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Characterization of Lactic Acid-Producing Bacteria Isolated from Rumen: Growth, Acid and Bile Salt Tolerance, and Antimicrobial Function

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Abstract: Lactic acid bacteria are some of the dominant bacteria in the rumen, and they have a high ability for lactic acid production. The present study aimed to screen and evaluate the performance of culturable rumen bacteria from Chinese Holstein dairy cows as a potential probiotic or inoculant for silage production, in order to isolate ruminal lactic acid bacteria and evaluate their potential as probiotics. Three strains of *Enterococcus avium* (*E. avium*, EA1-3); three strains of *Streptococcus lutetiensis* (*S. lutetiensis*, SL1-3); and six strains of *Streptococcus equinus* (*S. equinus*, SE1-6) were successfully identified from the rumen fluid using modified De Man Rogosa sharp medium supplemented with 0.325% lactic acid. *E. avium*, *S. lutetiensis* and *S. equinus* are clustered in the phylogenetic tree. All the 12 Gram-positive strains reached the plateau growth phase in 6–10 h, with an OD600 at about 1.8. Both gas and acid accumulation reached plateaus at about 10–12 h in all strains, and *S. equinus* showed the strongest capacity. The highest lactic acid accumulation was detected in *S. equinus* broth (up to 219.77 μmol/L). The growth of all isolates was inhibited at pH 4.0, and EA2, SL1, SL2, SL3 and SE2 were tolerant to 0.1%, 0.2% and 0.3% bile salt. In addition, the supernatants of the strains had inhibitory effects on *Escherichia coli* and *Staphylococcus aureus*. Specifically, the *S. equinus* strains exhibited the strongest inhibition of the pathogens. In conclusion, these 12 strains had good potential as silage inoculants or probiotics for edible animals, especially *S. equinus*.

Keywords: *Enterococcus avium*; *Streptococcus lutetiensis*; *Streptococcus equinus*; identification; lactic acid

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1. Introduction

Lactic acid bacteria have been isolated from various fermented foods due to their probiotic effects and are commonly used in food fermentation and preservation [1]. Additionally, the inclusion of lactic acid bacteria in silage decreases dry matter loss, inhibits the growth of unwanted microorganisms [2,3], and improves the quality of animal feed. Lactic acid bacteria are also known to produce the metabolites "bacteriocins", which are considered as a promising alternative to antibiotics. Bacteriocins produced by lactic acid bacteria can destroy harmful bacterial biofilms, dissipate inner membrane proton dynamics, inhibit the activity of the DNA-dependent RNA polymerase of harmful bacteria, or interact with thiol groups of harmful bacteria, resulting in bacterial DNA destruction, cell lysis, etc. [4,5]. Furthermore, lactic acid bacteria in silage can enhance the body's immunity or improve the intestinal barrier function by inhibiting the propagation of harmful microorganisms, reducing intestinal diseases in livestock and poultry [6].

Firmicutes is the dominant phylum in the rumen of dairy cows. The currently known lactic acid bacteria include 43 genera, totaling 373 species and subspecies, most of which are phylum Firmicutes [7], and the genus *Enterococcus* and genus *Streptococcus* are the main lactic acid producers in phylum Firmicutes [8]. These two genera comprise a ubiquitous group of lactic acid bacteria that is of great relevance to human life, as well as silage production. The majority of lactic acid bacteria are Gram-positive [9,10]. Sun et al. [11] isolated a high-purity L-lactic acid-producing *Enterococcus faecalis* S.156 from the rumen of

dairy cows, which can be used for producing L-lactic acid and reduce the operation cost of the fermentation process. In a study by Parks et al. [12], 51 strains of *Streptococcus bovis* were isolated from Holstein dairy cattle, Hanwoo and Korean native goat. Lauková [13] isolated lactic acid-producing *Staphylococcus* from the rumen of lambs, and the lactic acid production was 0.164–0.687 mol/L. Recent studies have shown that *S. equinus* may be equivalent to *Streptococcus bovis* and is, therefore, classified as the *Streptococcus bovis*/*Streptococcus equinus* complex (SBSEC) [14]. Similar research showed that *S. lutetiensis* was also a member of SBSEC, along with *S. equinus* and *S. bovis* [15]. In previous studies, *S. equinus* was the main lactic acid-producing flora when dairy cows experienced rumen acidosis [16], exhibiting fast growth and strong lactic acid production. Despite the extensive studies made in this regard, there still appears to be grey areas that have not been explored, taking cognizance of the fact that the rumen of different ruminant species and breeds contain several bacteria, including lactic acid bacteria. There is, however, paucity of information on lactic acid bacteria screened from the Chinese Holstein cow.

Before screening rumen-derived lactic acid bacteria for application in prebiotic or silage, growth capacity, acid production characteristics, bacteriostatic capacity, and acid bile salt tolerance need to be evaluated [17]. Therefore, the study seeks to screen culturable rumen bacteria from Chinese Holstein dairy cows and evaluate the performance of the identified bacteria as a potential probiotic or inoculant for silage production. Information gathered at the end of the study would offer the opportunity to identify economical and beneficial lactic acid bacteria from Chinese Holsten dairy cows for commercial use.

2. Materials and Methods

2.1. Rumen Fluid Inoculum and Culture Medium

Fresh rumen content was collected through ruminal fistula before morning feeding from three healthy Holstein dairy cows (BW = 600 ± 25 kg, Gaoyou Ranch, Yangzhou University, Yangzhou, China). The rumen fluid was filtered through four layers of sterilized gauze, pooled together in a thermos and transferred to the laboratory anaerobically at 39 °C. All the experiments on bacterial culture in this study were carried out in an anaerobic environment. The anaerobic gas formulation used was a mixture of hydrogen, carbon dioxide and nitrogen, and the volume ratio was 1:1:8.

