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Isolation and Screening of Lactic Acid Bacterial Strains with Antibacterial Properties from the Vaginas of Healthy Cows

Chao Cheng[1*], Qiaozhen Tian[2*], Shaofeng Su[3,4], Hui Zhang[1], Hongjie Guo[1], Pengfei Gao[5], Songhe Xu[1], Zhiguo Liu[6], Yanfen Ma[7], Zhenjun Li[6]

[1]School of Sciences Life, Jining Normal University, Jining, China
[2]Shanxi Province Datong University, Datong, China
[3]Inner Mongolia Academy of Agriculture and Animal Husbandry Sciences, Hohhot, China
[4]Inner Mongolia Agricultural University, Hohhot, China
[5]Vocational and Technical College of Ulanqab, Jining, China
[6]National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, China
[7]School of Agriculture, Ningxia University, Yinchuan, China
Email: liuzhiguo@icdc.cn, ma2999@163.com, Zhenjunli@icdc.cn

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Abstract

In the present study, eight strains were isolated from 20 cow vagina samples and identified using phenotype, biochemical analysis, sugar fermentation tests, and 16S rRNA sequence analysis. Among eight strains, only SQ0048 was identified as *Lactobacillus johnsonii* based on a series of biochemical testing (including the adhesion test, catalase test, bacteriocin production test, antibacterial test, and pH value), suggesting that its biological activity was superior to the other seven strains. Furthermore, SQ0048 had the lowest pH value (4.32) and the shortest fermentation time (8 h) compared with the other strains. The adhesion rate of SQ0048 was significantly higher than that of *Lactobacillus delbrueckii*, with an average adhesion number of 304 ± 2.67. The hydrogen peroxide production testing in SQ0048 was positive; in addition, bacteriocin gene of SQ0048, encoding an approximately 10-kDa product, was successfully cloned, expressed, and detected using the SDS-PAGE method. Meanwhile, SQ0048 had a weak inhibitory effect on *Staphylococcus aureus* and *Escherichia coli*. However, the expression products of the bacteriocin gene of SQ0048 had a very strong inhibitory effect on *S. aureus* and *E. coli,* with inhibition zone sizes of 18 ± 0.45 mm and 15 ± 0.60 mm, respectively. These data showed that SQ0048 has excellent antibacterial properties compared with other isolated

*These authors contributed equally to this work.

strains and is a potential probiotic candidate to improve the health of the vaginas of cows by inhibiting pathogenic microorganisms.

Keywords

Antibacterial Properties, Cow, Lactic Acid Bacteria, SQ0048, Vagina

1. Introduction

Pathogenic microorganisms can enter the uterus of postpartum cows via the vagina because of cervix expansion. This can cause endometritis and reduce fertility and milk production in cows [1]. Probiotics are well known because they are generally regarded as safe and can provide many health benefits, such as producing dairy products, food preservatives, and drugs [2] [3]. The normal microecological environment of the cow vagina is a complex and dynamic ecosystem inhabited by lactic acid bacteria (LAB) [4] [5] [6]. LAB are generally composed of beneficial strains that can protect the vaginal environment from invasion by potentially harmful microorganisms and increase the number of immune cells, further boosting immunity. Bacteria infecting the vagina of dairy cows mainly include *Escherichia coli* and *Staphylococcus aureus* [6]. MacIntyre DA. *et al.* (2015) perfused LAB (*Lactobacillus sakei* FUA 3089, *Pediococcus acidilactici* FUA 3140, and *Pediococcus anacidilactici* FUA 3138) isolated from the vaginas of cows, showing that the procedure could reduce the number of *E. coli*, reduce purulent vaginal discharge, and improve the animal's health [7]. Genís S *et al.* [8] injected LAB into the vaginas of cows to reduce the number of pathogenic bacteria and uterine infections, regulate local and systemic immune responses, and improve cow health. The anti-pathogenic activity of LAB appears to be related to the production of lactic acid, hydrogen peroxide, bacteriocin, and other active substances. It is also related to the competition for adhesion between LAB and pathogenic bacteria. Lactic acid protects the body tissues by maintaining a pH value between 4 and 4.5 that creates an unsuitable environment for pathogenic microorganisms [9]. Hydrogen peroxide (H_2O_2) is also a defense factor used by LAB against pathogenic microorganisms [10]. In addition, bacteriocins are proteins or protein complexes synthesized by the ribosomes of LAB, which have strong antibacterial ability. They can kill or inhibit some Gram-positive bacteria, Gram-negative bacteria, and certain fungi by preventing DNA and protein synthesis while also destroying the integrity of the cell wall. LAB shows strong adhesion to the stratified non-keratinized epithelium, which can repel or inhibit pathogenic microorganisms such as *Candida albicans*, *Gardnerella vaginalis*, *E. coli*, and *S. aureus* [11]. Thus, the objective of this study was to isolate and screen the LAB strain with superior antimicrobial qualities that could be used as a candidate strain to prevent and treat vaginal inflammation in dairy cows.

2. Materials and Methods

2.1. Ethics Statement

All animal experiments were conducted according to the practices and standards approved by the Animal Welfare and Research Ethics Committee of Jining Normal University (Inner Mongolia, China) (Approval ID: 41, 010, 620-1), and all efforts were made to minimize animal suffering.

