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Research Article

Research on Biofilm Formation Ability of Lactic Acid Bacteria under Different Conditions

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Abstract: In the study, *Lactobacillus plantarum* 18-2, *Leuconostoc citreum* 37 and *Leuconostoc citreum* 39, which were isolated from Inner Mongolia traditional dairy products, were used to detect their biofilm formation ability by the micro-titer plate biofilm assays. The results showed that the biofilm-forming ability of the three strains differed along with the alteration of their nutritional and environmental conditions. The optimal biofilm formation conditions of *L. plantarum* 18-2 were in pH5.6 MRS broth without any additives for 12 h at the temperature of 30°C Otherwise, *L. citreum* 37 was reported the best biofilm formation ability, when it's cultivated in pH 5.4 for 48 h at 30°C, while the culture in pH5.5 for 48 h at 30°C is most conducive to the biofilm forming of *L. citreum* 39. In addition, when sodium chloride or sucrose was added to MRS broth, the biofilm forming was inhibited as the concentration increased. Thus it can be seen that, *L. plantarum* and *L. citreum* 37 and 39 were capable of forming biofilm and the biofilm formation was strongly modulated by culture conditions and medium, including low pH; low temperature and high osmolality.

Keywords: Biofilm, conditions, lactic acid bacteria

INTRODUCTION

Attaching to the biological or non-biological surface, biofilm (hereinafter referred to as BF) consists of bacterial colonies and coated bacteria (Hall-Stoodley *et al*., 2004) and is widely applied in nature and industrial production, such as fermentation industry and sewage treatment system (Van Schaik and Abee, 2005). Comparing with dissociative bacteria, those in BF change significantly in their physiological status are strongly resistant to antibacterial agents (Donlan and Costerton, 2002). Biofilms of *Pseudomonas aeruginosa* were more resistant to heavy metals than planktonic bacteria (Teitzel and Parsek, 2003); Biofilms of *Burkholderia cepacia* and of *P. aeruginosa* were more resistant to antibiotics (Kubota *et al*., 2008). As we known, there were few reports have demonstrated the formation of lactic acid bacteria biofilms.

Lactic Acid Bacteria (LAB) are widely recognized to be the safest one applied to the food industry. Under the fermentation effect of LAB, the food is endowed with higher pH value, abundant nutrition and unique flavor and can be easily digested, improve microcirculation and prevent, cure or assist to cure various diseases. LAB metabolites are of high utility value. For instance, lactic acid is one of major materials for the food, pharmaceuticals and daily chemical industries; lactic acid bacteria bacteriocins can be used as the natural protective agent for foods, which not only do no harm to body health, but also have great nutritive value. Speranza *et al*. (2009) reported that BF from naturally fermenting LAB could be an approach to control the growth of *Listeria* in soft cheese. However, some lactic acid bacteria are the key risk factors for food deterioration. The study on BF formation plays a significant role in improving LAB suitability in fermentation agent production and preventing food materials, semi-finished and finished products from decaying.

In nature, bacteria have developed a variety of mechanisms of resistance to environmental stresses. One is the formation of biofilms. Many factors affect the BF generation on the agent surface, such as time, temperature, carbohydrate sources, pH value and osmotic pressure (Loo *et al*., 2000). In the study, we will explore the BF forming capability of 3 LAB strains under different conditions, as ways to improve their environmental suitability to make better use of the beneficial bacteria.

MATERIAL AND METHODS

Bacterial strains and media: Three lactic acid bacteria strains, *L. plantarum* 18-2, *L. citreum* 37 and 39, were

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separated from Inner Mongolian traditional dairy products (Chun-Lei *et al*., 2014) and kept in Inner Mongolia University.

MRS broth culture medium (made by Beijing Land Bridge Technology Co., Ltd.) was adopted pH to 6.2+0.2 and sterilized for 15 min at 121°C. MRS solid medium include 1.5- 2% agar in broth culture.

