at optimum conditions for 24 hours. After that, specimens were inoculated with the suitable agar. Viability in the artificial intestinal juice was counted at 24 h on MRS and M17 agar. The survival rate was calculated in the same manner as for the determination of the gastric acid resistance.

Antibiotic Sensitivity of Bacterial Species: The antibiotic sensitivity was evaluated by disc diffusion method[6] by using kanamycin (K; 30 mcg), gentamycin (CN; 30 mcg), chloramphenicol (C; 30 mcg), penicillin (P; 2 unit), erythromycin (E; 15 mcg), rifampin (RA; 5 mcg), neomycin (N; 30 mcg) and vancomycin (VA; 30 mcg) antibiotic discs. Fresh agar (1.5%) culture medium was prepared and 100 µl of the each fresh bacterial cultures was spread on the agar surface. Each antibiotic disc was replaced on the agar surface. The inhibition zone widths were measured after 24 hours of incubation at 37°C for *Lactobacillus* and 30°C for *Lactococcus*. The experiment was performed in triplicate.

Hydrophobicity of Bacterial Species: The bacterial cell surface hydrophobicity was investigated in accordance with previously published data[8]. Bacteria were cultured overnight and 3 ml of each culture was divided to different sterile falcon tubes. Falcon tubes were centrifuged at 4000 rpm at +4°C for 10 minutes. The supernatant was removed and 10 ml of phosphate urea magnesium sulfate buffer at pH 6.5 was added. The pellet was suspended and the centrifugation step was repeated 3 times. Initial cell absorbance value was determined using spectrophotometer at 450 nm. 0.6 ml of n-Hexane, n-Hexadecane and Xylene were added on the bacterial suspension slowly. The mixed solution was put into the water bath at 37 °C for 15 minutes within vortexing per 2 minutes. Then keep at room temperature for 25 minutes. The absorbance value of aqueous phase was determined via spectrophotometer at 450 nm. Their results were recorded and percent hydrophobicity was calculated by the using following formula:

Hydrophobicity % = ((OD_{450nm} N0-OD_{450nm} N1) / OD_{450nm} N0) x100

where OD_{450nm} N1= is the absorbance value for late bacteria concentration after applying chemicals and OD_{450nm} N0= is the absorbance value for initial bacteria concentration before applying chemicals.

Results

Survivals of bacteria in low pH: As observed in *L. bulgaricus, L. helveticus* represents a similar trend in terms

of growing in low pH. Its OD value was not affected by pH 6.3 and it grew slowly at pH 4.0, however, it did not struggle with pH 3.0 and pH 2.0 (Figure 2).

Similar tolerance features were monitored in *L. plantarum* at low pH. Even though its viability was not affected by pH 6.3 and pH 4.0, it could not resist pH 2.0 and pH.3.0. When its OD value was compared with the other strains at pH 4.0, it shows an increased survival rate (Figure 3).

The figure 4 showed that *L. lactis IL 1403* grew successfully at pH 6.9. However, contrary to the *Lactobacillus helveticus* ATCC 15009 *(L. helveticus)*, *Lactobacillus plantarum* ATCC 14917 *(L. plantarum)*, *Lactobacillus delbrueckii subsp. bulgaricus* ATCC 11842 *(L. bulgaricus)* and *L. lactis IL1403* pSIP, it indicated a lower survival rate measured as OD at pH 4.0 and could not persist at pH 3.0 and pH 2.0.

L. lactis IL 1403 strain included *pSIP* plasmid growth rates higher at pH 4.0 than *L. lactis IL 1403* at the same pH. Like the other species, its OD values at pH 6.9 were elevated in comparison with the pH 2.0 and pH 3.0. There was no viability at the extreme acidic condition (Figure 5).

Survivals of Bacteria in Simulated Gastric Acid and Intestinal Juice: The survival rate of *Lactobacillus helveticus* ATCC 15009 *(L. helveticus)*, *Lactobacillus plantarum* ATCC 14917 *(L. plantarum)*, *Lactobacillus delbrueckii subsp. bulgaricus* ATCC 11842 *(L. bulgaricus)*, *Lactococcus lactis IL1403 (L. lactis IL1403)*, *Lactococcus lactis IL1403 bearing* pSIP *plasmid (L. lactis IL403 pSIP)* with artificial gastric acid and intestinal juice is demonstrated in table 1. Our results show that all the evaluated bacteria could tolerate gastric acid juice and can reach the large intestinal area after 25 hours when initial number of the them is over 10^9. *L. plantarum* shows the reduced survival with 99.99996% death rate compared with the other investigated strains in simulated gastric juice after 5 hours.