2.2. Vaginal Samples

Twenty Holstein-Friesian breed adult cows were selected because of the convenience of feeding and management; that is, they were fed an ordinary diet alone for 30 days. Veterinarians diagnosed these selected cattle as having no history of metritis infection from Qing Chuang Ranch and Dairy Cooperative Ranch in Hohhot in Inner Mongolia in 2017. Then, the vulva was washed with povidone-iodine and normal saline. The back area of the vagina was wiped with a sterile cotton swab. The sterile cotton swab was then transported in a sterile test tube in an icebox and was rapidly transferred to the microbiology laboratory of Jining Normal University for conducting experiments.

2.3. Isolation and Identification of the LAB Strains

The LAB strains were isolated from the above experimental cotton swab. The sample was diluted in phosphate-buffered saline (PBS) and inoculated on yeast extract, peptone, and tryptone agar (Qingdao Hope Bio-Technology Co., Ltd., Qingdao, China), pH 6.6 (containing 0.2% bromocresol purple) culture, and then incubated at 39.5˚C for 12 - 24 h under anaerobic conditions. The yellow-edged colonies were selected as the LAB strain. The isolated strains were freeze-dried and stored in milk yeast extract (10% (w/v), skimmed milk powder, and 0.5% (w/v) yeast extract) at −80˚C [12].

Biochemical and sugar fermentation tests were performed using HBI Lactobacillus Biochemical Identification Strip (GB) (Qingdao Hope Bio-Technology Co., Ltd., Qingdao, China), and the phenotype was tested according to Bergey's Manual of Determinative Bacteriology [13]. The 16S rRNA sequence analysis was employed for further identification of these strains. According to the manufacturers' instructions, the DNA extraction was done using a Bacterial Genomic DNA extraction kit (Sigma-Aldrich, St. Louis, MO, USA). The universal primers of the 16S rRNA gene (27F and 1492R) were used, and the amplification program was performed based on previous reports [14]. Five microliters of the targeted amplified products were sequenced, and phylogenetic analysis was performed with the software MEGA 6.0 version.

2.4. Analysis of Acid Production by the Identified LAB Strains

Acid production was measured as previously described [9]. The identified strains were inoculated in LAPT broth medium at 39.5˚C for 0, 3, 5, 8, 10, 12, and 18 h in anaerobic conditions, the pH of all LAPT broth media in respective times was

detected using a pH meter. *Lactobacillus delbrueckii* ATCC 11842 was used as a positive control.

2.5. Cell Adhesion Analysis of the Identified LAB Strains

The adhesion ability of the identified strains to bovine vaginal epithelial cells (BVECs) was studied according to the procedure described before. The LAB strains were adjusted to n × 10^8 CFU/ml with DMEM/F12 (Dulbecco's modified Eagle medium: Nutrient Mixture F-12 (Gibco, Thermofischer Scientific, Illkirch, France), added into the BVECs, and cultured at 39.5˚C for 4 h in a CO_2 incubator [15]. The cells' culture solution was discarded, and the Wright-Giemsa procedure was performed on the cells. The cells were observed under a microscope, and the average number of bacteria adhering to each cell was calculated. *Lactobacillus delbrueckii* (ATCC 11842) and BVECs were used as positive or negative control. Each adherence assay was repeated three times, and the values were expressed as mean value ± standard deviation (SD).

BVECs were isolated, purified, cultured, and identified in accordance with the methods used previously [16]. The bovine vaginal tissue was washed using Dulbecco's phosphate buffered saline (DPBS) with streptomycin (100 U/ml) and penicillin (100 U/ml), and the digestion is carried out using 0.1% pronase at 4˚C for 16 h. The cells were harvested by centrifugation at 1500 rpm for 5 min. The separated cells were resuspended in DMEM/F-12 containing 15% fetal bovine serum (ExCell Biology, Shanghai, China), cultured in 5% CO_2 at 37˚C. Then, the purified BVECs were identified by immunofluorescence.

2.6. Analysis of Hydrogen Peroxide Production by the Identified LAB Strains

Hydrogen peroxide production of the strains with better acid-producing properties was determined using the catalase test [17]. TMB (3,3',5,5'-tetramethyl-benzidine) (Gibco, Thermofischer Scientific) was added to 100% ethanol, added to LAPT agar medium, and then sterilized at 121˚C for 15 min. When the temperature was 50˚C, peroxidase (Gibco, Thermofischer Scientific) was dissolved and added to the plate. The LAB strains were picked out and drawn at plate medium, then incubated at 39.5˚C for 36 h in an anaerobic environment. The plates were then taken out and left for 30 min to observe the color change under aerobic conditions. Similarly, *Lactobacillus delbrueckii* (ATCC 11842) was used as a positive control. Based on the color intensity, the strains were classified based on three levels of hydrogen peroxide production, including strong (blue), medium (brown), weak (light brown), or negative (colonies without discoloration) [17].

2.7. Detection and Analysis of the Bacteriocin of SQ0048

The bacteriocin gene of SQ0048 was detected via PCR. The DNA of the strain was extracted using a DNA extraction kit (Sigma Bacterial Genomic DNA extraction kit (Sigma-Aldrich, Louis, MO, USA). The primers were designed based

on the *Lactobacillus johnsonii* NCC 533 (NC_005362.1) sequence from GenBank with 99% similarity to the strain. *BamH* I and *Xho* I (NEB, Boston, MA, USA) restriction sites were added to the 5' end of the primer. FP (LafAY-F): 5'-CGCGGATCCATGAAACAATTTAATTATTTATCACA-3', RP (LafAY-R): 5'-CCGCTCGAGCTACTTTCTTATCTTGCCAAAA-3'.