Lactic acid bacteria cultivation: Suck up 200μL bacteria with a glycerin tube kept at the temperature of -80°C, inoculate into the tube with MRS broth medium and cultivate for 24 h at the temperature of 37°C. Conduct activation for three generations of strains with the same method, preserve the second generation and take the third generation out for experiment.

Biofilm Optical Density value (OD value) detection by the microtiter plate: The biofilm formation protocol was adapted from the protocol of Kim *et al*. (2009) with some modifications as follows. Put the activated 3rd generation of strains under the room temperature, collect 20μl bacterial suspension into a standard 24-well micro-titer dishes (3524-24 well cell culture cluster, Corning Incorporated) with 1mL MRS broth liquid medium per hole, inoculate 3 holes per strain and set aside blank control group.

1st measurement: take out strains after cultivating for a while (adjust upon variable requirements), measure OD_1 and OD_{1C} value at 600 nm by ELIASA (Glomax Multi ELIASA, Beijing Yuanpinghao Bio-tech Co., Ltd.).

2nd measurement: dispose of culture, wash off floating bacteria 3 times with distilled water, dry out the micro-titer plate for 2 h under the room temperature, add 1% crystal violet (Tianjin Damao Chemical Reagent Factory, AR) 1ml per well, place for 20 min, wash off 6-7 times dye solution on the pore wall with distilled water, till water becoming colorless. Dry the micro-titer plate for 30 min under the room temperature, add 95% ethyl alcohol (Tianjin Damao Chemical Reagent Factory, AR) 1ml per well, vibrate for 30 min and then measure OD_2 and OD_{2C} value at 600nm. Adhesion rate is set to be B and can be calculated as below:

$$B = \frac{OD_2 - OD_{2C}}{OD_2 - OD_{1C}}$$

OD_{1C} and OD_{2C} refer to the optical density value in blank control group. B<0.1 indicates non-adhesion, B≥0.1 for adhesive film, 0.1<B<1 for medium-intensity adhesive film and B>1 for intensive adhesive film.

The effect of different culture conditions on lactic acid bacteria BF formation rate:

The effect of different cultivation times on the BF formation: Suck up 20μl 3rd generation strains, inoculate into 1ml culture medium in 24-well micro-titer dishes (the initial pH of MRS broth was 6.3) and place in 37°C constant temperature incubator for 12 h, 24 h, 36 h, 48 h, 60 h and 72 h, respectively. Afterwards, take them out and measure OD values with the same method as 2.3 and calculate BF forming rate to determine the formation conditions of lactic acid bacteria BF. For each time point, the average OD was calculated from three independent measurements.

The effect of initial pH values on the BF formation: Use a digital acidometer (PHS-3C digital acidometer, Shanghai Yidian Instrument Co., Ltd.) to adjust the initial pH values of MRS broth culture to 5.0, 5.1, 0.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4 and 6.5 and put into 24-well micro-titer dishes respectively. Inoculate them with 20 μL 3rd-generation strains and incubated at 37°C for 48h. Afterwards, take out to measure OD value and calculate BF forming rate to determine the lactic acid bacteria BF forming.

The impact of culture temperature on the BF formation

Inoculate the three lactic acid bacteria strains into 24-well micro-titer dishes respectively and incubated at different culture temperatures (22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 35 and 37°C) for 48 h. Afterwards, measure BF forming rate by the same approach.

The effect of inoculum size on the BF formation: Dilute the three 3rd-generation strains with the doubling dilution method by 10^0, 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9 and 10^{10} times. Select 3 dilution solutions for the pour-plate culture of MRS solid medium to determine inoculum size. Afterwards, different diluted strain fluids are inoculated into 24-well micro-titer dishes for 48h, then measure OD value and calculate BF forming rate and determine the states of lactic acid bacteria BF formation.