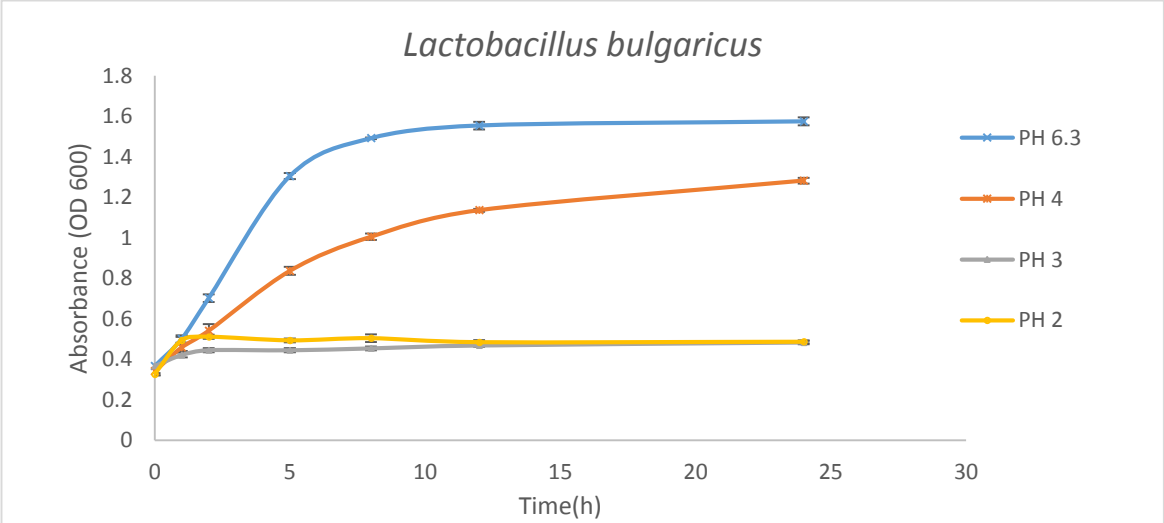

Figure 1: The survivals of *L. bulgaricus* at pH 2.0, pH 3.0, pH 4.0 and pH 6.3. The OD value was measured and recorded by the spectrophotometer at 60 min. interval for 24 hours

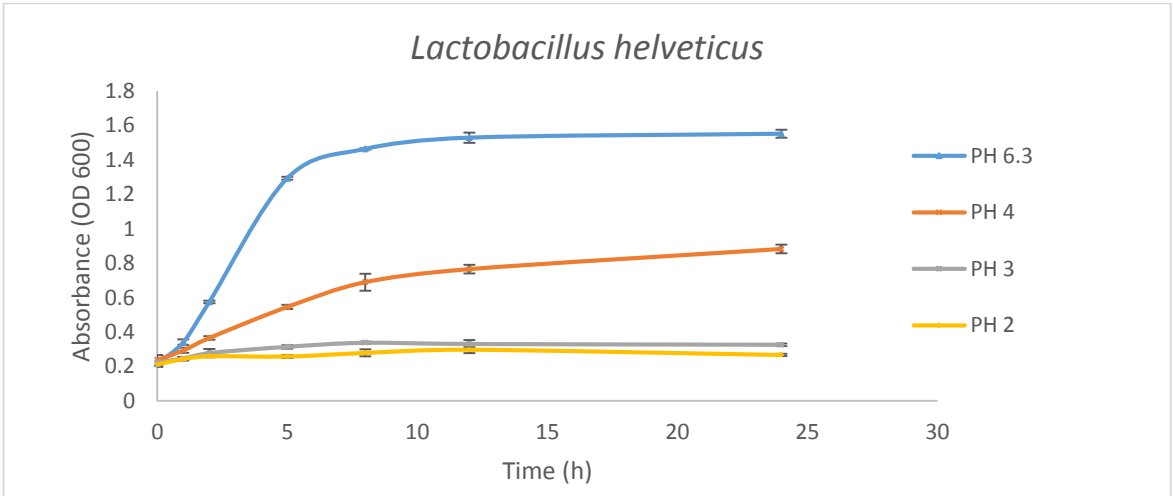

Figure 2: The survivals of *L. helveticus* at pH 2.0, pH 3.0, pH 4.0 and pH 6.3. The OD value was measured and recorded by the spectrophotometer at 60 min. interval for 24 hours.

