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In Vitro Characterization of Cell Surface Properties of 14 Vaginal *Lactobacillus* Strains as Potential Probiotics

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Abstract

Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host. Human-origin *Lactobacillus* is a preferable source of probiotic bacteria. This study screened 14 vaginal *Lactobacillus* strains as probiotic candidates by investigating probiotic-related cell surface characteristics including cell surface hydrophobicity (CSH), Lewis acidity/basicity, autoaggregation, and biofilm formation. Moderate to high CSH and autoaggregation, high basicity and low acidity were prevalent in the 14 tested strains. Biofilm formation varied in a large range among the 14 tested strains. CSH showed a high correlation with Lewis acidity and autoaggregation, while Lewis acidity was highly correlated with autoaggregation and biofilm formation. Four strains were selected as promising probiotic strains. This study was the first one to compare antibiotic sensitivity between biofilm-forming cells and planktonic cells of *Lactobacillus* species, and found that biofilm-forming cells of a *L. fermentum* strain had a significantly higher survival rate than planktonic cells in cefotaxime, cefmetazole and tetracycline, but were as sensitive to oxacillin and ampicillin as planktonic cells were.

Keywords

Lactobacillus, Probiotics, Cell Surface Hydrophobicity, Lewis Acidity, Lewis Basicity, Autoaggregation, Biofilm, Antibiotics

1. Introduction

Probiotics are live microorganisms which when administered in adequate

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amounts confer a health benefit on the host [1]. *Lactobacillus* spp. are widely used as probiotic bacteria, and their application in foods are generally recognized as safe (GRAS) [2]. Probiotic potential of *Lactobacillus* spp. is closely related to the cell surface characteristics including cell surface hydrophobicity (CSH), cell surface charge, and abilities of autoaggregation and forming biofilm, which are widely used for *in vitro* characterization and screening of probiotic strains [3]-[8].

Bacterial CSH influenced the strength of bacterial adhesion to the host tissues, so is important for probiotic bacteria to confer health benefit to the host [8]. It was believed that hydrophobic nature of cell surface could facilitate colonization and adhesion of bacteria to the epithelium of gastrointestinal tract of a host [9]. Some studies even showed correlation between CSH and adhesion ability in *Lactobacillus* [7].

Bacterial cell surface charge has also been shown to influence the strength of bacterial adhesion to the host [8]. Electron acceptor (*i.e.* Lewis acid) and electron donor (*i.e.* Lewis base) on two surfaces can interact with each other by forming a coordinate covalent bond. This interaction had been implicated in microbial adhesion, as well as in other interfacial phenomena such as phagocytosis and biofouling [10].

Aggregation ability has been suggested to be an important characteristic of many bacterial strains used as probiotics [8]. A good probiotic must possess high autoaggregation as well as strong hydrophobicity [9]. The ability of *Lactobacillus* to form multicellular aggregates can facilitate probiotic adhesion to intestinal cells and colonization to the intestines [7].

Biofilm formation by probiotic bacteria is a beneficial characteristic, because it could improve colonization and permanence over time in host mucosa, mechanically protect the mucosa, and prevent colonization of pathogens [5] [6], and it is also an important feature in food processing [11]. Besides, biofilm could also protect bacteria against antibiotics, which had been well demonstrated in pathogenic bacteria [12] [13]. Antibiotics could be encountered by probiotic bacteria in human body and during food processing, but how biofilm affects antibiotic sensitivity in probiotic bacteria, e.g. *Lactobacillus* species has been largely neglected so far.

Human-origin *Lactobacillus* is a preferable source of probiotic bacteria, and *Lactobacillus* isolates from human oral cavity [8] [14], breast milk [15] [16], stomach [17], feces or intestinal tract [18] [19] [20] have been studied on their probiotic characteristics. *Lactobacillus* plays an important role in maintaining the health of human vagina [4] [6]. *Lactobacillus* isolates from human vagina exhibited promising probiotic potential as suggested by high CSH, autoaggregation and biofilm formation [2]. This made vaginal *Lactobacillus* strains promising probiotic candidates. Investigation on probiotic-related cell surface characteristics is necessary for vaginal *Lactobacillus* isolates to be used in a probiotic food.