The effect of mixed culture on the BF formation: The 3rd-generation strains of 18-2, 37 and 39 were mixed in pairs in ratio of 1:1 for inoculation and measurement of BF formation. While inoculating, take 10μL mixture respectively into the micro-titer plate, incubated at 37°C for 48h to determine BF formation rate.

The effect of various additives on lactic acid bacteria BF formation rate:

The effect of sucrose on the BF formation: Bacteria were inoculated into 1 ml of MRS broth in micro-titer dishes containing sucrose at 2, 4, 8, 16, 32, 64 and 128 mg/mL, respectively. After static incubation for 48 h at 37°C under aerobic conditions, BF formation rate was measured.

The effect of various NaCl concentrations on the BF formation: To test osmolality impact on biofilm

formation, 3 strains lactic acid bacteria were incubated in MRS broth with various concentration of NaCl, such as 1.5, 3, 6, 12, 24, 48 and 96 mg/mL, respectively. After static incubation for 48 h at 37°C under aerobic conditions, BF formation rate was measured.

RESULTS AND DISCUSSION

Lactic acid bacteria BF forming rate under different culture periods: *L. plantarum* 18-2, *Leuconostoc citreum* 37 and 39 were cultured in MRS fluid medium respectively and their BF forming status were measured every 12 h. The BF forming rate was shown in Fig. 1.

From the overall tendency of adhesion rate in Fig. 1, the best BF forming rate of *Lactococcus* occurred after cultured for 48h. BF forming rates descend and then ascend along with the increase in culture time. It's reported that the BF formation process can be divided into 3 phases: 0-6 h adhesive period, 6-24 h for agglomeration period and 24-72 h for mature period. In accordance to such principles, the study set up a timeline for BF formation to inspect BF forming conditions. In the beginning 10h, there was no obvious adhesion was found until 12h. On 24h, the biofilm did not adhere to the bottom very well and was easily flowed away by washing, so the adhesion rate was remarkably lower than other time point. After then the BF forming rates increased meaning it reached the mature period, as was also reported for the BF formation of *Lactobacillus pentosus* (Ren *et al*., 2014).

Lactic acid bacteria BF forming rate under different initial pH values: The most favorable pH range for *Lactobacillus* growth is generally between 5.5 and 6.2 and *Lactobacillus* also can propagate under pH 5. However, *Leuconostoc* propagates well between pH 3.0 and 6.5 and its growth rate decrease when pH value is neutral or initially alkaline. To test the impact of pH to BF forming, 3 strains LAB was cultivated in MRS broth with different pH value, which were chosen that did not considerably influence growth in suspension (data not shown).

From Fig. 2, the LAB adhesive rate has the tendency of increasing first and then decreasing. At pH 5.6, 18-2 strain is reported with the optimal adhesive rate up to 1.0119; at pH 5.4, 37 is recorded with the best adhesion rate to 1.2687; and at pH 5.5, 39 registers the optimal adhesive rate to 1.4361. Above all, lactic acid bacteria BF forming was the best at approximately pH 5.5 and inhibited obviously near neutral pH value. As reported by Lebeer *et al*. (2007a), the biofilm formation by *L. rhamnosus* GG was inhibited at an initial pH of 4.0 in all media tested, in contrast to neutral pH in the control media. The differences could be attributable to the difference species.

Lactic acid bacteria BF forming rate under different cultivation temperatures: External temperatures play