The amplification program was as follows: 94˚C/5 min; 35 cycles of 94˚C/30 sec, 50˚C/30 sec, and 72˚C/1 min; and final extension 72˚C/10 min. The PCR product was purified and linked with pMD19-T vector (TaKaRa, Dalian, China) to construct a plasmid and was transformed into competent *E. coli Trans*1-T1 cells (TransGen Biotech, Beijing, China). The positive clones, pMD-LafAY and pET-28a (+), were digested by *BamH* I and *Xho* I, respectively, and ligated by T_4 ligase to form a recombinant plasmid (pET-LafAY). The recombinant plasmid (pET-LafAY) was transformed into competent *E. coli* BL21/DE3 cells (Supplement **Figure 1**). The constructed expression product with 1 mM IPTG (isopropyl *β*-D-thiogalactoside) (Thermo, Waltham, MA, USA) was cultured at 37˚C for 3 h in LB broth [18]. Then, the cells were centrifuged at 6000 r/min for 10 min at 4˚C. The bacteria were collected, resuspended, and broken. Bacteriocin gene expression was detected using SDS-PAGE. The SDS-PAGE experiment was performed

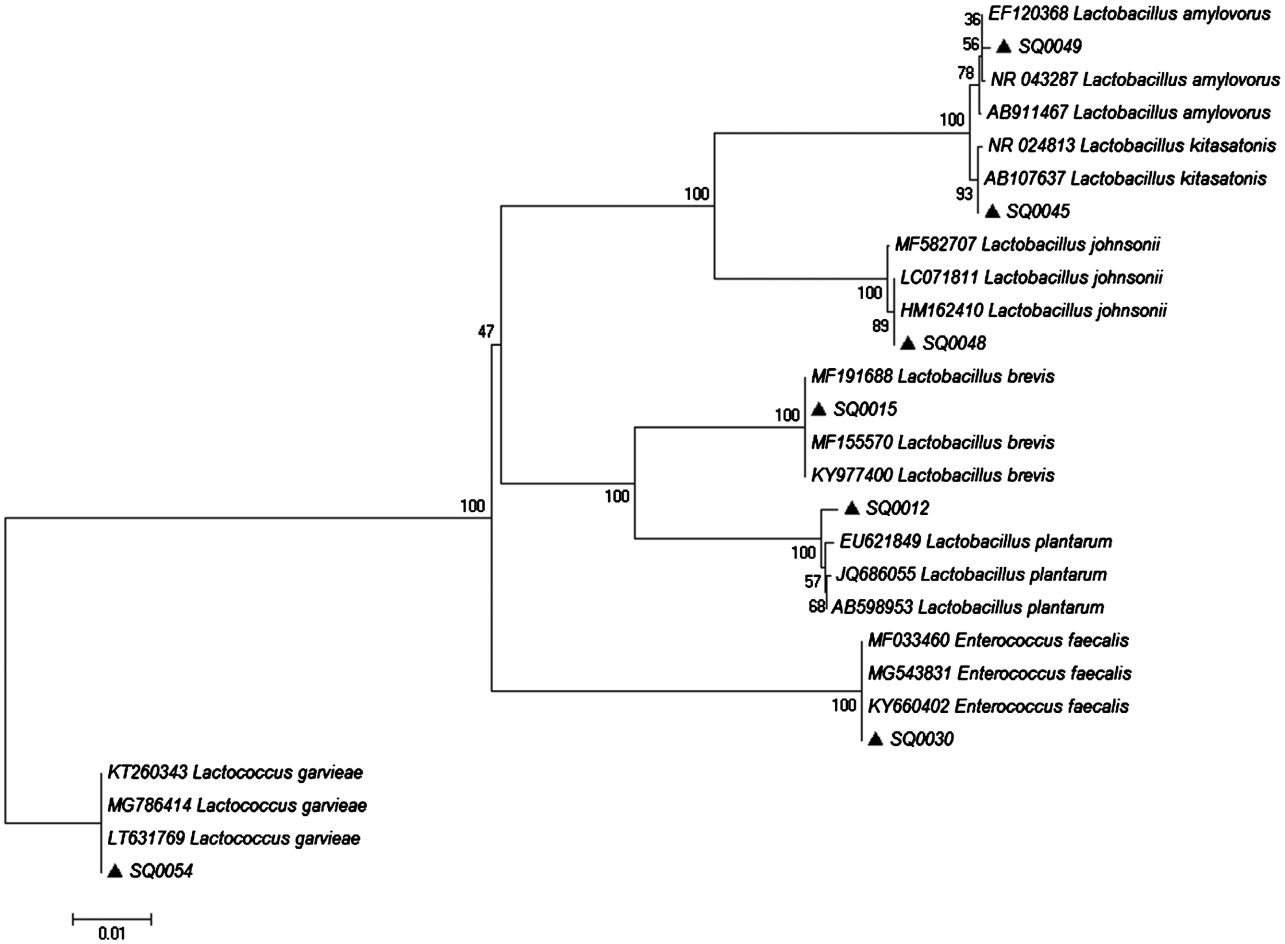

Figure 1. The phylogenetic tree based on the 16s rRNA gene in all tested strains.

according to the instructions of the SDS-PAGE Gel Preparation Kit (Thermo, Waltham, MA, USA).