This study aimed to screen 14 vaginal *Lactobacillus* strains as probiotic candidates, by *in vitro* investigation of probiotic-related cell surface characteristics

including CSH, Lewis acidity/basicity, autoaggregation, and biofilm formation. This study also aimed to provide valuable and novel reference information to understand the characteristics of vaginal *Lactobacillus* strains.

2. Materials and Methods

2.1. Bacterial Strains

The 14 vaginal *Lactobacillus* strains used in this study (*i.e. L. plantarum* strains KLB213, KLB234, KLB270, and KLB296, *L. fermentum* strains KLB231, KLB249, KLB261, KLB263, and KLB268, *L. salivatius* strain KLB265, *L. rhamnosus* strain KLB288 and unidentified strains KLB208, KLB223, and KLB255) were obtained from the *Lactobacillus* collection of So Lab of Inha University, Korea, and maintained in MRS broth with daily subculture as previously described [21].

2.2. CSH and Lewis Acidity/Basicity Assay

Microbial adhesion to solvents (MATS) method has been widely used to investigate microbial CSH and Lewis acidity/basicity [4] [10] [22] [23]. In this study, adhesion of 14 *Lactobacillus* strains to three solvents, *i.e.* hexadecane, chloroform and ethyl acetate was measured as indication of CSH, Lewis base (electron donor) and Lewis acid (electron acceptor) characteristics, respectively. Briefly, each strain was cultured in MRS broth at 37˚C to stationary phage. The bacteria were then harvested by centrifugation at 8000 × g for 5 min, washed twice, and resuspended in phosphate buffered saline (pH 7.0, PBS) to an OD_{600} of 1 (A_0). Three ml of this bacterial suspension was mixed 1 ml of hexadecane, chloroform or ethyl acetate by vortexing for 2 min, and then incubated at room temperature for 20 min to allow phage separation. OD_{600} of the aqueous phase (A_t) was measured. The percentage of bacterial adhesion to each solvent was calculated as $(1 - A_t/A_0) \times 100\%$. The assay was performed in triplicate, and the results were averaged.

2.3. Autoaggregation Assay

Autoaggregation ability of 14 *Lactobacillus* strains was assessed by phase separation method as previously described [24] with minor modifications. Each of the 14 *Lactobacillus* strains was grown at 37˚C in MRS broth to stationary phage. The cells were harvested by centrifugation at 8000 × g for 5 min, washed twice and resuspended in PBS to an OD_{600} of 0.5 (A_0). Four ml of this bacterial suspension was incubated at room temperature for 5 h, and OD_{600} of 1 ml aliquot of the upper phase (A_t) was measured. The autoaggregation percentage was expressed as $(1 - A_t/A_0) \times 100\%$. The assay was performed in triplicate, and the results were averaged.

2.4. Biofilm Formation Quantification

Biofilm formation by 14 *Lactobacillus* strains was quantified by crystal violet staining method as previously described [25] [26] with minor modifications.

Briefly, 100 µl culture of each strain at OD_{600} of 0.1 was inoculated per well in a flat-bottomed 96 well PVC plate and incubated at 37˚C for 40 h to allow biofilm formation. Planktonic bacteria were removed by gently rinsing twice with 100 µl PBS, and the plate was inverted to air dry for 30 min. The biofilm was stained with 50 µl of 0.1% crystal violet solution in ethanol for 45 min at room temperature. Unbound crystal violet was then removed and the well was rinsed twice with 100 µl PBS. The crystal violet bound in biofilm was dissolved with 200 µl of 95% ethanol at 4˚C for 30 min, and OD_{595} of 100 µl aliquot was measured as quantification of biofilm formation. The assay was performed in triplicate, and the results were averaged.

The *L. fermentum* strain KLB261, which exhibited high CSH and abilities of autoaggregation and biofilm formation (see the section of results), was selected to assess antibiotic susceptibility.