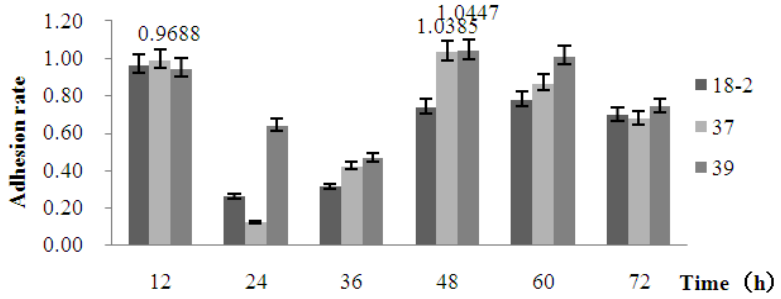

Fig. 1: Lactic acid bacteria BF forming result under different cultivation time

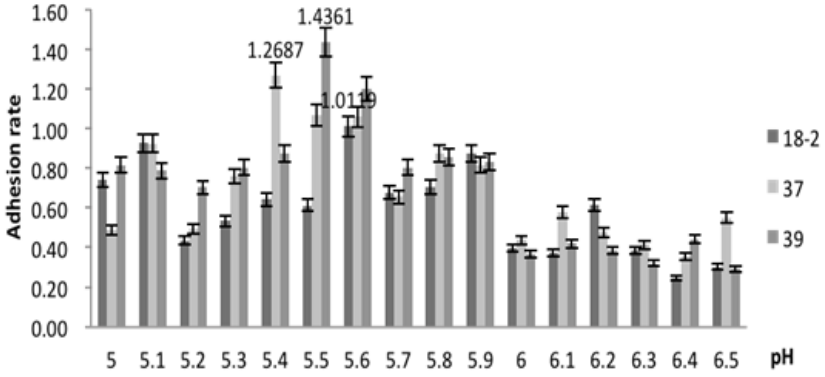

Fig. 2: Lactic acid bacteria BF adhesion result under different pH values

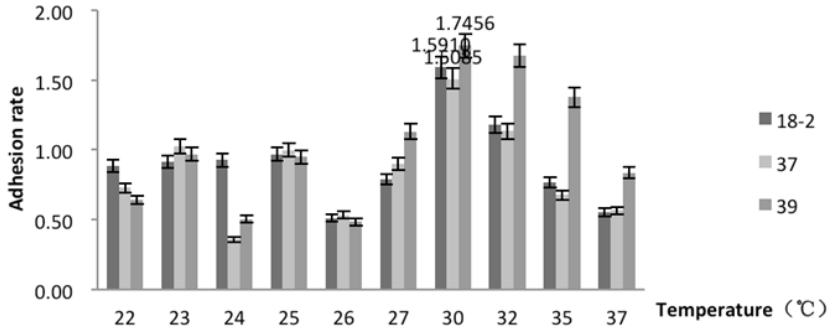

Fig. 3: Lactic acid bacteria BF adhesion result under different temperatures

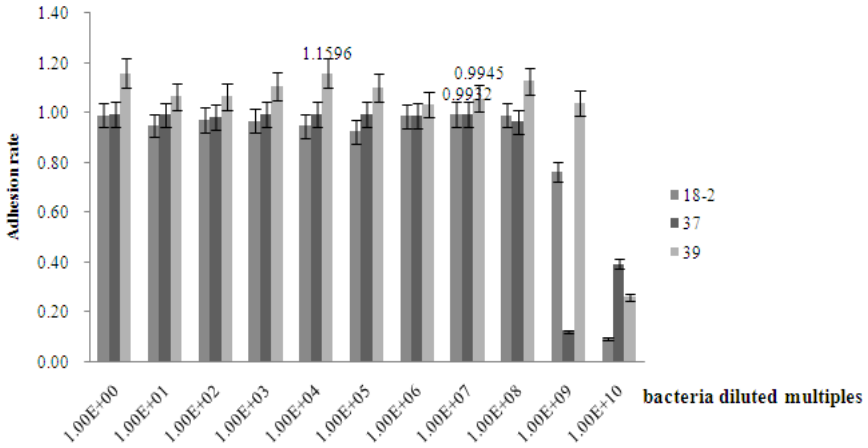

Fig. 4: Lactic acid bacteria BF adhesion result under different inoculum sizes

significant role in the bacteria propagation and their metabolites, cause the changes in temperature can affect the expression of metabolite genes. The three strains were cultured at various cultivating temperature for 48 h at pH 5.5 and displayed changing biofilm forming ability as Fig. 3 shown.