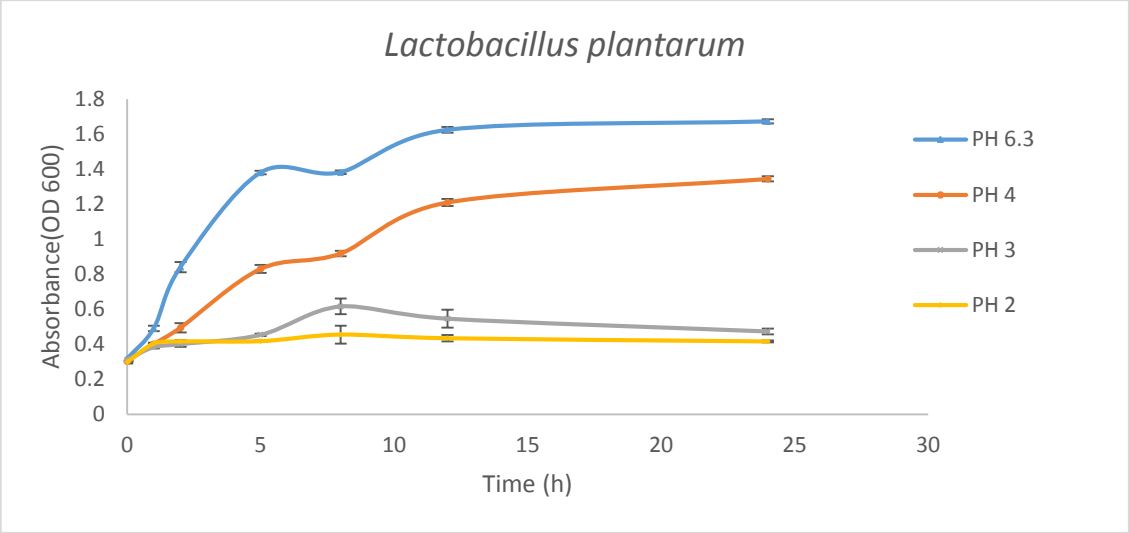

Figure 3: The survivals of *L. plantarum* at pH2.0, pH 3.0, pH 4.0 and pH 6.3. The OD value was measured and recorded by the spectrophotometer at 60 min. interval for 24 hours.