2.8. Antibacterial Activity Analysis of the SQ0048

This experiment was performed using the Oxford cup method [19]. The method of evaluating the antibacterial activity of LAB against pathogenic microbial strains was described in previous studies [20] [21]. The bacterial liquids of LAB, *E. coli* (ATCC11303), and *S. aureus* (ATCC9144) were separately centrifuged, washed, and adjusted to the concentration to n × 10^8 CFU/ml with PBS. *E. coli* (ATCC11303) or *S. aureus* (ATCC9144) was added onto Mueller-Hinton Agar (containing 1% glucose). Sterile Oxford cups were placed regularly at equal distances. Furthermore, LAB suspension was added into the cup and allowed to stand for 2 h, then incubated at 39.5˚C for 24 h. The PBS was used as a negative control, and Penicillin G (0.12 μg/ml; Batch number: 130437-201005; Biological Product Testing Institute, China) or Streptomycin (10 μg/ml; Batch number: 130307-201009; Biological Product Testing Institute, China) [22] was separately used as the positive control of *E. coli* (ATCC11303) or *S. aureus* (ATCC9144). Vernier calipers were used to measure the diameter of the bacteriostatic zone. The judgment criteria were inhibition range < 6 mm (refers to negative (−)), 6 - 10 mm (mild inhibition (+)), 10 - 14 mm (strong inhibition (++)), and >14 mm (very strong inhibition (+++)), which was based on the method of Abolfazl Davoodabadi *et al.* and Oxford cup size [20] [21]. The antibacterial activity of the previously obtained bacteriocin gene was tested using the experimental steps described above. All experiments were repeated three times under the same experimental conditions.

2.9. Statistical Analysis

The experimental data were analyzed using analysis of variance (ANOVA) in SPSS and GraphPad Prism 6. $^*P < 0.05$ indicates a significant difference; $^{**}P < 0.01$ indicates an extremely significant difference.

3. Results

3.1. Isolation and Identification of the LAB Strains

The eight strains were isolated from 20 samples and were further identified based on morphology, biochemical characteristics, sugar fermentation, and 16S rRNA gene sequencing (**Table 1** and **Table 2**). Among the eight strains, three were Gram-positive cocci, and the remaining five were gram-positive bacilli. All eight strains were immobile facultatively anaerobic, and the results of four biochemical experiments (including the catalase test, litmus milk test, indole test, and gelatin liquefaction test) were all negative. The results of all eight strains tested for sugar fermentation and 16S rRNA gene sequencing are shown in **Table 1** and **Table 2**, respectively. The isolated strains were finally classified as follows: SQ0012 *Lactobacillus plantarum*, SQ0015 *Lactobacillus brevis*, SQ0030

Table 1. Sugar fermentation feature in eight isolated LAB strains.

Tests	SQ0012	SQ0015	SQ0030	SQ0041	SQ0045	SQ0048	SQ0049	SQ0054
Arabic candy	−	+	−	−	−	−	−	−
Cellobiose	+	−	+	+	+	+	+	+
Seven leaves Gan	W	−	W	−	−	W	W	W
Fructose	+	+	+	+	+	+	+	+
Galactose	+	−	+	W	+	+	+	+
Glucose	+	+	+	W	+	+	+	+
Gluconate	−	−	−	−	+	−	−	−
Lactose	W	−	+	+	−	+	+	−
Maltose	+	+	+	W	+	+	+	W
Mannitol	−	−	W	W	−	−	−	W
Mannose	+	−	+	W	+	+	+	+
Pine three sugar	−	−	−	−	−	−	−	−
Honey	−	+	−	−	W	−	+	−
Cotton candy	−	−	−	−	+	−	−	−
Rhamnose	−	−	−	−	−	−	−	−
Ribose	−	+	−	−	−	−	−	W
Salicin	+	−	+	−	+	+	−	+
Sorbitol	−	−	+	−	−	−	−	−
Cane sugar	−	−	−	+	+	+	+	−
Trehalose	−	−	+	−	+	+	+	+
Xylose	−	−	−	−	−	−	−	−

Note: "+" indicates positive; "−" indicates negative; "W" indicates weak positive.

Table 2. Similarity profile of 16sRNA gene sequences of the eight LAB strains based on BLAST.

Key	Similarity	GenBank number	Interpretations
SQ0012	99%	EU621849	*L. plantarum*
SQ0015	99%	MF191688	*L. brevis*
SQ0030	99%	MF033460	*Enterococcus faecalis*
SQ0041	99%	MF033460	*Enterococcus faecalis*
SQ0045	100%	AB107637	*L. kitasatonis*
SQ0048	99%	HM162410	*L. johnsonii*
SQ0049	99%	NR043287	*L. amylovorus*
SQ0054	99%	MG786414	*L. garvieae*

Enterococcus faecalis, SQ0045 *Lactobacillus kitasatonis*, SQ0054 *Lactococcus garvieae*, SQ0048 *Lactobacillus johnsonii*, and SQ0049 *Lactobacillus amylovorus*. According to the analysis of identification results, SQ0030 and SQ0041 were the same strain. Moreover, SQ0048 formed a monophyletic clade with LC071811 *Lactobacillus johnsonii* and HM162410 *Lactobacillus johnsonii,* with a bootstrap value of 89%, and SQ0015 was monophyletic with KY977400 *Lactobacillus brevis,* MF191688 *Lactobacillus brevis,* and MF155570 *Lactobacillus brevis,* with a bootstrap value of 100%. SQ0012 formed a monophyletic clade with EU621849 *Lactobacillus plantarum,* JQ686055 *Lactobacillus plantarum,* and AB598953 *Lactobacillus plantarum,* with a bootstrap value of 100%. SQ0030 formed a monophyletic clade with MG543831 *Enterococcus faecalis*, MF033460 *Enterococcus faecalis,* and KY660402 *Enterococcus faecalis*, with a bootstrap value of 100%. SQ0049 formed a monophyletic clade with NR043287 *Lactobacillus amylovorus* and EF120368 *Enterococcus faecalis,* with a bootstrap value of 56%; SQ0045 formed a monophyletic clade with NR024813 *Lactobacillus kitasatonis* and AB107637 *Lactobacillus kitasatonis,* with a bootstrap value of 93%. SQ0054 was monophyletic with KT260343 *Lactococcus garvieae,* MG786414 *Lactococcus garvieae,* and LT631769 *Lactococcus garvieae*. The phylogenetic tree of strains is shown in **Figure 1**.