From the overall tendency of Fig. 3, as temperature increases, the BF forming rate firstly ascends and then descends. At the temperature of 30°C, the three strains present the best adhesive rates as 1.5085, 1.5910 and 1.7456 respectively. In relation to the impact of temperature to BF forming, there were several different reports. Ren *et al*. (2014) reported the cultivating temperature did not influence much on the BF forming of *L. pentosus*. Otherwise, Xie *et al*. (2011) found the low temperature was not benefit for BF forming to LAB isolated from spoiled food but 37 and 42°C were the favorite temperature.

Lactic acid bacteria BF forming rate under different inoculum sizes: From Fig. 4, as bacteria mother liquor is diluted, bacteria propagation goes weaker, yet BF formation was stable without any decreases before 10^8 dilutions. While by 10^9, BF adhesive rate decreases sharply and BF cannot be formed basically at 10^{10}. It was the bacteria density dependent test that could initially confirm the biofilm-forming ability was regulated to Quorum Sensing (QS). QS System refers to an information transition mechanism between cells, to control bacteria flora performance by compounding and decomposing Auto-Inducer (AI) concentrations (Fuqua *et al*., 1994). When AI concentration reaches certain threshold value along with bacteria flora density, the specific gene expression is initiated. As results, the BF forming ability showed a certain density dependent effect, but the confident certify need further date. QS System maybe adjusts its specific gene expression to secure the formation of functional BF. For LAB, in the recycling of S-adenosylmethionine (SAM), the LuxS enzyme catalyzes the conversion of S-ribosylhomocysteine, yielding AI-2 and homocysteine. Previously, we have shown that the *L. rhamnosus* GG *luxS* mutant CMPG5412 is affected in biofilm formation in AOAC medium (Lebeer *et al*., 2007b).

Lactic acid bacteria BF forming rate under different mixed culture: The natural bacteria biofilm was not formed by single species but mixed species. In the period of biofilm development, maturation and the release of bacteria from mature biofilms, complex sequential mechanisms is required for which cross-species cell-to-cell communication and/or interspecies quorum sensing (QS) might be important factors (Abee *et al*., 2011).

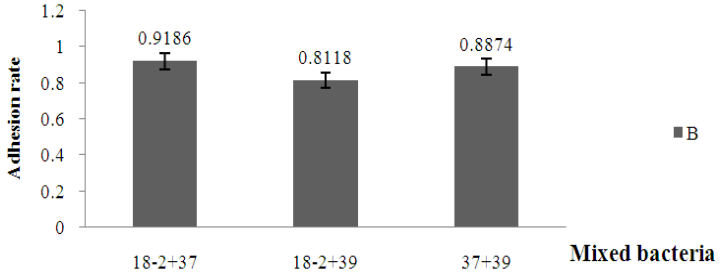

Fig. 5: Lactic acid bacteria BF adhesion result under different mixed culture

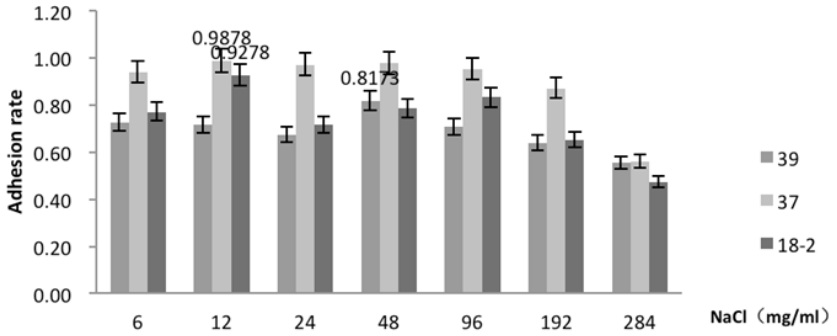

Fig. 6: Lactic acid bacteria BF adhesion result under different NaCl concentration