The modified De Man Rogosa sharp (MRS) medium (MMRS): MRS powder (China Haibo Biotechnology Co., Ltd., Qingdao, China) was dissolved in the modified Menke artificial buffer [18], then 0.325% (*v*/*v*) DL-lactic acid (China Shanghai Aladdin Technology Co., Ltd., Shanghai, China) was added. The medium was mixed on a magnetic stirrer (Dalong Xingchuang Experimental Instrument (Beijing) Co., Ltd., Beijing, China) and flowed with carbon dioxide for over 2 h to remove the oxygen. The basal medium was aliquoted into Hungate tubes (8 mL each). To guarantee anaerobic fermentation, the gas was pumped out by a vacuum pump (AP-01D) (Auto Science Instrument Co., Ltd., Tianjin, China) for 2 min, the carbon dioxide storage tank was opened and CO_2 streamed in for 10 s, which was then repeated for 3 cycles. The medium was autoclaved (121 °C, 15 min) and placed in a 4 °C refrigerator for storage. Before inoculating, a volatile fatty acid (VFA) mix (acetic acid: propionic acid: butyric acid = 60:25:15, *v*:*v*:*v*) and vitamin solution [19] was added to provide extra nutrients.

2.2. Enrichment, Isolation and Purification

Three hundred microliters of rumen fluid was inoculated into MMRS medium (8 mL) and cultured at 39 °C anaerobically. Then, 3 generations were passed (0.3 mL of cultures were transferred to fresh medium in 72-h intervals) to enrich the lactic acid bacteria [19,20].

Before isolation, MMRS agar (15 g/L) plates were placed in the anaerobic glove box incubator (Shanghai Yuejin Medical Instrument Co., Ltd., Shanghai, China) for 2 days to remove the oxygen. One hundred microliters of enrichment culture was spread onto the surface of agar plates and cultured at 39 °C for 3 days [21]. Growth was observed every day. The colonies with different morphology were selected, and the individual colony was added

into an anaerobic culture tube containing 8 mL of MRS for purifying. After incubation for 3 days, Gram staining was performed [22] using Gram staining solution (Bickman Biotechnology Co., Ltd., Changde, China), and photographed at 1000× magnification with Olympus micro-imaging system BX53M (Olympus Corporation, Tokyo, Japan) under mineral oil. The non-contaminated pure culture of the strain was selected for identification and characterization. One milliliter of pure strain was mixed with glycerin (1:1, *v*/*v*) anaerobically and stored at −80 °C [23].

2.3. DNA Sequencing and Identification

The pure strain culture after 24-h of growth was centrifuged at 12,000× *g* for 10 min at 4 °C, then the supernatant was discarded. The pellet was resuspended with 1 mL of sterile PBS buffer (pH = 6.8) and DNA was extracted using a DNA extraction kit (Tiangen Biochemical technology Co., Ltd., Beijing China). The DNA purity and concentration were determined by an ultramicro spectrophotometer (Nanjing Wuyi Technology Co., Ltd., Nanjing, China), and then used as a template for PCR amplification. Primer design: specific primers with a barcode were synthesized according to the V3-V4 region of the bacterial 16S rRNA gene, and the universal primer sequences were 27F and 1492R, 5′-AGA GTT TGA TCC TGG CTC AG-3′and 5′-TAC GGY TAC CTT GTT ACG ACTT-3′, respectively. The PCR mixture contained 12.5 μL of 2 × Taq PCR Master Mix (Tiangen Biochemical Technology Co., Ltd., Beijing, China); 1 μL of forward primer 27F and 1 μL of reverse primer 1492R; 600 ng of target DNA; and sterile ddH_2O supplemented to 25 μL. The reaction procedure was pre-denaturation at 94 °C for 3 min; 94 °C denaturation for 30 s; annealing at 55 °C for 30 s; and 72 °C extension for 1 min. This was performed for 30 cycles. Finally, the PCR amplification process was completed at 72 °C for 10 min.

The amplified products were detected by regular agarose G-10 (Solarbio Biotechnology Co., Ltd., Beijing, China) (2%, *w*/*v*) electrophoresis and sent to Songon Biotech Co., Ltd. (Shanghai, China) for sequencing. The nucleotide sequences were analyzed for sequence identity by BLAST in the GenBank of NCBI (https://blast.ncbi.nlm.nih.gov, 21 October 2021). To obtain the GenBank accession numbers, the 16S rRNA sequences of strains were uploaded into NCBI databases (https://submit.ncbi.nlm.nih.gov, accessed on 21 October 2021).

Three homologous strains (GenBank accession numbers MW876164, MT275465 and AB680295, respectively) were selected and aligned by ClustalW to construct a neighbor-joining tree using the bootstrap method (1000 bootstrap replications), after which a phylogenetic tree was constructed.

2.4. Growth, Gas and Organic Acid Production

Activated culture: the stored strains were thawed in the refrigerator at 4 °C. Three hundred microliters of bacterial solution with the OD600 at 1.60 was added into Hungate tubes with 8 mL of MRS broth (MRS powder was dissolved in distilled water; pH = 6.8, autoclaved) and cultured at 39 °C for 24 h. Then, 3 replicates per strain and 0.3 mL of the activated culture of each replicate were inoculated into 8 mL of MRS broth and incubated for 24 h in Hungate tubes. The OD600 value was determined by the UV/visible spectrophotometer (V-1200) (Fragrant Instrument (Shanghai) Co., Ltd., Shanghai, China). Gas production was measured by a digital pressure gauge (Model DPG1000B15PSIG-5, Cecomp Electronics, Libertyville, IL, USA) and calculated according to the equation utilized in a previous study [24]. After the absorbance and gas pressure measurements, the tube was placed on ice to stop the fermentation; the pH value was recorded immediately using a PHS-25 model pH meter (Shanghai Yidian Scientific Instrument Co., Ltd., Shanghai, China). Referring to Zhang's method [25], 1 mL of culture was transferred into a sterile 1.5 mL microfuge tube and centrifuged at 12,000× *g* for 2 min at 4 °C. To denature and remove protein, the supernatant was mixed with 0.2 mL 20% metaphosphoric acid containing 60 Mm of crotonic acid (5:1, *v*:*v*), refrigerated overnight at −20 °C, centrifuged again after thawing, and filtered using 0.22 μm of aqueous phase membrane (syringe filter) (Shanghai

Anpu Experimental Technology Co., Ltd., Shanghai, China). The deproteinized supernatant was used for organic acid determination.