3.2. Analysis of Acid Production by the Identified LAB Strains

Our experiments showed that the seven strains had a lower pH in a short period than the positive control (**Table 3**). The pH values of all strains were lower than 4.5 within 12 h. The pH value of SQ0012 reached 4.45 ± 0.01, SQ0045 reached 4.5 ± 0.01, SQ0048 reached 4.32 ± 0.01, and SQ0049 reached 4.41 ± 0.02 at 8 h, and then the pH value did not decrease. SQ0054 reached 4.45 ± 0.01 at 10 h. The pH of SQ0015 reached 4.47 ± 0.01 and SQ0030 reached 4.5 ± 0.00 at 12 h. The experimental data are expressed as mean ± SD derived from three repetitions. Their fermentation pH or fermentation time was lower than *L. delbrueckii.* The pH of *L. delbrueckii* reached 4.52 ± 0.01 at 12 h.

Table 3. pH values of LAB isolates in different fermentation times (n = 3) ($\bar{X} \pm S$).

Key	Acid-producing time (h)						
	0	3	5	8	10	12	18
SQ0012	6.6 ± 0.00	5.51 ± 0.03	4.97 ± 0.01	4.45 ± 0.01	4.47 ± 0.02	4.48 ± 0.01	4.48 ± 0.01
SQ0015	6.6 ± 0.00	5.64 ± 0.01	5.17 ± 0.00	4.92 ± 0.01	4.65 ± 0.01	4.47 ± 0.01	4.47 ± 0.02
SQ0030	6.6 ± 0.00	5.86 ± 0.02	5.22 ± 0.01	4.98 ± 0.00	4.77 ± 0.01	4.49 ± 0.01	4.50 ± 0.00
SQ0045	6.6 ± 0.00	5.5 ± 0.06	4.86 ± 0.02	4.50 ± 0.00	4.5 ± 0.01	4.51 ± 0.02	4.51 ± 0.02
SQ0048	6.6 ± 0.00	5.62 ± 0.02	4.78 ± 0.01	4.32 ± 0.01	4.36 ± 0.02	4.37 ± 0.02	4.37 ± 0.02
SQ0049	6.6 ± 0.00	5.78 ± 0.01	4.95 ± 0.02	4.41 ± 0.02	4.45 ± 0.03	4.45 ± 0.04	4.46 ± 0.02
SQ0054	6.6 ± 0.00	5.54 ± 0.03	5.02 ± 0.02	4.85 ± 0.00	4.45 ± 0.01	4.47 ± 0.04	4.47 ± 0.02
L. delbrueckii	6.6 ± 0.00	5.92 ± 0.01	5.35 ± 0.01	5.08 ± 0.00	4.83 ± 0.01	4.52 ± 0.01	4.55 ± 0.02

3.3. Cell Adhesion Analysis of the Identified LAB Strains

Based on the cell adhesion analysis, two of the seven strains (SQ0048 and SQ0049) had higher adhesive properties than the positive control group (*L. delbrueckii*) (Table 4 and Figure 2). The adhesion values of SQ0048 and SQ0049 were significantly higher than that of the positive control group (304 ± 2.67 vs 149 ± 5.72, $P < 0.01$; 196 ± 5.4 vs 149 ± 5.72, $P < 0.05$, respectively). The adhesion values of SQ0012 and SQ0015 were extremely lower than that of the positive control group (37 ± 4.8 vs 149 ± 5.72, $P < 0.01$; 5 ± 0.78 vs 149 ± 5.72, $P < 0.01$, respectively). However, no adhesion activity was observed in the other four strains (SQ0030, SQ0041, SQ0045, and SQ0054) (Table 4 and Figure 2).

3.4. Analysis of Hydrogen Peroxide Production by the Identified LAB Strains

An obvious color (brown) change was observed in the SQ0048 strain, and the color in positive control strains was blue (Supplement Figure 2). No color change was observed in the other strains. Thus, these data indicated that SQ0048 had a medium positive reaction to H_2O_2; moreover, *L. delbrueckii* had a strong positive reaction to H_2O_2 (Supplement Figure 2).

3.5. Bacteriocin Detection Analysis of SQ0048

SQ0048 showed a stronger adhesion effect and produced more hydrogen peroxide than the other seven strains. Furthermore, a specific 400-bp product of the bacteriocin gene of SQ0048 was detected (Supplement Figure 3); a 100% sequence similarly was found in the bacteriocin gene among a positive clone strain of bacteriocin SQ0048 and *Lactobacillus johnsonii* NCC 533 (NC_005362.1). Furthermore, a

Table 4. Adhesion trait of eight strains of LAB to BVECs (n = 3, $\bar{X} \pm SD$).