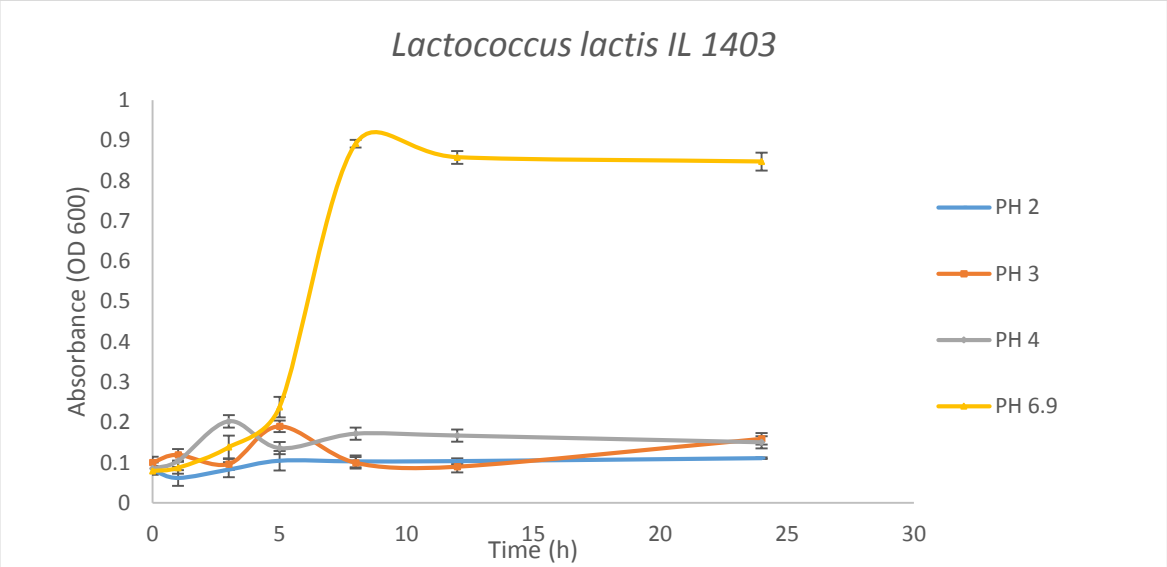

Figure 4: The survivals of *L. lactis* IL 1403 at pH 2.0, pH 3.0, pH 4.0 and pH 6.9. The OD value was measured and recorded by spectrophotometer at 60 min. interval for 24 hours.

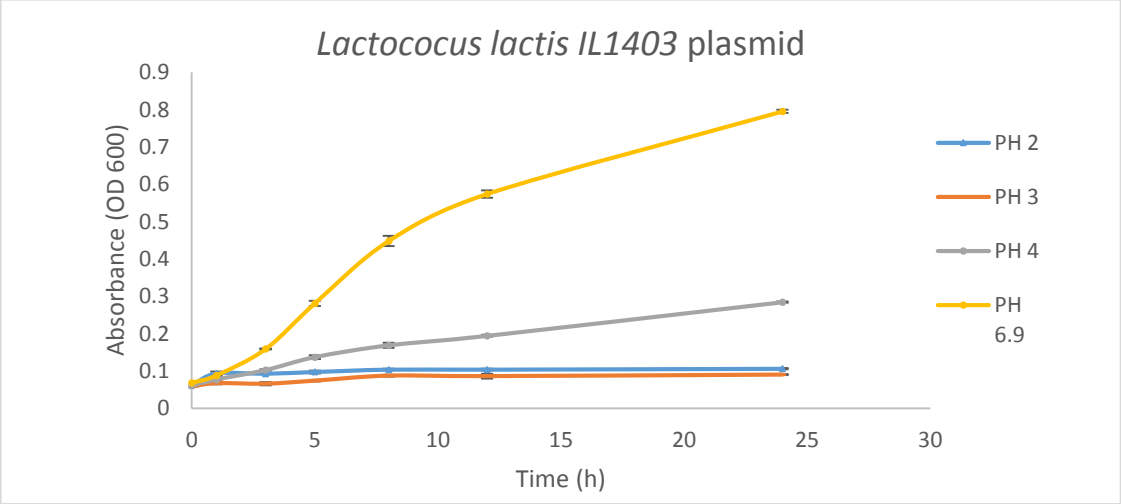

Figure 5: The survivals of *L. lactis* IL1403 pSIP at pH 2.0, pH 3.0, pH 4.0 and pH 6.9. The OD value was measured and recorded by the spectrophotometer at 60 min. interval for 24 hours

Table 1
The survival rate in simulated gastric juice at pH 2.0 and stimulated intestinal juice at pH 8.

Bacterial survivals in artificial gastric acid and intestinal juice						
Bacterial strains	**Initial bacterial colony numbers**	**The colony numbers after 5 hours in gastric acid juice in PH 2 pepsin**	**Death rate (%), during pepsin, after 5 hours**	**The colony numbers after 24 hours in intestinal juice in PH 8 trypsin**	**Death rate (%), during trypsin, after 24 hours**	**Death rate (%), totally after 29 hours.**
Lactobacillus bulgaricus	960 X 10^9 CFU/10ml	10 X 10^3 CFU/10ml	%99.99895	46 X 10^0 CFU/10ml	%99.54	%99.999995
Lactobacillus helveticus	4 X 10^9 CFU/10ml	410X 10^2 CFU/10ml	%99.98975	60 X 10^0 CFU/10ml	%99.85	%99.999985
Lactobacillus plantarum	314 X 10^9 CFU/10ml	10 X 10^1 CFU/10ml	%99.99996	3 X 10^0 CFU/10ml	%97.00	%99.999999
Lactococcus lactis IL 1403 plasmid	54,8 x 10^9 CFU/10ml	74 X 10^2 CFU/10ml	%99.99864	6 X 10^2 CFU/10ml	%91.89	%99.999890
Lactococcus lactis IL 1403	1920 x 10^9 CFU/10ml	335 X 10^2 CFU/10ml	%99.98255	296 X 10^2 CFU/10ml	%11.64	%99.984583