The concentration of lactic acid was determined by a lactic acid assay kit (Nanjing Jiancheng Technology Co., Ltd., Nanjing, China) and VFAs were measured using Agilent J&W GC columns (GC-9800) gas chromatograph (Shanghai Kechuang Chromatographic Instrument Co., Ltd., Shanghai, China). The determination was performed on a CP-WAX capillary column (30 mm × 0.53 mm × 1 μm). The temperature of the gasification chamber was 200 °C, and the FID detector temperature was 200 °C. The heating rate of the column temperature was 3 °C/min, which finally reached 150 °C [26].

2.5. Resistance to Acid and Bile Salt

To determine the resistance to acid: three replicates per strain and 0.3 mL of each replicate of cultures with the OD600 at 1.60 were added to Hungate tubes containing 8 mL of MRS broth adjusted with hydrochloric acid to 6.8, 6, 5 and 4, respectively. The concentration of hydrochloric acid was 3 M. The Hungate tubes were incubate anaerobically at 39 °C for 24 h, then, the OD600 value was measured. The culture medium without an inoculated strain was used for zero calibration. The resistance to bile salt was determined by adding 0.3 mL of cultures to Hungate tubes containing 8 mL of MRS broth with bile salt concentrations at 0%, 0.1%, 0.2% and 0.3%, respectively. The Hungate tubes were cultured anaerobically at 39 °C for 24 h, and the OD600 values were measured. Culture medium without an inoculated strain was used for zero calibration.

2.6. The Bacteriostasis of the Post-Culture Medium

According to the methods of Talib et al. [27], the diameter of a bacteriostatic circle equal to 7.8 mm is defined as resistant (−); 10–20 mm as moderately susceptible (+); and 21–30 mm and inhibition zones > 31 mm as very susceptible. The supernatants of the 12 strains, and three replicates per strain were obtained by centrifugation at 12,000× *g* for 10 min at 4 °C, followed by filtration with 0.22 μm of aqueous phase membrane (syringe filter) (Shanghai Anpu Experimental Technology Co., Ltd., Shanghai, China). *Staphylococcus aureus* (ATCC25923) and *Escherichia coli* (ATCC8739) (Shanghai Beinuo Biotechnology Co., Ltd., Shanghai, China) were used as indicator bacteria to conduct bacteriostasis experiments with the Oxford cup diffusion method [28]. The 0.1 mL of storage solution of the indicator bacteria was cultured in 20 mL of LB broth (Haibo Biotechnology Co., Ltd., Qingdao, China) at 37 °C for 24 h, then diluted 10^8 fold, and 0.1 mL of culture was streaked onto LB agar (Haibo Biotechnology Co., Ltd., Qingdao, China). Next, three Oxford cups were set and filled with 0.2 mL of the supernatant of the strains, then incubated at 37 °C. After 24 h, the size of the inhibition zones was measured.

2.7. Data Analysis

The experimental data were plotted using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA). SPSS 24.0 (IBM Corp., Armonk, NY, USA) was used for a one-way analysis of variance (ANOVA). A post hoc multiple comparison with LSD's significant test at a significance level of 0.05 was used to establish the significance between the experimental groups. Prior to conducting the ANOVA, Levene's test was used to ensure that the assumption of the homogeneity of variances was met ($p > 0.05$). MEGA 11.0 (Mega Limited, Auckland, New Zealand). It was also used to construct the bacterial phylogenetic tree.

3. Results

3.1. 16S rRNA Sequencing Identification and Homologous Analysis of E. avium, S. lutetiensis and S. equinus

The electrophoresis results of the amplified fragments of the 12 isolated Gram-positive lactic acid strains presented about 1400 bp with narrow and bright bands. The NCBI GenBank accession numbers of EA1-3 were ON182136, ON182137 and ON182140, respectively;

SL1-3 were ON182138, ON182149 and ON182158, respectively; and SE1-6 were ON182139, ON182142, ON182150, ON182155, ON182156 and ON182157, respectively. In the phylogenetic tree, EA1-3 and *E. avium* strain D26-1 are clustered on the same branch; SL1-3 and *S. lutetiensis* strain XJB-82 are clustered on the same branch; and SE1-6 and *S. equinus* strain NBRC 12253 are clustered on the same branch (Figure 1).

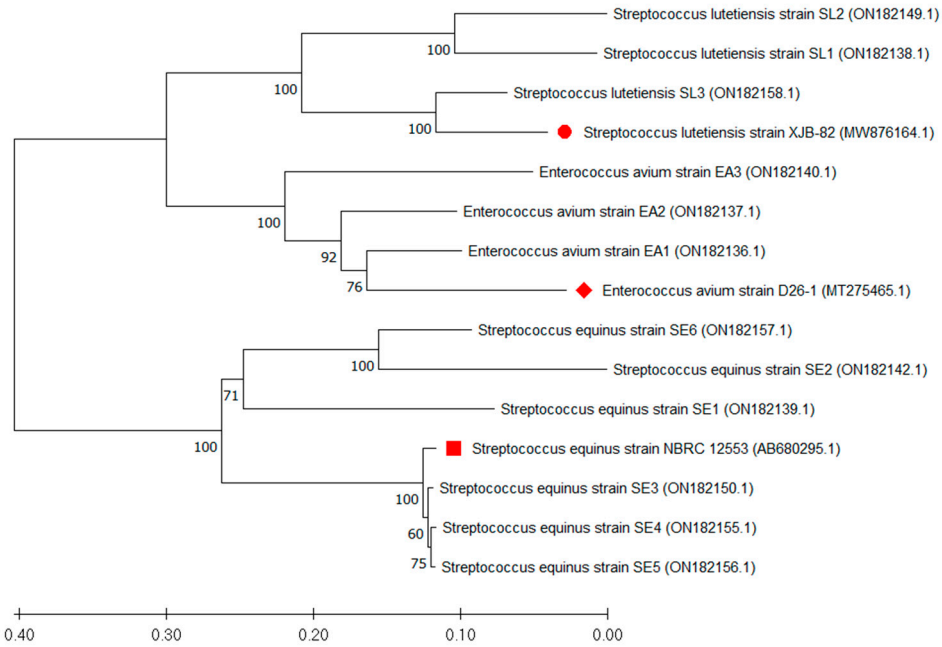

Figure 1. Neighbor-joining phylogenetic tree of strains of *Enterococcus avium*, *Streptococcus lutetiensis* and *Streptococcus equinus*. Red color indicates model strains.