Strains	SQ0012	SQ0015	SQ0030	SQ0045	SQ0048	SQ0049	SQ0054	*L. delbrueckii*
size	37 ± 4.8**	5 ± 0.78**	0 ± 0*	0 ± 0**	304 ± 2.67**	196 ± 5.4*	0 ± 0**	149 ± 5.72

Note: Each value represents the mean value standard deviation (SD) from three trials under same experimental conditions. **Indicates the extremely significant difference compared with *L. delbrueckii* ($P < 0.01$). *Indicates the significant difference compared with *L. delbrueckii* ($P < 0.05$).

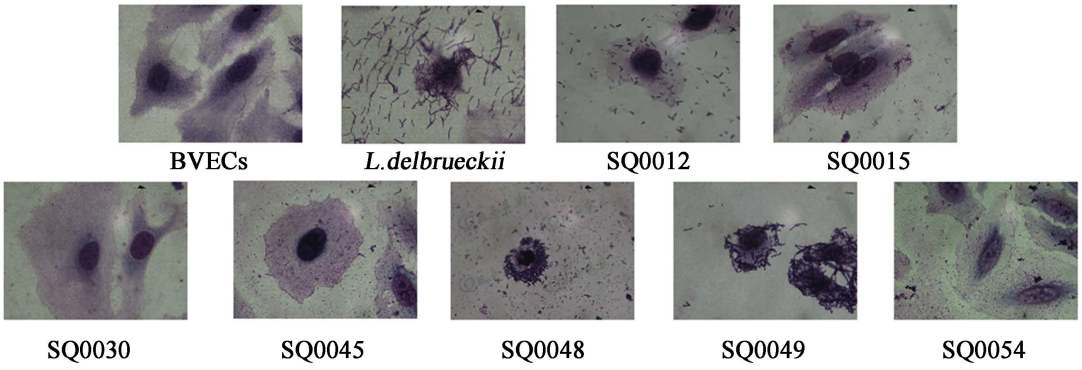

Figure 2. Adhesion profile of seven LAB to bovine vaginal epithelial cells (10 × 100).

clear 10-kDa band was detected in the SQ0048 strain (Supplement **Figure S4**).

3.6. Antibacterial Effect

SQ0048 had a weak inhibitory effect on *S. aureus* and *E. coli* (**Table 5**; **Figure 3**). However, the expression products of the bacteriocin gene of SQ0048 had strong inhibitory effects on *S. aureus* and *E. coli* (**Table 5**; **Figure 3**). Furthermore, the expression level of the antibacterial gene of SQ0048 was significantly higher than both the positive control group and the blank control group (PBS) ($P < 0.01$).

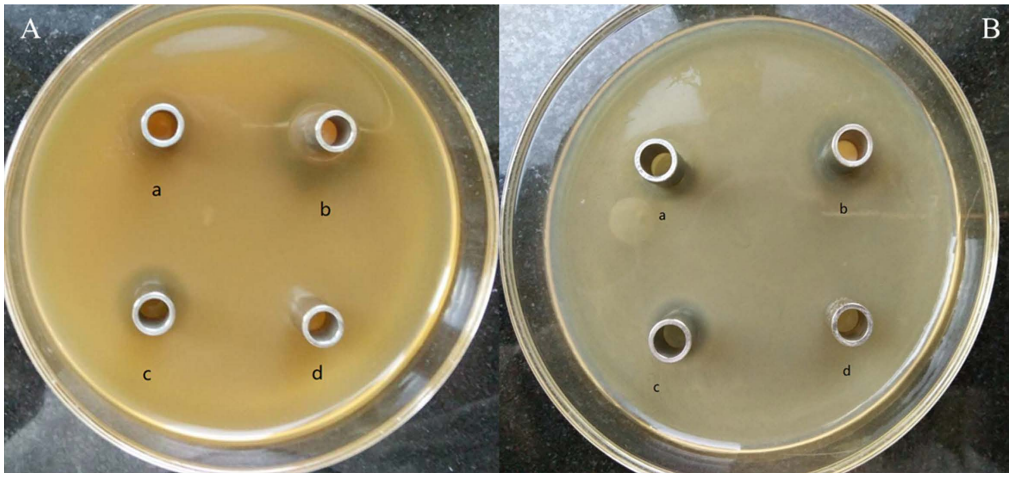

Figure 3. The antimicrobial activity of the SQ0048 and its bacteriocin gene expression products. (A): a: The SQ0048 inhibited the growth of *Staphylococcus aureus*. b: The expression products of the bacteriocin gene of SQ0048 inhibited the growth of *Staphylococcus aureus*. c: Streptomycin (10 μg/ml) inhibited the growth of *Staphylococcus aureus*. d: PBS inhibited the growth of *Staphylococcus aureus*. (B): a: SQ0048 inhibited the growth of *E. coli*. b: The expression products of the bacteriocin gene of SQ0048 inhibited the growth of *E. coli*. c: Penicillin G (0.12 μg/ml) inhibited the growth of *E. coli*. d: PBS inhibited the growth of *E. coli*.

Table 5. Antimicrobial activity analysis of SQ0048 bacteriocin under different conditions (n = 3, $\bar{X} \pm SD$).