2.5. Determination of Minimal Inhibitory Concentrations of Antibiotics

Minimal inhibitory concentrations (MICs) of 5 antibiotics (oxacillin, cefotaxime, cefmetazole, ampicillin, tetracycline) on the *L. fermentum* strain KLB261 was determined using a previously described microdilution procedure [27] with minor modifications. Briefly, 100 µl culture at OD_{600} of 0.1 was inoculated per well in a flat-bottomed 96 well PVC plate containing a series of concentrations of an antibiotic. After incubation at 37˚C for 40 h, the lowest concentration at which no visible growth was observed was determined as MIC. The assay was performed in triplicate and the results were averaged.

2.6. Comparison of Antibiotic Susceptibility between Biofilm-Forming Bacteria and Planktonic Bacteria

Susceptibility to 5 antibiotics (oxacillin, cefotaxime, cefmetazole, ampicillin, tetracycline) was compared between biofilm-forming cells and planktonic cells of the *L. fermentum* strain KLB261 using a method described by Ishida *et al.* [28] with minor modifications. KLB261 was cultured at 37˚C for 40 h in MRS broth with a 1.8 cm × 0.8 cm PVC sheet for biofilm formation. To assess the antibiotic susceptibility of biofilm-forming bacteria, the PVC sheet was taken out from the culture, washed gently with PBS, and incubated at 37˚C in PBS containing an antibiotic at its MIC for either 0 or 24 h. The PVC sheet was then transferred to 1 ml fresh PBS and vortexed vigorously to suspend the biofilm-forming bacteria. The suspension was serially diluted, plated on MRS agar, and CFU was counted. To assess the antibiotic susceptibility of planktonic bacteria, the planktonic bacteria in the culture from which the PVC sheet had been taken out was harvested by centrifugation, washed twice, and resuspended in 1 ml PBS containing an antibiotic at its MIC. After incubation at 37˚C for either 0 or 24 h, the suspension was serially diluted, plated on MRS agar, and CFU was counted. The survival rate of biofilm-forming or planktonic bacteria was calculated by dividing the CFU at 24 h by the CFU at 0 h of antibiotic challenge. The assay was performed

in triplicate and the results were averaged.

2.7. Statistical Analysis

The value arrays of CSH, Lewis acidity, Lewis basicity, autoaggregation, and biofilm formation obtained from 14 *Lactobacillus* strains were paired, and correlation coefficient of each pair was calculated as indication of the strength of correlation. Differences in survival rate for each antibiotic between biofilm-forming cells and planktonic cells of the *Lactobacillus* strain KLB261 were assessed by performing ANOVA after determination of normality and variance homogeneity. The significance level was set at $P < 0.05$.

3. Results

3.1. CSH and Lewis Acidity/Basicity of 14 *Lactobacillus* Strains

CSH and Lewis acidity/basicity of 14 vaginal *Lactobacillus* strains were quantified by MATS assay. Five strains (KLB208, KLB213, KLB223, KLB255, KLB261, and KLB265) showed high CSH (≥60%), 4 strains (KLB213, KLB249, and KLB296) were found to have moderate CSH (10% - 60%), and 5 strains (KLB231, KLB263, KLB268, KLB270, and KLB288) presented low CSH (≤10%) (Table 1). Most strains tested turned out to be strong electron donors, showing high affinity for chloroform (≥80%), except that KLB263 showed a moderate affinity (43%), and KLB268 (11%) and KLB270 (5%) lacked affinity for chloroform (Table 1). By contrast, most strains in this study exhibited low affinity for ethyl acetate (≤40%), indicating they were weak electron acceptors, except that KLB223,

Table 1. CSH, Lewis acidity, Lewis basicity, autoaggregation, and biofilm formation of *Lactobacillus* strains.