The 16S rRNA sequences analysis showed that EA1-3 were identified as *E. avium* with a similarity level at 99%, 97% and 99%, respectively; SL1-3 were identified as *S. lutetiensis* with a similarity level at 97%, 98% and 99%, respectively; and SE1-6 were identified as *S. equinus* with a similarity level at 99%, 98%, 97%, 99%, 99% and 98%, respectively.

3.2. Growth

The lag growth phase of all *E. avium* isolates was observed over the first 4 h of culture (Figure 2a). In contrast, EA1 entered the logarithmic phase at 6 h, and the OD600 was 1.86 at 24 h; EA2 and EA3 entered the logarithmic phase until 8 h, with OD600 values of 1.86 and 1.72, respectively, at 24 h. The growth of EA1 was better than that of the other strains during the period of 0–8 h, and the growth of EA2 was better after 10 h. There were no significant differences among the three strains during incubation ($p > 0.05$).

SL1 and SL2 arrived in the logarithmic growth phase at 2 h and 4 h of culture, respectively. Then, they reached the plateau phase at 6 h, with OD600 values at 24 h of 1.75 and 1.84, respectively. The lag growth phase of SL3 was observed in the first 4 h of culture. This strain reached the plateau phase at 6 h, and the final value of OD600 was 1.83 (Figure 2b). There were no significant differences among the three strains during incubation ($p > 0.05$).

The logarithmic growth phase of SE2, SE5 and SE6 was observed in the first 4 h of culture (Figure 2c). Then, SE1 entered the plateau phase at 8 h, while SE4 reached the plateau phase at 6 h. The plateau phases of the SE2, SE3, SE5 and SE6 strains were seen at 2–6 h of culture. The final OD600 values of SE1 SE2, SE3, SE4, SE5, and SE6 were 2.03, 1.96, 1.97, 1.86, 1.93, and 1.75, respectively. There were no significant differences among SE1, SE2 and SE3 ($p > 0.05$), and no significant differences among SE2, SE3 and SE4 ($p > 0.05$). Other pairwise comparisons showed significant differences ($p < 0.05$). The OD600 of SE1 was significantly higher than that of SE4, SE5 and SE6 ($p < 0.05$).

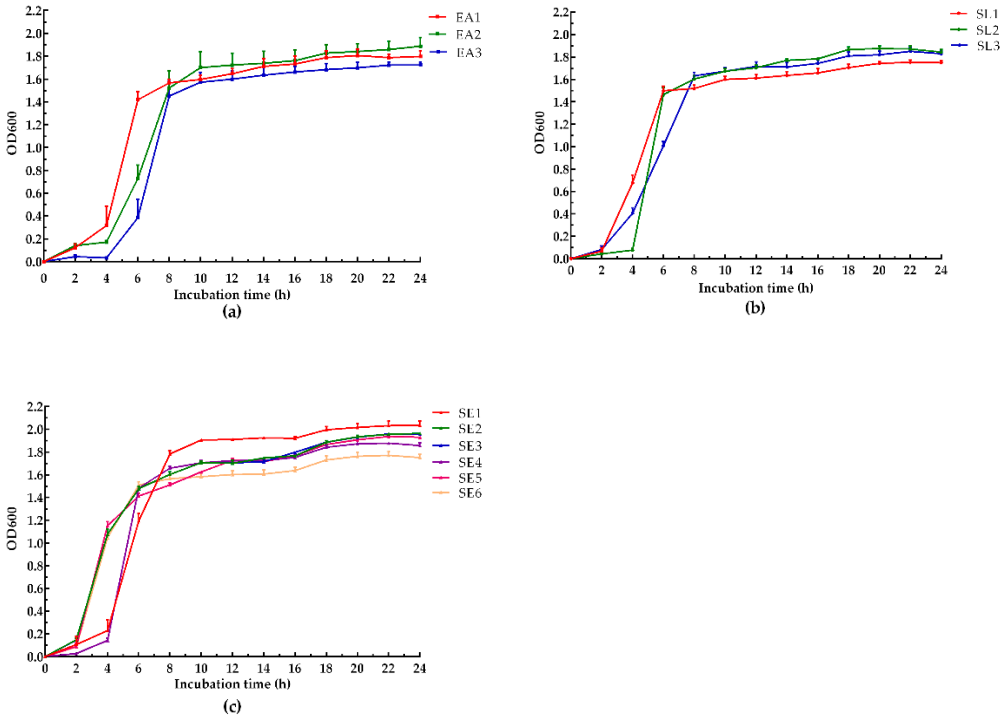

Figure 2. Growth curves (means ± SD, n = 3) of three strains of (**a**) *Enterococcus avium* (EA1-3); (**b**) three strains of *Streptococcus lutetiensis* (SL1-3); and (**c**) six strains of *S. equinus* (SE1-6).

3.3. Gas Production

All 12 strains produced gas and the curve trends were similar. With incubation, about half of the total gas was produced in the first 2 h, and the gas volume increased until 12 h (Figure 3). There was no significant difference within the strains in *E. avium* or *S. lutetiensis* ($p > 0.05$), respectively. The average gas production at 24 h of *E. avium* was 7.97 mL and that of *S. lutetiensis* was 7.87 mL. In *S. equinus*, the gas production of SE2 was the highest at 24 h (8.19 mL), which was significantly different from that of SE1, SE4 and SE6 ($p < 0.05$). The average gas production of SE1, SE3, SE4, SE5 and SE6 was 6.99 mL, 7.81 mL, 7.27 mL, 7.93 mL and 7.57 mL, respectively.

3.4. VFAs and Lactic Acid Concentrations

The highest concentration of acetic acid in the supernatant was EA2 (56.37 mmol/L) and the lowest was SE6 (41.39 mmol/L) ($p < 0.05$) (Table 1). The highest propionic acid concentration in the supernatant was SL3 (15.19 mmol/L) and the lowest was EA2 (8.83 mmol/L) ($p < 0.05$). In the supernatant, the butyric acid concentration of SE5 was the highest (0.34 mmol/L), and that of SE3 and SL2 were the lowest (0.13 mmol/L) ($p < 0.05$). The lactic acid concentration of SE3 was the highest (219.77 mmol/L), and that of SL1 was the lowest (31.17 mmol/L) ($p < 0.05$). The ratio of lactic acid to acetic acid (LA/ACA) in the supernatant was highest in SE2 (4.30) and lowest in SL1 (0.63) ($p < 0.05$).

