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The nature of adhesion factors which lie on the surfaces of *Lactobacillus* adhering to cells

Xueyan Lin, Zhonghua Wang*, Zhongxiang Niu*, Jun Peng, Yun Wang

College of Animal Science and Technology, Shandong Agricultural University, Tai'an, China
Email: linxueyan@sdau.edu.cn, *zxniu@sdau.edu.cn, *zhwang@sdau.edu.cn

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ABSTRACT

***Lactobacillus* adheres to intestinal epithelial cells and yeast fungus cells with the aid of adhesion factors expressed on its cell surface. To identify adhesion factors nature on the surface of *Lactobacillus*, an adhesion experiment was carried out by pre-treating the *Lactobacillus* supernatant with different concentrations of bovine serum albumin, trypsin and 100˚C for 10min. Additionally, intestinal epithelial cells were treated with sodium iodate, trypsin and sugar inhibition tests to characterize the receptors in *Lactobacillus* that interact with intestinal epithelial cells. It was demonstrated that *Lactobacillus* adhesion ability was decreased ($P < 0.01$) after treating the supernatant with different concentrations of bovine serum albumin, trypsin and 100˚C for 10 min respectively. The adhesion factor on the surface of *Lactobacillus* cells was identified as a D-mannose glycoprotein. This observation was confirmed after treating intestinal epithelial cells with sodium iodate and trypsin, and sugar inhibition tests. Wild type *Lactobacillus* can agglutinate yeast fungus cells but after being exposured to mannose, agglutination to yeast fungus cells is lost or reduced. Results from this study we also got that inactivated and live bacteria that similarly adhere to intestinal epithelial cells.**

Keywords: *Lactobacillus*; Adhesion; Intestinal Epithelial Cells; Adhesion Factors Nature

1. INTRODUCTION

The cell surface of *bifidobacterium* cells consists of LTA (lipid teichoic acid) and protein while adhesion substances on the surface of *Lactobacillus* cells are not well characterized and likely consist of protein [1]. It was demonstrated that adhesion substances on the *bifidobacterium* cell surface was present in the supernatant. Based on this conclusion, we isolated the supernatant in *Lactobacillus* cultures by high speed centrifugation, and performed various measures to identify the unknown adhesion substance [2-5]. Earlier research suggests that the *bifidobacterium* adhesion receptor needed for attachment to intestinal epithelial cells is a glycoprotein. Similarly, our study identifies the *Lactobacillus* receptor that allows for efficient attachment to intestinal epithelial cells.

*Corresponding authors.

2. MATERIALS AND METHODS

2.1. CaCo-2 Cells

A human colon cancer cell line, which is equivalent to intestinal epithelial cells, was purchased from the Shanghai Cell Research Institute.

CaCo-2 cell culture: DMEM media containing 10% bovine serum (penicillin 1,000,000 IU/L, streptomycin 100 μg/L) (pH: 7.0 ~ 7.2); flushing liquor PBS (pH 7.4); cell digestive juice (0.02% EDTA: D-Hanks = 10:1) (not including Ca^{2+}, Mg^{2+}); Passing was done when oblate cells overgrew in the culture flasks and was done by adding 3 ml digestive juice and digesting for 20 ~ 30 min. Digestion was stopped when the cell wall fell off, at which point we incubated the culture flask for 3 min, and flushed the flask with 10% DMEM. Cells were collected after removing the digestive juice.

2.2. *Lactobacillus* Bacterial Strain

Bacterial strain got from Bacteria Research Institute of Shandong Agricultural University. The $SD_n A_3$ bacterial strains, which adhesion ability were validated as efficient in a previous experiment, were cultured at 37˚C for 2 days. ("A" stands for bacterial strains derived from SPF chicken craws)

2.3. The Method for Identifying Adhesion Factors Expressed on the Surface of *Lactobacillus* Cells

Culture supernatants were acquired after the *Lactobacillus thalli* suspension was centrifuged at 5000 rpm for 30 min.

1) The number of *Lactobacillus* cells that adhered to intestinal epithelial cells were counted after incubating cultures with different concentrations of trypsin (37˚C, 30 min). The concentrations of trypsin selected were as follows: 0, 5, 10, 15 and 20 mg/ml.

2) The number of *Lactobacillus* cells that adhered to intestinal epithelial cells were counted after incubating cultures with different concentrations of bovine serum albumin (37˚C, 30 min). The concentrations of bovine serum albumin selected were as follows: 0, 5, 10, 15 and 18 mg/ml.