Strain	Adhesion to Hexadecane[1] (% ± S.D.)	Adhesion to Chloroform[2] (% ± S.D.)	Adhesion to Ethyl Acetate[3] (% ± S.D.)	Autoaggregation (% ± S.D.)	Biofilm formation (OD_{595} ± S.D.)
KLB208	66.31 (±15.78)	89.90 (±1.89)	12.96 (±1.61)	57.06 (±8.28)	0.35 (±0.08)
KLB213	59.90 (±3.98)	90.44 (±4.48)	32.76 (±1.77)	85.61 (±7.47)	0.16 (±0.06)
KLB223	75.30 (±6.40)	81.46 (±8.75)	93.40 (±2.00)	88.56 (±3.48)	0.58 (±0.09)
KLB231	3.40 (±1.84)	81.96 (±5.99)	15.85 (±5.09)	68.00 (±5.90)	0.59 (±0.05)
KLB234	40.00 (±3.89)	95.59 (±0.47)	28.43 (±1.84)	68.85 (±9.83)	0.09 (±0.04)
KLB249	21.22 (±7.27)	90.29 (±9.39)	26.49 (±1.70)	37.30 (±1.16)	0.21 (±0.05)
KLB255	68.46 (±8.40)	82.23 (±6.78)	64.08 (±19.3)	79.13 (±6.61)	0.57 (±0.09)
KLB261	72.33 (±11.58)	89.91 (±1.61)	85.13 (±3.63)	84.70 (±5.29)	0.63 (±0.03)
KLB263	5.57 (±0.79)	43.37 (±2.13)	12.60 (±2.08)	60.86 (±7.26)	0.09 (±0.04)
KLB265	82.62 (±8.58)	84.58 (±4.01)	90.66 (±2.38)	85.75 (±2.47)	0.68 (±0.09)
KLB268	4.15 (±4.97)	10.94 (±5.61)	16.16 (±2.80)	46.89 (±16.97)	0.07 (±0.04)
KLB270	3.43 (±4.22)	5.41 (±2.35)	11.37 (±1.80)	40.03 (±12.01)	0.04 (±0.01)
KLB288	1.41 (±0.78)	98.24 (±1.39)	14.62 (±1.19)	32.95 (±6.93)	0.33 (±0.08)
KLB296	31.27 (±3.58)	84.39 (±6.04)	34.77 (±1.58)	76.26 (±8.01)	0.44 (±0.12)

[1]as indication of CSH; [2]as indication of Lewis basicity; [3]as indication of Lewis acidity; S.D. = standard deviation.

KLB255, KLB261, and KLB265 displayed high affinity for ethyl acetate (≥60%) (Table 1).

3.2. Autoaggregation Ability of 14 *Lactobacillus* Strains

Among the 14 tested vaginal *Lactobacillus* strains, 9 strains (KLB213, KLB223, KLB231, KLB234, KLB255, KLB261, KLB263, KLB265, and KLB296) exhibited high autoaggregation (≥60%), while the other 5 strains (KLB208, KLB249, KLB268, KLB270, and KLB288) showed autoaggregation of a moderate level (30% - 60%) (Table 1).

3.3. Biofilm Formation Ability of 14 *Lactobacillus* Strains

Ability of 14 vaginal *Lactobacillus* strains to form biofilm was quantified by crystal violet staining method and the results were summarized in Table 1. Five strains (KLB223, KLB231, KLB255, KLB261, and KLB265) exhibited high biofilm formation (≥0.5), and other 5 strains (KLB208, KLB213, KLB249, KLB288, and KLB296) formed biofilm on a moderate level (0.1 - 0.5). The other 4 strains (KLB234, KLB263, KLB268, and KLB270) lacked biofilm formation ability (<0.1).

3.4. MICs of Antibiotics on Strain *L. fermentum* KLB261

MICs of oxacillin, cefotaxime, cefmetazole, ampicillin, and tetracycline on *L. fermentum* strain KLB261 were determined by microdilution procedure to be 1.25 μg/ml, 5 μg/ml, 50 μg/ml, 0.125 μg/ml and 12.5 μg/ml, respectively.

3.5. Antibiotic Susceptibility of Biofilm-Forming Cells and Planktonic Cells of *L. fermentum* KLB261

Susceptibility of biofilm-forming cells and planktonic cells of *L. fermentum* KLB261 to oxacillin, cefotaxime, cefmetazole, ampicillin, and tetracycline at their respective MIC was assessed, and the results were presented in Figure 1. Biofilm-forming cells of KLB261 were significantly more tolerant than their planktonic counterparts to cefotaxime, cefmetazole, and tetracycline, but were as sensitive to oxacillin and ampicillin as planktonic cells were.