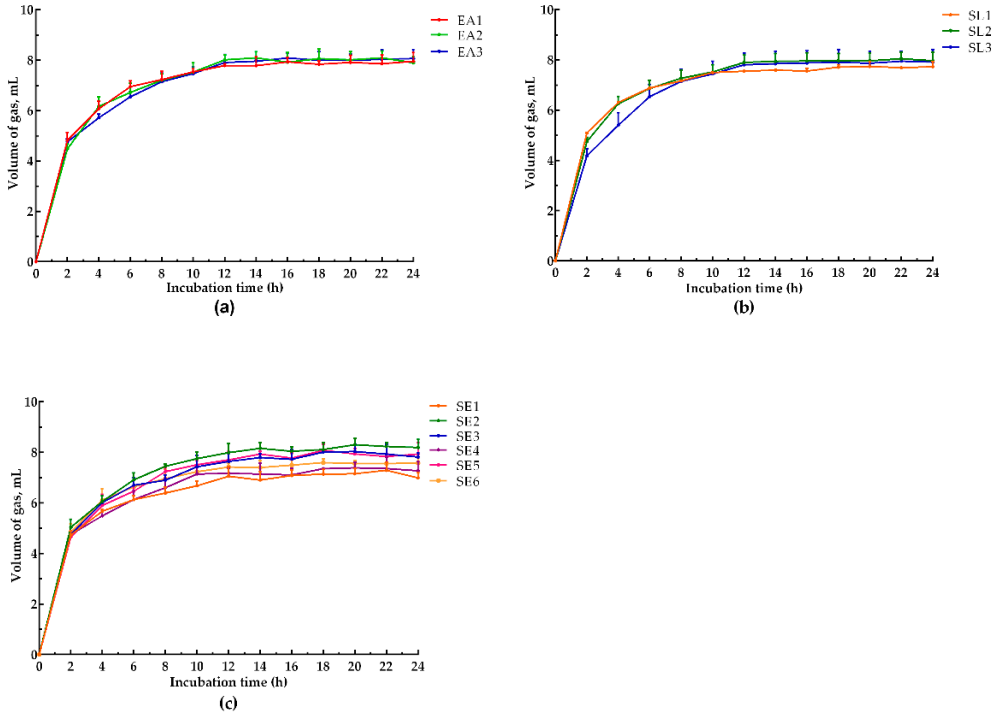

Figure 3. Gas production (means ± SED, n = 3) of three strains of (**a**) *Enterococcus avium* (EA1-3); (**b**) three strains of *Streptococcus lutetiensis* (SL1-3); and (**c**) six strains of *Streptococcus equinus* (SE1-6).

Table 1. Volatile fatty acid and lactic acid concentrations in the culture medium of strains of *Enterococcus avium*, *Streptococcus lutetiensis* and *Streptococcus equinus* at 24 h.

Species	Strain Code	Acetic Acid	Propionic Acid	Butyric Acid	Lactic Acid	LA/ACA [1]
Enterococcus avium	EA1	47.11 ^{F}	11.3 ^{CDE}	0.14 ^{C}	80.63 ^{F}	1.71 ^{F}
	EA2	56.37 ^{A}	8.83 ^{E}	0.20 ^{C}	76.24 ^{G}	1.35 ^{G}
	EA3	53.88 ^{B}	13.08 ^{ABCD}	0.20 ^{C}	120.11 ^{E}	2.23 ^{E}
Streptococcus lutetiensis	SL1	49.56 ^{DE}	10.79 ^{DE}	0.19 ^{C}	31.17 ^{J}	0.63 ^{F}
	SL2	50.97 ^{CD}	11.62 ^{BCDE}	0.13 ^{C}	45.13 ^{I}	0.89 ^{H}
	SL3	47.73 ^{EF}	15.19 ^{A}	0.15 ^{C}	70.55 ^{B}	1.48 ^{G}
Streptococcus equinus	SE1	47.59 ^{EF}	14.9 ^{AB}	0.76 ^{B}	145.53 ^{C}	3.06 ^{B}
	SE2	48.13 ^{EF}	14.43 ^{ABC}	0.14 ^{C}	207.04 ^{B}	4.30 ^{A}
	SE3	52.26 ^{BC}	10.69 ^{DE}	0.13 ^{C}	219.77 ^{A}	4.21 ^{A}
	SE4	51.64 ^{BCD}	11.2 ^{CDE}	0.34 ^{C}	130.03 ^{D}	2.52 ^{D}
	SE5	47.53 ^{EF}	10.9 ^{DE}	1.1 ^{A}	120.21 ^{E}	2.53 ^{D}
	SE6	41.39 ^{G}	13.51 ^{ABCD}	0.22 ^{C}	117.01 ^{E}	2.83 ^{C}
SEM		0.79	0.45	0.06	11.61	1.16
p-value		<0.01	0.01	<0.01	<0.01	<0.01

$^{A–J}$ Means (mmol/L, n = 3) in columns and with the same superscript letter are not significantly different ($p > 0.05$); [1] LA/ACA = lactic acid/acetic acid ratio.

3.5. pH Changes

The pH value began to decrease from 2 h. During 8 to18 h of incubation, all bacteria reached the plateau stage of pH value. In Figure 4a, the pH of EA1 dropped faster than the other two stains, visually. All reached the plateau stage at 10 h, and the lowest pH was 4.75 in EA1, while the average of EA2 and EA3 was 5.45 and 5.25, respectively. In Figure 4b, the pH of SL3 dropped faster than the other two stains, visually, SL2 had the lowest final pH 5.32, while the average of SL1 and SL3 was 6.16 and 5.97, respectively. In Figure 4c, the pH of SE2 and SE3 dropped faster than other stains, visually, and the pH of SE2 and SE3 were the lowest at the plateau stage ($p < 0.05$) (3.71 and 3.62, respectively),

and the average final pH of the other four stains SE1, SE4, SE5 and SE6 was 4.89, 5.02, 5.01 and 5.16, respectively.