Table 2
The zone of inhibition radius of different antibiotics on *Lactobacillus helveticus* ATCC 15009 *(L. helveticus), Lactobacillus plantarum* ATCC 14917 *(L. plantarum), Lactobacillus delbrueckii subsp. bulgaricus* ATCC 11842 *(L. bulgaricus), Lactococcus lactis* IL1403 *(L. lactis IL1403), Lactococcus lactis* IL1403 *bearing pSIP plasmid (L. lactis IL403 pSIP)*

Antibiotic sensitivity					
Zone of inhibition (mm)	***Lactobacillus bulgaricus***	***Lactobacillus helveticus***	***Lactobacillus plantarum***	***Lactococcus lactis IL 1403 pSIP***	***Lactococcus lactis IL 1403***
RA5	17.66 ±0.47	11.33 ±1.24	14.33 ±0.94	13.33 ±0.94	8.66 ±0.47
C30	11.33 ±0.94	21.66 ±1.69	19 ±0.81	19.33 ±2.49	33 ±0.81
CN30	7.33 ±1.24	8 ±2.44	19.66 ±0.94	22 ±1.63	18.66 ±0.47
P2	12.66 ±0.94	15.33 ±3.77	14.66 ±1.24	2.66 ±0.94	35.66 ±0.47
E15	14 ±2.44	2.66 ±0.47	14.66 ±0.47	0 ±0	31.33 ±2.05
N30	2.66 ±0.47	18.33 ±1.24	4 ±0	11.33 ±1.88	12 ±0.81
K30	1.83 ±0.84	0 ±0	0 ±0	0 ±0	11 ±0
VA30	0 ±0	12 ±1.41	0 ±0	0 ±0	21.33 ±0.47

However, in artificial intestinal juice, *L. helveticus* has the lower survival proportion with 99.85% death rate after 24 hours among tested organism. As *L. lactis IL1403* has the highest viability value in pH 8.0 with trypsin, *L. lactis 1403* pSIP cannot survive as much as *L. lactis IL1403* at the same condition.

Antibiotic sensitivity: Kanamycin (K; 30 mcg), gentamycin (CN; 30 mcg), chloramphenicol (C; 30 mcg), penicillin (P; 2 unit), erythromycin (E; 15 mcg), rifampin (RA; 5 mcg), neomycin (N; 30 mcg) and vancomycin (VA; 30 mcg) antibiotics were used to investigate the resistance of the *Lactobacillus helveticus* ATCC 15009 *(L. helveticus), Lactobacillus plantarum* ATCC 14917 *(L. plantarum), Lactobacillus delbrueckii subsp. bulgaricus* ATCC 11842 *(L. bulgaricus), Lactococcus lactis IL1403 (L. lactis IL1403) and Lactococcus lactis IL1403 bearing* pSIP *plasmid (L. lactis IL403 pSIP).*

All the examined bacteria are sensitive to the RA, C, CN, P and N (Table 2). When analyzed in terms of the sensitivity to the rifampin, *L. bulgaris* has the lowest combat percentage with 17.66 mm inhibition zone, but it is resistant against vancomycin (Table 2).

Contrary to the *L. bulgaris*, *L. helveticus* is not sensitive against vancomycin but can struggle with kanamycin (Table 2). As mentioned for *L. bulgaris* and *L. helveticus*, *L. plantarum is* sensitive to gentamycin, chloramphenicol, penicillin, erythromycin, rifampin and neomycin but resists aganist both vancomycin and kanamycin (Table 2). Table 2 shows the determined zone of the inhibition radius for *L. lactis IL 1403*. Our results demonstrated that *L. lactis IL1403* is responsive to the gentamycin, chloramphenicol, penicillin, erythromycin, rifampin, neomycin, vancomycin and kanamycin (Table 2).