4. Discussion

4.1. CSH and Lewis Acidity/Basicity of 14 *Lactobacillus* Strains

Lactobacillus strains with high CSH were preferable in probiotic application, as a hydrophobic cell surface could facilitate colonization and strengthen adhesion of bacteria to the epithelium of gastrointestinal tract of a host [7] [8] [9]. This study revealed the prevalence of moderate to high affinities for hexadecane and chloroform, and low affinity for ethyl acetate among the tested *Lactobacillus* strains, but these three characteristics did not always coincide in the same strains. This result indicated that hydrophobic and negatively charged cell surfaces were prevalent in the tested strains, and that these bacteria might play a role of electron

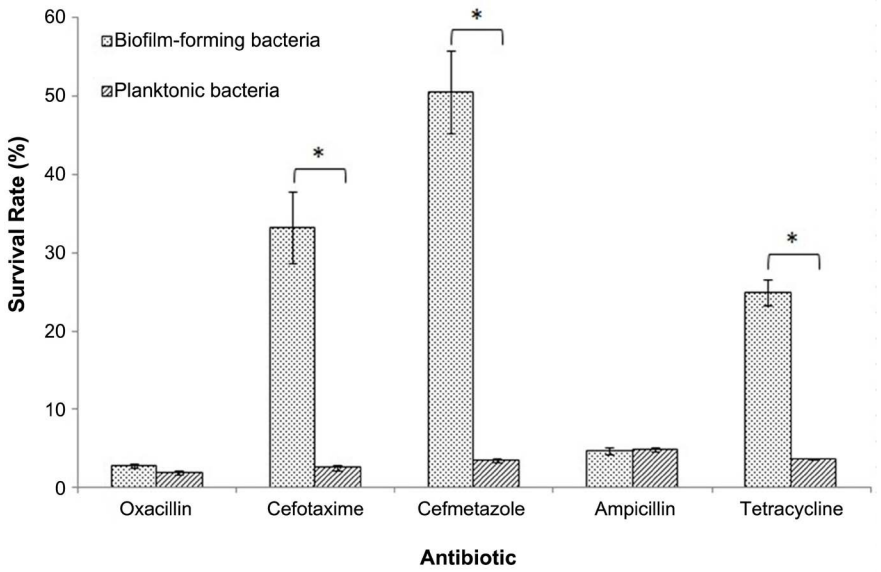

Figure 1. Antibiotic susceptibility of biofilm-forming cells and planktonic cells of strain *L. fermentum* KLB261. The asterisks between two columns indicate significant difference.

donor in interfacial interactions. The observed prevalence of moderate to high CSH in vaginal *Lactobacillus* isolates was consistent with the studies by Pino *et al.* [2] and Ocana *et al.* [29]. It was disagreed in different studies on whether correlation between CSH and Lewis acidity/basicity exists. Pelletier *et al.* found that strains with affinity for chloroform also had affinity for hexadecane, and not for ethyl acetate [30], but Ocana *et al.* suggested that there is no strong correlation between the affinities for hexadecane and for chloroform [29]. In the current study, analysis of data from the 14 *Lactobacillus* strains suggested that CSH was highly correlated with Lewis acidity, and moderately correlated with Lewis basicity, but Lewis acidity and basicity were not correlated (**Table 2**).

4.2. Autoaggregation Ability of 14 *Lactobacillus* Strains

Lactobacillus strains with higher autoaggregation ability were considered more desirable in terms of application in probiotic foods, as multicellular aggregates can facilitate colonization and adhesion of probiotic bacteria to intestinal cells [7] [8] [9]. Autoaggregation ability was prevalent in the 14 *Lactobacillus* stains tested in this study. This result was consistent with the prevalence of high autoaggregation in *Lactobacillus* strains from human vaginas reported by Bouridane *et al.* [31] and Pino *et al.* [2], but showed a higher percentage of strains with autoaggregation activity than other studies [32] [33] [34] [35]. Correlation between autoaggregation and CSH of *Lactobacillus* strains was also controversial in different studies. Kmet *et al.* demonstrated this correlation in vaginal *Lactobacillus* strains [34], but it was denied by Bouridane *et al.* [31]. In the current study, analysis of data from the 14 *Lactobacillus* strains suggested that autoaggregation was highly correlated with CSH and Lewis acidity, but not correlated with Lewis basicity (**Table 2**).