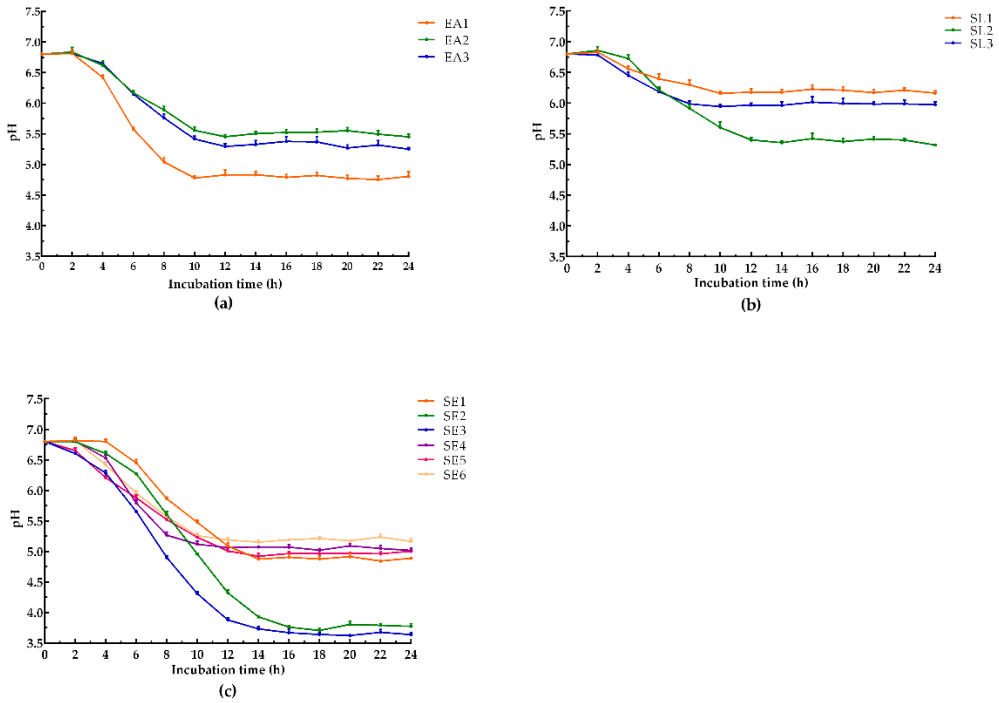

Figure 4. pH curves (means SD, n = 3) of three strains of (**a**) *Enterococcus avium* (EA1-3); (**b**) three strains of *Streptococcus lutetiensis* (SL1-3); and (**c**) six strains of *Streptococcus equinus* (SE1-6).

3.6. Acid Resistance

Compared with pH 6.8, EA1 and EA2 strains had similar OD600 in pH 6.0 and 5.0 broth at 24 h of incubation (Table 2). However, the OD600 values were significantly decreased in pH 4.0 broth ($p < 0.01$). The OD600 of SL1 in pH 4.0 broth was significantly lower than other pH treatments ($p < 0.01$). The pH 6.0 broth had a higher OD600, while pH 4.0 broth had a lower value in SL2 and SL3 cultures ($p < 0.01$). The absorbance of SE1, SE2, and SE6 broth with pH 6 and 5 were similar with the control, while pH 4.0 broth was significantly decreased ($p < 0.05$). Incubation in the pH 6.0 broth for SE3, SE4, and SE5 increased the OD600 values compared to the control, while values for the pH 5.0 cultures decreased, and pH 4.0 broth had the lowest values ($p < 0.01$). No matter pH = 6.8, pH = 6.0 or pH = 5.0, the OD600 value of SL2 was higher, and when pH = 4, its OD600 value was significantly higher than that of other strains ($p < 0.05$), indicating that SL2 was more tolerant to different pH than other strains.

Table 2. Growth (OD600) of *Enterococcus avium*, *Streptococcus lutetiensis* and *Streptococcus equinus* after 24 h of growth in media at different pH.

Strain Code	pH 6.8 Control	pH 6	pH 5	pH 4	SEM	*p*-Value
Enterococcus avium						
EA1	1.75 ^{Aa}	1.71 ^{BCDa}	1.78 ^{Aa}	0.29 ^{Bb}	0.20	<0.01
EA2	1.72 ^{Aa}	1.63 ^{CDa}	1.59 ^{Cda}	0.47 ^{Abb}	0.19	0.01
EA3	1.68 ^{Aa}	1.65 ^{BCDab}	1.51 ^{Deb}	0.26 ^{Abc}	0.18	<0.01
Streptococcus lutetiensis						
SL1	1.66 ^{Aa}	1.62 ^{Cda}	1.71 ^{ABCa}	0.06 ^{Bb}	0.20	<0.01
SL2	1.69 ^{Ab}	2.35 ^{Aa}	1.74 ^{Abb}	0.15 ^{Abc}	0.25	<0.01
SL3	1.67 ^{Ac}	2.12 ^{Aba}	1.75 ^{Ab}	0.11 ^{Bd}	0.23	<0.01

Table 2. *Cont.*

Strain Code	pH 6.8 Control	pH 6	pH 5	pH 4	SEM	*p*-Value
Streptococcus equinus						
SE1	1.65 ^{Aa}	1.76 ^{Bca}	1.70 ^{ABCa}	0.75 ^{Ab}	0.14	<0.01
SE2	1.68 ^{Aa}	1.25 ^{Da}	1.55 ^{Dea}	0.33 ^{Abb}	0.21	0.03
SE3	1.36 ^{Bb}	2.13 ^{Aba}	1.70 ^{ABCb}	0.12 ^{Bc}	0.23	<0.01
SE4	1.70 ^{Ab}	2.08 ^{ABCa}	1.62 ^{BCDb}	0.19 ^{Abc}	0.22	<0.01
SE5	1.73 ^{Ab}	1.89 ^{ABCa}	1.45 ^{Ec}	0.14 ^{Bd}	0.21	<0.01
SE6	1.67 ^{Aa}	1.98 ^{ABCa}	1.80 ^{Aa}	0.41 ^{Abb}	0.20	<0.01
SEM	0.13	0.06	0.02	0.28		
p-value	0.02	<0.01	<0.01	0.04		

$^{A–E}$ Means (n = 3) in columns and with the same superscript letter are not significantly different ($p > 0.05$); $^{a–e}$ Mean (n = 3) in rows and with the same superscript letter are not significantly different ($p > 0.05$).