3) The number of *Lactobacillus* cells that adhered to intestinal epithelial cells were counted after incubating cultures at 100˚C for 10 min.

Fresh liquid culture medium (MRS) was marked as a control I. Each treatment group was combined with *Lactobacillus* cells after rinsing with PBS and centrifuging 5 times prior to performing the adhesion experiment. *Lactobacillus* thalli structures were rinsed with PBS (PH 7.4) and centrifuged 5 times (control II).

Adhesion experiment: Intestinal epithelial cells were added each well of a 24-well plate (with the cover-glass slip placed inside) and were allowed to adhere for 2 - 3 days. $SD_n A_3$ (200 μl) bacterial strains in each well were given different treatments and maintained at a concentration of 1×10^7/ml. Cells were then transferred to an incubator for 4 - 24 h, rinsed with PBS (pH 7.4) 6 times after removing the cover-glass slip, air-dried, fixed with carbinol, given a Gram stain test and counted under the microscope (20 eye captures; 40 counted cells; counted by 2 technicians).

2.4. The Method for Identifying Adhesion Factors Nature on the Surface of Intestinal Epithelial Cells

Intestinal epithelial cells were added to 24-well plates (with a cover-glass placed inside) and allowed to adhere for 2 - 3 days. *Lactobacillus* cells (200 μl) were added to each well and maintained at a concentration of 1×10^7/ml. Cells were then transferred to an incubator for 2 - 24 h, rinsed with PBS (pH 7.4) 6 times after removing the cover-glass slip, air-dried, fixed with carbinol, given a Gram stain test and counted under the microscope (20 eye captures; 40 counted cells; counted by 2 technicians). The average and standard deviations for each sample were also calculated.

2.4.1. Disposal of Cells with Sodium Periodate

The adhesion experiment was carried out with different concentrations of sodium periodate (37˚C, 30 min), which was added to CaCo-2 cells after rinsing with PBS (pH 7.4) once. 0.2 M acetate buffer (pH 4.6) was used to dilute sodium periodate and used as a control. The variable concentrations of sodium periodate were used were as follows: 0, 20, 40, 60, 80 and 100 mg/ml.

2.4.2. Treatment of Cells with Trypsin

The adhesion experiment was carried out with different concentrations of trypsin (37˚C, 30 min), which was added to CaCo-2 cells after rinsing with PBS (pH 7.4) once. 0.2 M acetate buffer (pH 4.6) was diluted with trypsin and used as a control. The different concentrations of trypsin used were as follows: 0, 5, 10, 15, 20 and 25 mg/ml.

2.4.3. Sugar Inhibition Tests

Adhesion experiments were done after adding glucose, lactose, sorbose, sucrose, D-fructose or D-mannose (at a final concentration of 20 mg/ml) to Lactobacillus suspensions. As a control, PBS (pH 7.4) was added to suspensions.

Adhesion experiment: Intestinal epithelial cells were added to 24-well plates (with a cover-glass slip inside) and allowed to adhere for 2 - 3 days. *Lactobacillus* cell suspensions (200 μl, each with two replicates) were treated with various sugar concentrations and transferred to an incubator for 4 - 24 h after removing the cover-glass slip. Suspsensions were then rinsed with PBS (pH 7.4) 6 times, air-dried, fixed with carbinol, given a Gram stain test and counted under a microscope (20 eye captures; 40 counted cells; counted by 2 technicians). The averages and standard deviations were also calculated.

2.5. Characterizing the Effect of *Lactobacillus* on Yeast Fungus Agglutination

One sample of *Lactobacillus* cells was treated with 0.2 M mannose while the other sample was not. Cells were then monitored for yeast fungus agglutination induced by *Lactobacillus*.

2.6. The Effect of Inactivated and Live Bacteria on *Lactobacillus* Adhesion Ability

Treatment: SD_nA_3 strains were derived from chicken intestinal epithelial cells after culturing for 2 days and suspensions were maintained at a concentration of 1×10^8 Cfu/ml. The supernatants were isolated after centrifuging at 5000 rpm for 15 min. pH 7.4 PBS buffer was used to rinse bacteria and the acquired suspensions were grouped as either live bacteria or inactivated bacteria. The inactivated bacteria group consisted of cells placed in a 65˚C water bath for 30 min.