However, *L. lactis IL1403* pSIP persists to growing against erythromycin as it carries resistance gene on pSIP plasmid, it also resists against vancomycin and kanamycin (Table 2) not observed in *L. lactis IL1403* (Table 2).

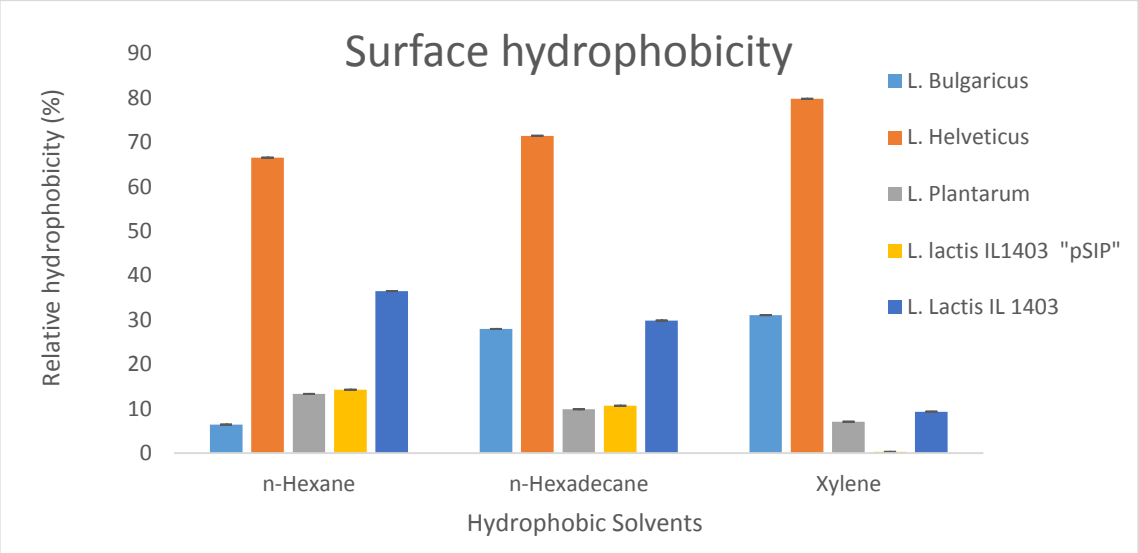

Figure 6: The surface hydrophobicity for each bacteria in n-hexane, n- hexadecane, xylene. The hydrophobicity is ranging from 79.80% to 0.29%.

Hydrophobicity: The cell surface hydrophobicity is used as a measurement of the ability to adhere to the cell monolayer. In this research, we have found that hydrophobicity proportion differs among the tested LAB and ranged from 79.80 to 0.29 % (Figure 6). The greatest hydrophobicity feature was observed by the *L. helveticus* for n-hexane, n-hexadecane and xylene at 66.51%, 71.46% and 79.80% respectively (Figure 6).

Discussion

The investigation of the probiotic features of LAB for implementation in preservation of food products and human health has a great interest recently. As probiotics are widely ingested orally, so they must be capable to resist through the gastrointestinal tract. Therefore, survival and growth rate in different pH have been shown important criteria to pass through the stomach and intestinal system for probiotic bacteria. In this study, we investigated the ability *of L. helveticus, L. plantarum, L. bulgaricus, L. lactis IL1403* and *L. lactis IL1403* pSIP to grow at different pH conditions ranging from 6.9 to the 2.0 (Figure 1-5).

All the tested bacterial strains have shown good growth rate in their suitable condition on pH 6.3 or pH 6.9. However, as the pH values decrease, their growth rates have been reduced. pH 3.0 and pH 2.0 showed strong blocking effect on the replication of the strains. The high hydrogen concentration has negative effect on the bacterial growth.