3.7. Bile Salt Resistance Test

The resistance of isolated strains to bile salt was shown in Table 3. Compared with 0% bile salt supplementation (control), the OD600 value of EA1 was lower in all bile salt levels, and no difference was observed in EA2 broth, while the OD600 value of EA3 was lower in 0.2% and 0.3% bile salt levels ($p = 0.04$) compared to the control and 0.1% bile salt. There was no difference in the SL1 OD600 values with different bile salt levels; however, SL2 broth showed lower OD600 values with all bile salt supplementation levels, and lower absorbance was also observed in 0.3% levels in SL3 cultures compared to the other treatments ($p < 0.05$). There was no significant difference in the absorbance of SE2 compared with the control group, while SE1, SE3, SE4 and SE6 incubation had lower absorbance in all treatments compared to the control cultures ($p < 0.05$). The bile salt concentration at 0.2% and 0.3% significantly decreased the absorbance of SE5 broth ($p = 0.04$). Among all 12 strains, SL3 had higher OD600 values at different bile salt concentrations. Therefore, SL3 had better resistance to different bile salts than other strains.

Table 3. Growth (OD600) of *Enterococcus avium*, *Streptococcus lutetiensis* and *Streptococcus equinus* cultures after 24 h of growth under different bile salt conditions.

Strain Code	Bile Salt				SEM	*p*-Value
	Control	0.1%	0.2%	0.3%		
Enterococcus avium						
EA1	1.65 ^{BCa}	1.44 ^{CDb}	1.39 ^{BCDb}	1.40 ^{Bb}	0.03	<0.01
EA2	1.58 ^{BC}	1.52 ^{BC}	1.51 ^{ABC}	1.51 ^{B}	0.05	0.98
EA3	1.46 ^{Ca}	1.41 ^{CDa}	1.28 ^{Db}	1.32 ^{Bb}	0.023	0.04
Streptococcus lutetiensis						
SL1	1.43 ^{C}	1.43 ^{CD}	1.35 ^{CD}	1.47 ^{B}	0.04	0.78
SL2	1.83 ^{ABa}	1.53 ^{ABCb}	1.61 ^{Ab}	1.43 ^{Bb}	0.05	<0.01
SL3	1.80 ^{ABa}	1.61 ^{ABa}	1.62 ^{Aa}	1.57 ^{Ab}	0.05	0.02
Streptococcus equinus						
SE1	1.86 ^{ABa}	1.62 ^{ABb}	1.51 ^{ABCb}	1.52 ^{Bb}	0.05	<0.01
SE2	1.59 ^{BC}	1.62 ^{AB}	1.44 ^{ABCD}	1.48 ^{B}	0.03	0.14
SE3	1.86 ^{ABa}	1.59 ^{ABb}	1.53 ^{ABb}	1.41 ^{Bb}	0.05	<0.01
SE4	1.84 ^{ABa}	1.55 ^{Db}	1.56 ^{ABb}	1.49 ^{Bb}	0.08	0.01
SE5	1.95 ^{Aa}	1.67 ^{Aab}	1.38 ^{BCDb}	1.53 ^{Bb}	0.08	0.04
SE6	1.81 ^{ABa}	1.65 ^{ABb}	1.57 ^{ABbc}	1.54 ^{Bc}	0.04	<0.01

Table 3. *Cont.*

Strain Code	Bile Salt				SEM	*p*-Value
	Control	0.1%	0.2%	0.3%		
SEM	0.04	0.02	0.02	0.03		
p-value	<0.01	<0.01	<0.01	0.03		

$^{A–D}$ Means (n = 3) in columns and with the same superscript letter are not significantly different ($p > 0.05$); $^{a–c}$ means (n = 3) in rows and with the same superscript letter are not significantly different ($p > 0.05$).

3.8. Bacteriostatic Performance Assay

Escherichia coli was resistant and moderately susceptible (+) to the supernatant of EA1-3, SL1 and SL3, SE1-6, and the diameters of the inhibition zones ranged from 10 to 20 mm (Table 4). The bacteriostatic circle diameter of SE1 was the largest ($p < 0.01$) among them. *Staphylococcus aureus* was resistant and moderately susceptible to the supernatants of EA1-3, SL1-3, SE1-6, and the diameters of the inhibition zones were within 10–20 mm. The antibacterial circle diameter of SE2 was the largest ($p < 0.01$) among them.

Table 4. *Escherichia coli* and *Staphylococcus aureus* zones of inhibition by supernatants from *Enterococcus avium*, *Streptococcus lutetiensis* and *Streptococcus equinus* cultures.

Strain Code	Diameter Zone Inhibition (mm) [1]	
	Escherichia coli	***Staphylococcus aureus***
Enterococcus avium		
EA1	12.60 ^{BCD} (+)	12.79 ^{CD} (+)
EA2	12.87 ^{BC} (+)	13.70 ^{CD} (+)
EA3	15.27 ^{B} (+)	14.20 ^{C} (+)
Streptococcus lutetiensis		
SL1	12.07 ^{CD} (+)	12.47 ^{CD} (+)
SL2	(-)	13.93 ^{CD} (+)
SL3	10.70 ^{DE} (+)	11.80 ^{D} (+)
Streptococcus equinus		
SE1	16.00 ^{A} (+)	13.33 ^{CD} (+)
SE2	12.83 ^{BC} (+)	17.30 ^{A} (+)
SE3	14.43 ^{AB} (+)	13.83 ^{CD} (+)
SE4	14.90 ^{C} (+)	16.53 ^{AB} (+)
SE5	14.43 ^{AB} (+)	14.60 ^{BC} (+)
SE6	12.20 ^{CD} (+)	13.40 ^{CD} (+)
SEM	0.34	0.31
p-value	<0.01	<0.01

$^{A–E}$ Means (n = 3) in columns and with the same superscript letter are not significantly different ($p > 0.05$); [1] resistant (-; inhibition zone < 10 mm); moderately susceptible (+; 10–20 mm).