Suspensions of live bacteria and inactivated bacteria were centrifuged and rinsed with PBS 3 times, the supernatant removed, PBS (pH 7.4), the *Lactobacillus* cultured supernatant and fresh MRS culture liquids were used to dilute the suspension of live and inactivated centrifuged bacteria individually (via the gradient dilution

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method). Bacterial adhesion ability was quantified at different bacterial suspension concentrations.

2.7. Statistics

Between treatment were assessed utilizing with the ANOVA procedure and Duncan's Multiple Range Test was used for multiple comparisons analyze the Strains effects adherence to the cells.

3. RESULTS

3.1. The Effect of Different Concentrations of Trypsin on the Adhesion Ability of *Lactobacillus* (Table 1)

It was demonstrated in **Table 1** that *Lactobacillus* supernatants treated with trypsin decreased the ability of *Lactobacillus* cells to adhere to CaCo-2 cells. The number of CaCo-2 cells that adhered to *Lactobacillus* decreased with increasing concentrations of trypsin.

3.2. Effects on the Adhesion Ability of *Lactobacilus* Due to Treatment with Different Concentrations of Bovine Serum Albumin (Table 2)

It was demonstrated in **Table 2** that *Lactobacillus* supernatants treated with bovine serum albumin decreased the ability of *Lactobacillus* to adhere to CaCo-2 cells. The number of CaCo-2 cells that adhered to *Lactobacillus* decreased with increasing concentrations of bovine serum albumin.

Table 1. *Lactobacillus* adhesion levels after treating *Lactobacillus* supernatants fluid with different concentrations of trypsin.

trypsin concentration (mg/ml)	the number of *Lactobacillus* adhering to each cell
0	20.20 ± 1.70^{c}
5	12.30 ± 1.05^{b}
10	5.20 ± 0.80^{a}
15	5.00 ± 0.91^{a}
20	4.00 ± 1.09^{a}

Annotate: Different superscripts a, b, c denote that the difference is notable ($p < 0.01$).

Table 2. *Lactobacillus* adhesion level after treating *Lactobacillus* supernatants with different concentrations of bovine serum albumin.

bovine serum albumin concentration (mg/ml)	the number of *Lactobacillus* adhering to each cell
0	20.20 ± 1.70^{a}
5	9.30 ± 1.52^{b}
10	7.30 ± 0.91^{b}
15	8.30 ± 1.05^{b}
18	5.90 ± 0.85^{b}

Annotate: Different superscripts a, b denote that the difference is notable ($p < 0.01$).

3.3. The Effect on *Lactobacillus* Adhesion Ability after Heating with Up to 100˚C (Table 3)

It was demonstrated in **Table 3** that after treating the supernatants with high temperature, the adhesion ability of *Lactobacillus* decreased. A likely explanation is that there is an adhesion substance (possibly protein) in the *Lactobacillus* supernatant.

3.4. The Effect on *Lactobacillus* Attachment to CaCo-2 Cells Treated with Different Concentrations of Sodium Periodate (Table 4)

It was demonstrated in **Table 4** that in contrast with the control group, the adhesion ability of *Lactobacillus* was decreased after CaCo-2 cells were treated with sodium periodate. The receptor on the surface of CaCo-2 cells is likely a glycoprotein. Sodium periodate breaks the carbon-carbon bond of the hydroxyl group of sugar, and likely damages the adhesion factor.

3.5. Differences in the Adhesion Ability of *Lactobacillus* after CaCo-2 Cells Were Treated with Different Concentrations of Trypsin (Table 5)

It was demonstrated in **Table 5** that in contrast with the

Table 3. *Lactobacillus* adhesion level after treating *Lactobacillus* supernatant fluid with 100˚C.

treatment	the number of *Lactobacillus* adhering to each cell
100˚C	9.60 ± 0.69^{b}
37˚C	20.20 ± 1.70^{c}
PBS Control	4.40 ± 0.54^{a}
MRS Control	6.70 ± 1.13^{ab}

Annotate: Different superscripts a, b, c denote that the difference is notable ($p < 0.01$).

Table 4. *Lactobacillus* adhesion level after CaCo-2 cells were treated with different concentrations of sodium periodate.

sodium periodate concentration (mg/ml)	the number of *Lactobacillus* adhering to each cell
0	20.20 ± 2.25^{b}
20	16.60 ± 2.12^{b}
40	8.20 ± 1.31^{a}
60	7.50 ± 0.73^{a}
80	7.30 ± 1.37^{a}
100	5.50 ± 0.91^{a}

Annotate: Different superscripts a, b denote that the difference is notable ($p < 0.01$).