Table 2. Correlation coefficient between any two of CSH, Lewis acidity, Lewis basicity, autoaggregation, and biofilm formation of *Lactobacillus* strains.

	CSH	Lewis basicity	Lewis acidity	Autoaggregation
Lewis basicity	0.51	-	-	-
Lewis acidity	0.81	0.35	-	-
Autoaggregation	0.77	0.39	0.75	-
Biofilm formation	0.60	0.53	0.74	0.61

4.3. Biofilm Formation Ability of 14 *Lactobacillus* Strains

Lactobacillus strains with ability of forming biofilm were considered good probiotic candidates, as biofilm formation of probiotic bacteria is an important feature in food processing [11], and also provide health benefit by improving colonization and permanence of probiotic bacteria in host mucosa, mechanically protecting the mucosa, and preventing colonization of pathogens [5] [6]. Biofilm formation varied in a large range among the tested vaginal *Lactobacillus* stains in this study. Similar results were previously found in vaginal *Lactobacillus* strains [2] [36] [37] and *Lactobacillus* strains of other origins [16], but most vaginal *Lactobacillus* strains were found to be weak biofilm producers by Malik *et al.* [35]. On the other hand, a study by Klimko *et al.* suggested the absence of correlation between high hydrophobicity and intense biofilm formation [16], but analysis of data from the 14 *Lactobacillus* strains in the current study suggested that biofilm formation was highly correlated with Lewis acidity, and moderately correlated with CSH, Lewis basicity and autoaggregation (**Table 2**).

4.4. Correlation among CSH, Lewis Acidity/Basicity, Autoaggregation, and Biofilm Formation of 14 *Lactobacillus* Strains

Although this study found high correlation coefficients (>0.7) in the pairs of CSH vs Lewis acidity, CSH vs autoaggregation, Lewis acidity vs autoaggregation, and Lewis acidity vs biofilm formation, there was no consensus on correlations among these cell surface characteristics in previous studies yet [29] [30] [31] [34]. Due to the limited quantity of *Lactobacillus* strains investigated this study, values of and relationships between different cells surface characteristics might not unveil the whole story of *Lactobacillus* spp., so there is a need for comprehensive analysis of cell surface characteristics when screening *Lactobacillus* strains to provide optimal probiotic functions for applications in food.

4.5. Antibiotic Susceptibility of Biofilm-Forming Cells and Planktonic Cells of Strain KLB261

Resistance of biofilm against antibiotics had been well documented in pathogenic bacteria [12] [13], but was rarely studied in probiotic bacteria. This was the first study to compare antibiotic susceptibility between biofilm-forming cells and planktonic cells of *Lactobacillus* species, and suggested differential protective ef-

fects of biofilm on *Lactobacillus* species against different antibiotics. Biofilm enhanced the tolerance of *L. fermentum* KLB261 against cefotaxime, cefmetazole, and tetracycline, but did not provide protection against oxacillin and ampicillin. It is equally important to find the antibiotics which a probiotic biofilm is tolerant against and sensitive to, so that probiotic strains can be selected or modulated during food processing and in human body. Protective mechanisms of biofilm include acting as a barrier against antibiotic penetration, interaction of antimicrobials with biofilm matrix components, reduced growth rates and various actions of specific genetic determinants of antibiotic resistance and tolerance [12] [13].

5. Conclusion

Lactobacillus strains KLB223, KLB255, KLB261, and KLB265, which were distinguished by their high CSH (≥60%), high autoaggregation (≥60%) and high biofilm formation (≥0.5), were selected as promising probiotic strains for further study, which would include adhesion to epithelial cells, safety evaluation, and animal trial. In addition, this study revealed correlations between different cells surface characteristics in lactobacilli (CSH vs Lewis acidity, CSH vs autoaggregation, Lewis acidity vs autoaggregation, and Lewis acidity vs biofilm formation). Another important finding of this study was the differential protective effects of biofilm on *Lactobacillus* species against different antibiotics. There is still a need for comprehensive analysis of cell surface characteristics when screening vaginal *Lactobacillus* strains to provide optimal probiotic functions for applications in food.

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Conflicts of Interest

The authors declare no conflicts of interest.

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