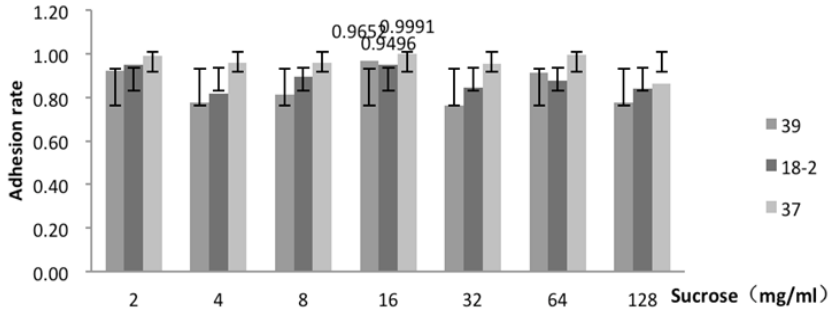

Fig. 7: Lactic acid bacteria BF adhesion result under different sucrose concentration

From Fig. 5, we can see that no significant difference in BF formation rate when being cultured by paired mixture, but smaller than those cultured separately. That may result from bacteria strains compete with each other and the adjustment of QS system, causing decreased BF formation rate. However, 18-2 and 37 mixed strains were reported with the best BF forming rate among mixed bacteria.

Lactic acid bacteria BF forming rate under different NaCl concentration: *Leuconostoc* usually can live with osmotic pressure, growing in the 4-6% NaCl culture medium. From Fig. 6, NaCl can significantly inhibit the adhesive rate of three strains respectively. As NaCl concentration increases, the adhesive rate changes from firstly increasing to decreasing. The highest bacteria adhesive rate among the three strains occurs when NaCl concentration is 12 mg/mL, but the highest adhesion rate (about 0.8-0.9) was obviously lower than that of mediums without NaCl. Osmotic pressure is an important stressful condition for bacteria biofilm. It was also reported that high osmolality highly modulate biofilm formation of *L. rhamnosus* GG, but the effect depends on the microenvironment (i.e., the culture medium) (Lebeer *et al*., 2007a).

Lactic acid bacteria BF forming rate under different sucrose concentration: From Fig. 7, when sucrose is added to culture medium, the adhesive rate of the three strains is reported lower than that without sucrose added, which may be the utilization effect of disaccharides not as good as that of monosaccharide. As the sucrose concentration increases, there is no significant difference in adhesive rate of the three strains. The influence of some growth medium components on BF formation was also mentioned by Lebeer *et al*. (2007a). They found *L. rhamnosus* GG did not form biofilms in the standard MRS medium. When glucose was omitted from MRS medium, biofilm formation could be observed. Otherwise, in contrast to

L. rhamnosus GG, no biofilm formation in glucose-depleted MRS medium could be observed for the other *L. rhamnosus* and *L. casei* strains tested.

CONCLUSION

The BF adhesive rate of lactic acid bacteria, *Lactobacillus plantarum* 18-2, *Leuconostoc citreum* 37 and 39, was detected under different conditions. It concluded that without any additives, *L. plantarum 18-2* strains had the highest adhesive rate, when cultivated for 12 h in pH 5.6 at 30°C, while 48 h cultivation time, in around pH 5.5 at 30°C are more favorable for the BF formation of *L. citreum* 37 and 39. Thus it can be seen that BF forming is determined by certain internal and external conditions. As the BF forming conditions are different between *L. citreum* 37 and 39 and *L. plantarum* 18-2, species also holds a key to BF formation could be species-dependent.

When NaCl and sucrose are added to culture medium, BF formation of LAB was apparently impacted, especially their concentration increased. In addition, BF adhesive rate of mixed culture of strains was lower than that of single strains possibly as a result of competition among them. The results in this study indicate the significance of studying biofilms of LAB in the food industry, which can lead to the development of reliable techniques for controlling LAB contaminants from raw ingredients or for offering common reference for improving LAB survival ability to benefit people's lives.

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