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Table 5. *Lactobacillus* adhesion levels after CaCo-2 cells were treated with different concentrations of trypsin.

trypsin concentration (mg/ml)	the number of *Lactobacillus* adhering to each cell
0	20.20 ± 2.15^{a}
5	14.10 ± 1.29^{b}
10	10.90 ± 0.84^{b}
15	13.20 ± 1.35^{b}
20	13.30 ± 1.46^{b}
25	12.70 ± 1.04^{b}

Annotate: Different superscripts a, b denote that the difference is notable ($p < 0.01$).

control group, the adhesion ability of *Lactobacillus* decreased after CaCo-2 cells were treated with trypsin (it acts on peptide sequences). These results suggest that the receptor that is expressed on the CaCo-2 cell surface is a type of protein.

3.6. The Effect on *Lactobacillus* Adhesion Ability after Treatment of CaCo-2 Cells with Different Sugars for 4 h (Table 6)

It was demonstrated in **Table 6**, **Figures 1** and **2** that the difference in adhesion ability was not significant after *Lactobacillus* was treated with glucose, lactose, sorbose, sucrose and D-fructose; yet the difference in adhesion ability was significant after *Lactobacillus* was treated with D-mannose，demonstrating that the adhesion receptor of SD_nA_3 strains may be the D-mannose protein receptor.

3.7. Treatment Effects on *Lactobacillus* Agglutination to Yeast Fungus

When *Lactobacillus* was treated with 0.2 M mannose, its agglutination to yeast fungus cells weakened compared to untreated *Lactobacillus* cells (**Figures 3** and **4**).

3.8. The Effect of Inactived Bacteria and Live Bacteria Introduction on *Lactobacillus* Adhesion Ability (Table 7)

It was demonstrated in **Table 7** that live and dead *Lactobacillus* cells have similar adherence levels. Adherence matter is in the 2 days cultured supernatant. This observation suggests that the protein in the supernatant retains its adhesion ability regardless of whether it is activated or inactivated.

4. DISCUSSION

1) Previous research suggests that dead *Lactobacillus* and inactivated *bifidobacterium* have similar adhesion ability compared to live bacteria. This observation is in

Table 6. Adhesion levels of *Lactobacillus* after CaCo-2 cells were treated with different sugars.

sugar	the number of *Lactobacillus* adhering to each cell
glucose	15.20 ± 0.90^{a}
lactose	15.60 ± 0.79^{a}
sorbose	14.40 ± 0.56^{a}
sucrose	14.50 ± 0.45^{a}
D-fructose	16.20 ± 0.90^{a}
D-mannose	5.10 ± 1.12^{b}
control	16.10 ± 0.96^{a}

Annotate: Different superscripts a, b denote that the difference is notable ($p < 0.01$).

Table 7. Live and inactived *Lactobacillus* adhesion ability to intestinal epithelial cells.

treatment	the number of *Lactobacillus* adhering to each cell
live lactobacillus in PBS	7.30 ± 0.60^{a}
live lactobacillus in MRS	12.60 ± 3.15^{b}
live lactobacillus in supernatant	20.90 ± 1.83^{c}
inactived lactobacillus in PBS	6.40 ± 0.65^{a}
inactived lactobacillus in MRS	12.00 ± 1.75^{b}
inactived lactobacillus in supernatant	23.00 ± 2.31^{c}
inactived lactobacillus 10^{-1}	12.10 ± 1.08^{b}
inactived lactobacillus 10^{-2}	7.30 ± 0.87^{a}

Annotate: Different superscripts a, b, c denote that the difference is notable ($p < 0.01$).