4. Discussion

Lactic acid plays an important role in the food, pharmaceutical, chemical, feed and cosmetic industries [29]. Currently, reported sources of lactic acid bacteria are extensive, including plants, animals and the environment [17,30,31]. Since rumen is a natural fermenter, rumen lactic acid bacteria have been extensively studied. *Enterococcus avium*, *S. lutetiensis* and *E. equinus* which were isolated in this study are common lactic acid-producing bacteria in the rumen.

For silage, adding lactic acid bacteria could reduce pH; increase lactic acid concentration and inhibit the production of mycotoxins [32]; improve the growth performance of calves [33]; and inhibit the attachment of *Escherichia coli*, *Klebsiella ent* of *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* [34]. However, a problem that arises and needs to be solved is whether lactic acid bacteria can rapidly colonize after addition, and

whether lactic acid bacteria can be induced to play a better supportive role [35]. In this study, *E. avium* EA2, *S. lutetiensis* SL2 and SL3, and *S. equinus* SE1 had faster growth rates, which means that these strains may rapidly proliferate to become the dominant floras and competitively inhibit the growth of pathogenic bacteria [36]. Previous studies [37] showed that *E. avium* entered the logarithmic growth phase at 2 h and the plateau phase at 8 h, which was similar to this study. In contrast, the growth performance of *S. lutetiensis* and *S. equinus* are poorly understood.

Low pH is related to the success of silage during the silage process and preservation [38]. Even if lactic acid bacteria were inoculated on moldy corn straw, silage quality could still be guaranteed when the pH of silage products reached 3.44–3.66 [32]. For any high-quality silage, the ideal pH value is about 3.8–4.2 [39]. In this study, the pH of SE3 declined to 3.62 at 16 h and the pH of SE2 declined to 3.71 at 18 h, which are in line with the silage conditions.

Lactic acid is produced by catalysis by lactate dehydrogenase following the consumption of soluble sugar [40]. As a feed additive, lactic acid is also a critical factor in ensuring feed quality. Studies have shown that the inclusion of lactic acid at 20 to 40 g/kg of dry matter to silage contributes to achieving a lower pH because it is approximately a 10- to 12-fold stronger acid than other major acids [38]. Recent research has shown that after 90 days of ensiling with *Lactobacillus buchneri*, the lactic acid content could reach 106 g/kg, and after 90 days of ensiling with *Lactobacillus plantarum*, the lactic acid/acetic acid content could reach 6.82 (g/kg:g/kg) [32]. Lactic acid/acetic acid can significantly reflect the quality of silage fermentation [41]. Under the laboratory conditions (39 °C, MRS medium, cultured for 24 h) in this study, the highest lactic acid content of the 12 strains of lactic acid bacteria was 219.77 mmol/l, and the highest lactic acid/acetic acid ratio was 4.30. These conditions are conducive to improving the quality of silage. Organic acids in feed fermentation, including VFAs, greatly affect the production performance of edible animals [42]. Acetic acid and propionic acid can inhibit yeast and mold growth and improve fermented feed's aerobic stability [43,44]. EA2 had the highest acetic acid concentration in this study, with an average acetic acid content of 56.37 mmol/L, significantly different from other strains. SL3 had the highest propionic acid concentration, with an average propionic acid concentration of 15.19 mmol/L, significantly different from other strains. However, high concentrations of acetic acid and propionic acid represent more loss of dry matter in fermented feed [38]. Butyric acid is also an unwelcome product in feed fermentation, as an increase in butyric acid led to the loss of nutrients in fermented feed [45]. In this study, the butyric acid concentration of SE5 was the highest at 1.1 mmol/L, which was significantly different from that of other strains.

During the fermentation, lactic acid could be further converted to acetic acid, ethanol and gas by acetyl coenzyme A [46]. These by-products can lead to the loss of feed's dry matter, but the ethanol that is produced plays an essential role in the aerobic stability of silage [47], while the gas is a form of nutrient loss. In this study, all fermentation produced gas, and the gas volume ranged from 7.8 to 8.2 mL; EA3 had the most and SE1 produced the least gas at 24 h. In the study of Kaewpila, et al. [48], the addition of lactic acid bacteria improved the in vitro digestibility and reduced the production of methane. More gas emissions may induce more waste in the silage process or food preservation and will lead to the greenhouse effect [49].

The optimal pH value for the growth of lactic acid bacteria is between 6.2 and 8.5 [50], while intestinal bile salts play an essential role in fat digestion in animals [51]. However, bile salts can lead to the oxidative damage of bacterial DNA and the expression of OxyR and SoxRS regulators, which induce the expression of antioxidant activities in response to O_2^- and H_2O_2 stress, respectively [52]. Therefore, acid and bile salt tolerance tests evaluate the survival ability of isolates in the gastrointestinal tract. In this study, the fact that all strains grew poorly at pH 4.0 means that they cannot survive in the stomach. That EA2, SL1 and SE2 had better bile salt tolerance in this study means that they may present probiotic function in the intestine. Missotten et al. [53] reported that there are differences in acid

tolerance and bile salt tolerance between the bacteria of different species and different strains of the same species, which is consistent with the results of this study. Different resistance capacities may be due to strain-specific properties which need to be studied in the future.

The metabolites produced by lactic acid bacteria have broad-spectrum bacteriostasis and contribute to them being an ideal substitute for antibiotics [54]. A previous study showed that cell-free supernatant of lactic acid bacteria reduced the adhesion ability of pathogenic bacteria and inhibited the growth of harmful bacteria [55]. In this experiment, the supernatant of all strains presented an inhibition of *E. coli* and *S. aureus*. Therefore, the identified strains may also play an important role in preventing disease occurrence in cows.

5. Conclusions

In conclusion, the 12 strains identified from the rumen grew rapidly, and the OD600 could reach about 1.6 in 8 h; among the isolates, the pH value of SE3 could reach 3.62, and the concentration of lactic acid could reach 219.77 mmol/L. All 12 strains had good tolerance to the environment with a pH of 5, and all the other strains had good tolerance to 0.2% bile salt, except EA3, SE1 and SE5. The 12 strains also had good inhibitory effects on *Escherichia coli* and *Staphylococcus aureus*, except SL2. All 12 strains, especially SE2 and SE3, could be used as potential research strains for silage and probiotics.

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