Groups	Diameter (mm) (n = 3, $\bar{X} \pm SD$)	
	Staphylococcus aureus (ATCC9144)	*Escherichia coli* (ATCC11303)
SQ0048	10 ± 0.23 (+)	8 ± 0.51 (+)
Expression of bacteriocins gene of SQ0048	18 ± 0.45 (+++)**	15 ± 0.60 (+++)**
Penicillin G	12 ± 0.2 (++)	–
Streptomycin	–	12 ± 0.21 (++)
PBS	0 (–)	0 (–)

Note: The SQ0048 with inhibition zone < 6 mm, 6 - 10 mm, 10 - 14 mm and >14 mm were classified as negative (–), mild (+), strong (++) and very strong (+++) inhibition, respectively; each value represents the mean value ± standard deviation (SD) from three trials; the comparisons were established with the blank ($P < 0.01$); *: The antibacterial diameter was compared between the expression product of SQ0048 bacteriocin gene and antibiotics. *: Significant difference ($P < 0.05$),**: Extremely significant difference ($P < 0.01$).

4. Discussion

In this study, the probiotic antibacterial properties of the eight LAB strains isolated from 20 vaginal samples of healthy cows were investigated, and further experimental results suggested that biological characteristics of SQ0048 *L. johnsonii* were superior to other seven strains. LAB strains are recognized as the dominant species in healthy vaginal microbial flora [23]. A lack of LAB is considered to disrupt the balance of vaginal microbial flora and reduce the immune barrier function, which can lead to reproductive tract inflammation, parasitic diseases, and fungal and chlamydial infections [24]. Our results were similar to a previous report that LAB has an important role in maintaining the biological function of the vagina, such as hydrogen peroxide production, inhibition of pathogenic bacteria, and adherence to vaginal epithelial cells of cows [8] [25]. The acid production capacity of SQ0048 was the strongest (4.31 - 4.33 for 8 h) compared to other strains tested. Another study shows that LAB can keep the pH value between 4 and 4.5, thus creating an environment unsuitable for the survival of pathogenic microorganisms [26]. LAB can decrease the extent of the endometrium inflammation of cows via lowering pH to inhibit *Escherichia coli* [27]. Free lactic acid can penetrate the cell wall of pathogens and reduce the pH, which would inhibit the growth of pathogenic bacteria and destroy the metabolic processes necessary for bacterial growth [26].

In this study, SQ0048 had stronger adhesion to bovine vaginal cells than other strains. Similarly, LAB showed strong adhesion to the layered non-keratinized epithelium, which can repel and inhibit pathogenic microorganisms such as *C. albicans*, *Gardnerella*, *E. coli, Streptococcus, Streptococcus agalactiae*, and *S. aureus* [28]. Another related report showed that the adhesion activity of *C. albicans* and *Trichomonas vaginalis* to the vaginal epithelial cells was reduced by 50% in the presence of LAB, while more than 60% of other bacteria were replaced by exogenous LAB [29]. Moreover, previous report confirmed that the strong adhesion capacity of LAB was closely related to its antibacterial activity [30]. Hydrogen peroxide (H_2O_2) is also a defense factor used by LAB against pathogenic microorganisms [31]. A strong H_2O_2 production capacity was observed in SQ0048. LAB cannot use the cytochrome system to convert the oxygen end to H_2O_2 because it lacks a heme, but it can directly use the flavin protein to directly convert oxygen to H_2O_2 [31], and excessive quantities of H_2O_2 can inhibit or destroy other bacterial strains.

In this study, the bacteriocin gene of SQ0048 was successfully cloned and expressed, and our results suggest that SQ0048 had various antibacterial properties, which is consistent with the activity of other LAB bacteriocins against pathogenic bacteria [32] [33] [34]. Bacteriocins are effective against the most common pathogens, including *Gardnerella vaginalis*, *E. coli*, and *Candida albicans* [35]. The antibacterial activity of bacteriocins is similar to antibiotics. Moreover, most microorganisms are intolerant to bacteriocins [28]. However, only a few strains and limited analyses were used in the present study. Therefore, the elucidation of the pertinent mechanisms remains essential. According to our experimental re-

sults, SQ0048 was considered a potential probiotic candidate because it could produce high levels of acid and hydrogen peroxide, inhibiting pathogenic microorganisms. It also has a strong adhesion ability to the vaginal epithelial cells of cows, as well as bacteriocin genes and antibacterial activity, but *in vitro* experiments were needed to verify its antibacterial properties.

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Availability of Data and Materials

All of the data generated or analyzed during this study are included in this published article, and the supplementary information files will be freely available to any scientist wishing to use them for non-commercial purposes upon request via e-mail.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Abbreviations