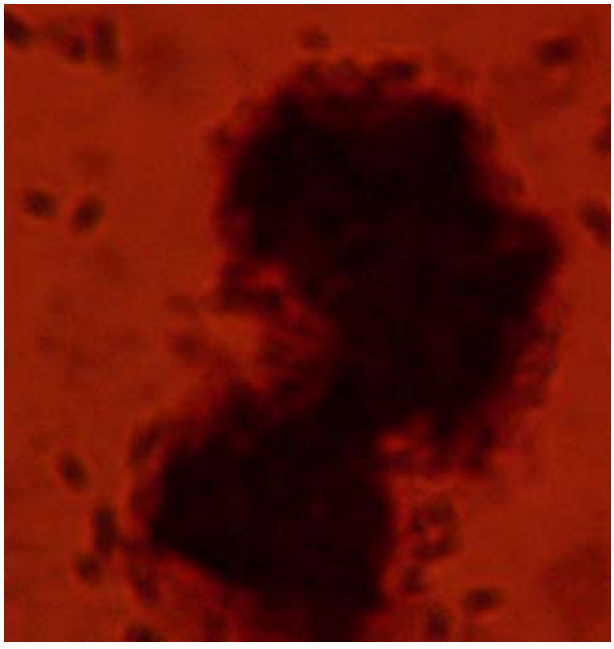

Figure 1. *Lactobacillus* can firmly adhere to CaCo-2 cells prior to treatment with D-mannose.

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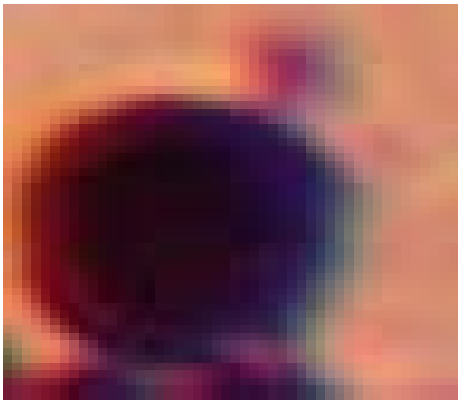

Figure 2. *Lactobacillus* adhesion to CaCo-2 cells decreased after treatment with D-mannose.

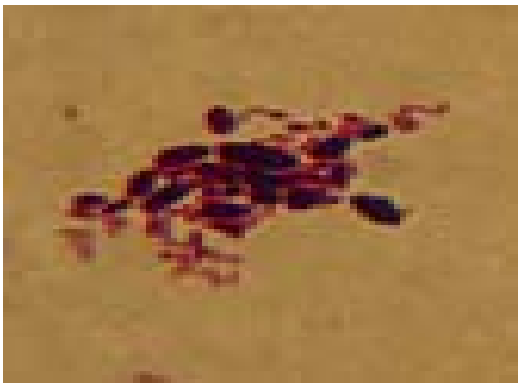

Figure 3. Untreated *Lactobacillus* can agglutinate to yeast fungus cells.

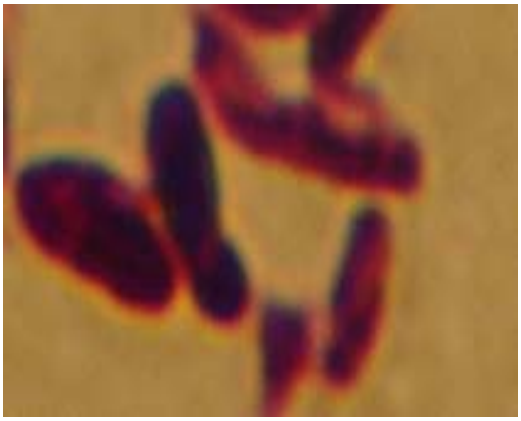

Figure 4. *Lactobacillus* agglutination to yeast fungus cells decreases after treatment with D-mannose.

agreement with our findings.

2) Our experimental results demonstrate that *Lactobacillus* adhesion ability decreased when CaCo-2 cells were incubated with digestive juice containing trypsin but was not true for CaCo-2 cells digested with digestive media containing no trypsin. This result demonstrates that the receptor expressed on the intestinal epithelial cells that is responsible for successful *Lactobacillus* attachment is likely a protein.

3) Gusilsc (1999) report that certains strains of *Lactobacillus* can agglutinate yeast fungus cells treated with glutaraldehyde and glycine but the agglutination is inhibited when *Lactobacillus* is treated with 0.2 M mannose [3]. This result is consistent with the findings of our study.

5. CONCLUSION

Our results indicate that the substance on the surface of *Lactobacillus*, that promotes adhesion to intestinal epithelial cells, is likely a kind of protein. Based on our findings we also predict that the adhesive substance is present in *Lactobacillus* supernatants and that the recaptor for this substance is likely a D-mannose glycoprotein receptor expressed by intestinal epithelial cells. *Lactobacillus* cells were able to agglutinate yeast fungus cells, *Lactobacillus* receptor above in it is related to mannose, we reveal that both inactived bacteria and live bacteria have a similar ability to adhere to intestinal epithelial cells.

6. ACKNOWLEDGEMENTS

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