It causes high protonation in cell and blocking important pathway responsible for bacterial replication such as protein and ATPase synthesis[2]. We have also observed that while the *L. lactis IL1403* did not grow at pH 4.0, *L. lactis IL1403 pSIP* has an ability to persist in same condition. However, initial bacterial concentration of *L. lactis IL1403* and *IL1403 pSIP* differed in this experiment. Further investigation is needed to understand effect of the plasmid on low pH survival rate.

The gastric protease, low pH in gastric juices, various enzymes, bile acids and other substances in intestinal juice hinder the development of microorganisms. Therefore, gastrointestinal tract resistance is a significant indicator for the determination of potential probiotic. In the current study, it was found that *L. lactis IL1403* has 3.52 Log (CFU/ml) after 5 hours in simulated gastric acid juice that Log value was 11.28 (CFU/ml) initially. In addition, *L. helveticus* shows the similar properties with *L. lactis IL1403* in same environment (*L. helveticus* has 8 Log (CFU/ml) value initially. After 5 h in the most extreme condition, it has 3.61 Log (CFU/ml)). Consistent with our findings, a strong survival proportion of the *L. helveticus* and *L. lactis* in artificial gastric acid juice was reported[12]. Even though *L. lactis IL1403* has a similar behaviour also in simulated intestinal juice and has the most strong resistance among the tested organisms, reduced viability was demonstrated by the same research[12].

Antibiotic sensitivity is a significant factor in safety of probiotics usage. It is considered that the LAB can transfer its resistant genes to pathogenic bacteria inside of the intestinal area. The tested microorganisms showed different characteristic against K, CN, C, P, E, RA, N and VA antibiotic discs. The *Lactococcus lactis IL1403* was sensitive to erythromycin but *Lactococcus lactis IL1403* pSIP showed tolerance to same antibiotic because of the erythromycin resistant gene carrier of pSIP[16]. *L bulgaricus, L. helveticus, L. plantarum, L. lactis IL 1403 and L. lactis IL 1403 pSIP were* sensitive to RA, C, CN, P, CN respectively. The LAB bacteria are known to sensitive to be chloramphenicol[5,17,22,25].

The present study is consistent with this literature because all tested bacteria are sensitive to chloramphenicol. It was mentioned that *Lactobacillus* is sensitive to glycopeptide antibiotics such as vancomycin[28]. Even though our results do not uphold the assertion of natural resistance against

vancomycin in all *Lactobacillus*, the *L. helveticus* and *L. lactis IL 1403* were found sensitive to vancomycin among tested bacteria as observed earlier[4,5]. We also determined that *L. plantarum* was resistant to K and VA and could not tolerate rest of the antibiotics. However, another investigation showed that *L. plantarum* has antibiotic sensitivity to erythromycin, penicillin G and rifampicin[7]. These relatively incompatible results can be caused by the strain and source of the organism evaluated.

Bacterial hydrophobicity was determined to evaluate the attachment properties of microorganisms to the hydrocarbon surface which is a measure of adhesion to epithelium cells in gut[31]. The hydrophobic character depends on the strain and organism specify and is effected by different factors such as aging, chemical structure of the surface, even composition of culture medium[33] and experimental method[21]. In our study, the highest hydrophobicity score was determined for *L. helveticus* for n-hexane, n- hexadecane and xylene at 66.51%, 71.46% and 79.80% respectively. There was comparison of hydrophobicity properties determined in xylene of *L. plantarum* strains ranging from 16.90 to 96.62[29].

It was also shown that the *L. plantarum Lp996* was isolated from Argentina cheese having 2.19 % cell hydrophobicity value[34]. However, we found the lowest 66.51±0.012 score for n-hexane for *L. plantarum*. In addition, surface hydrophobicity is effected by plasmid, we evaluated the hydrophobic feature of *L. lactis IL1403* to compare it with pSIP carriers. Although some researchers revealed that hydrophobicity properties for *L. lactis* range between 14.9 and 31.3% and between 48 and 88% respectively[30,9], our results are compatible for *L. lactis IL1403*.

Conclusion

This investigation uncovered an extensive heterogeneity in probiotic features among the tested microorganisms. Because of their probiotic effect, these strains may assist to promote health of hosts, protect hosts from intestinal pathogens and maintain the natural balance of intestinal microflora during antibiotic treatments in accordance with their probiotic features.

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