LAB: lactic acid bacteria

PCR: polymerase chain reaction

BVECs: Primary bovine vagina epithelial cells

IPTG: isopropyl β-D-thiogalactoside

Supplementary

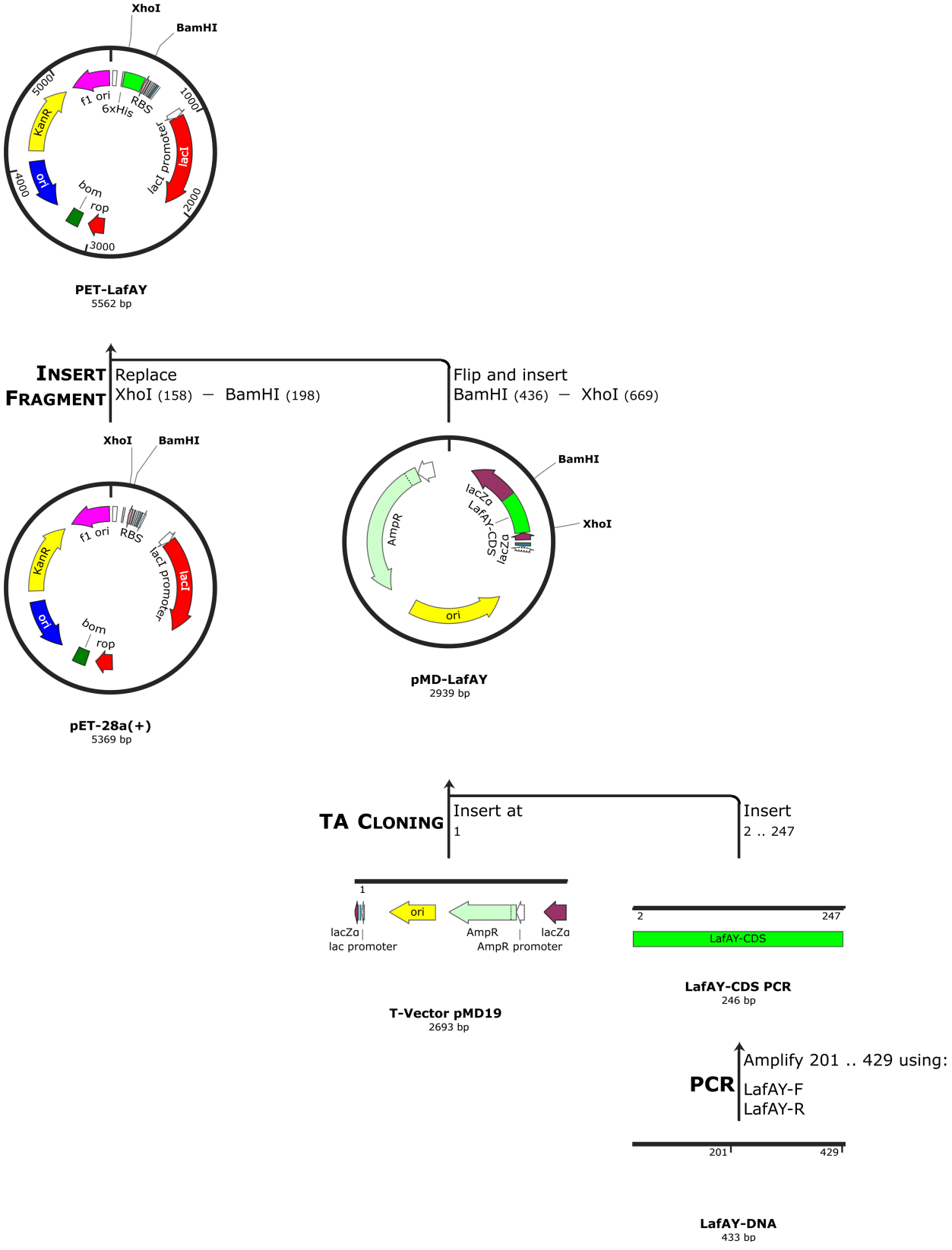
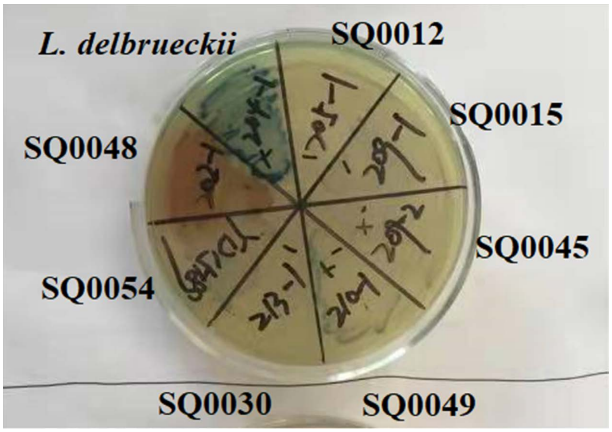


Figure S1. The approach in construction of recombinant plasmid pET-LafAY expressing bacteriocin.



Note: The hydrogen peroxide production performance of the strains was classified into four levels: strong (blue), medium (brown), weak (light brown), and negative (colonies without discoloration).

Figure S2. The hydrogen peroxide production performance for the seven isolated LAB strains.

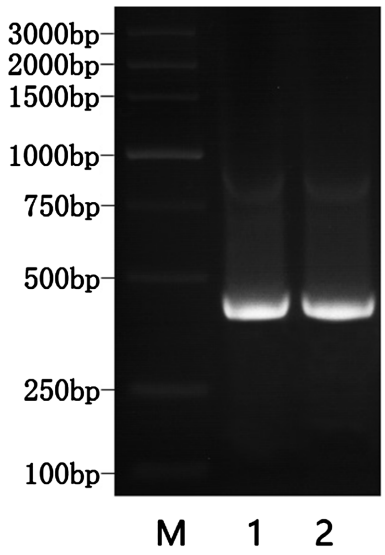


Figure S3. The PCR product sizes of the bacteriocin gene of SQ0048. (Note: lane s1 and 2 showed the bacteriocins gene of SQ0048; M: Marker [200 kDa]).

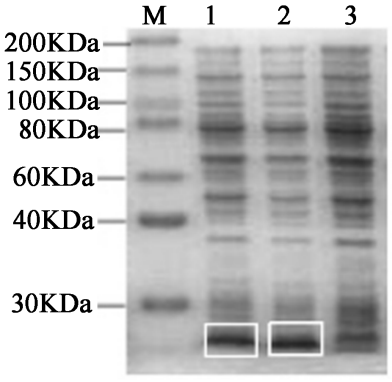


Figure S4. The experimental expression profile of the SQ0048 bacteriocin gene.