INTRODUCTION

Lactic acid bacteria (LAB) are a group of Gram-positive, non-sporulating, anaerobic or facultative aerobic cocci or rods, which produce lactic acid as one of the main fermentation products of the metabolism of carbohydrates. LAB play a critical role in food production and health maintenance. There is an increasing interest in these species to reveal the many possible health benefits associated with them. Moreover, the actions of LAB are species and strain specific, and depend on the number of bacteria available in the gastrointestinal tract. In this study, LAB species isolated from the rumen and colon of Bali cattle (Bos sondaicus) which have the characteristics of a high ability to adapt to unfavorable environments, are predicted to find LAB species with superior abilities.

Research on lactic acid bacteria (LAB) from the digestive tract of Bali cattle has been carried out by several researchers. A study of the antimicrobial activity of isolate 9A from the colon of Bali cattle proved that this species was able to inhibit the growth of Streptococcus aureus 37.56-61.47% and through analysis of the 16S rRNA gene, it was identified as Streptococcus bovis. Furthermore testing of its potential as a source of probiotics also found that the extracellular protein from Streptococcus bovis 9A is potential to be used as a beef bio preservative through its ability to extend the shelf life of meat at 4°C for 10 days. Its ability was characterized by inhibition of the pH-increasing in beef compared to control. Phenotypic testing of LAB isolate 9A using the API 50 CHL kit has also been carried out by Ekamelinda et al who identified it as Lactobacillus fermentum. The results of this identification are slightly different when compared to the results of previous studies conducted by Widyadnyana et al.

Another researcher, Francisca et al., has also tested the bio preservative potential of LAB Streptococcus bovis 9A on beef through the Eber test. Meanwhile, the resistance test of isolate 9A to low pH and Sodium Deoxycholate (NaDC) has also been carried out by Febrianti et al. All of the studies above strengthen the potency of Streptococcus bovis 9A isolated from gastrointestinal tract of bali cattle as a probiotic candidate.

Furthermore, testing for low pH and bile acids is a test that must be carried out for a LAB species before being applied as a source of probiotics. In its application in the gastrointestinal tract, LAB will experience both of these conditions because they have to pass through the stomach with a low pH and in the small intestine they will meet with bile acids. On the other hand, the study of the resistance of LAB isolates from the rumen of Bali cattle to low pH and bile acid conditions has never been studied.

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Based on these considerations, this study aimed to determine the probiotic potential of lactic acid bacteria isolated from the rumen and colon of Bali cattle by testing their resistance to low pH and bile salts.

**METHODS**

**Isolate Cultivation**
The samples used in this study were 10 rumen LAB isolates isolated from the rumen of Bali cattle, namely: SR11, SR12, SR13, SR14, SR16, SR17, SR18, SR21, SR22, and SR23. Isolates stored in the freezer at -20°C were thawed and then grown on de Man, Regosa, and Sharpe media (liquid MRS and incubated for 48 hours at 37°C in an anaerobic atmosphere.

**Low pH Test**
The cultivated LAB isolates were then tested for resistance to low pH, namely at pH 2, 3, and 4. A total of 100 μL of LAB isolates that had previously been cultivated were put into an Eppendorf tube. Each of the Eppendorf tubes contained 900 μL of liquid MRS media whose pH had been adjusted (pH 2, 3, and 4), then incubated in a water heater at 37°C for 3 hours. Next, the suspension was centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded. The bacterial pellet obtained was washed twice using 300 μL of saline by vortexing and centrifuged again. Then the pellets obtained were suspended, then 50 μL of this suspension was inoculated into 5 ml of liquid MRS medium with a neutral pH for further incubation at 37°C for 24 hours in an anaerobic atmosphere. The resistance of LAB to low pH is shown by the growth of LAB as measured using a spectrophotometer at a wavelength of 660 nm (OD 660 nm). If the absorbance value (A) < 0.1 then the bacterial strain is considered not resistant to low pH, and if A ≥ 0.1 then the BAL strain is resistant to NaDC.

**Bile Salts Test**
A resistance test of LAB isolates against bile salts/NaDC was carried out by culturing isolates from the stock into 5 ml of liquid MRS medium with a pH of 7.2. 50 μL of each isolate grown into a test tube containing 5 mL of liquid MRS with the treatment of each tube, including control (liquid MRS medium without sodium deoxycholate (NaDC), treatments 1, 2, and 3 respectively 10, 20, and 30 μL of NaDC were added so that in each treatment the final NaDC concentrations of 0.2, 0.4, and 0.6 mM were obtained. Furthermore, all tubes were incubated at 37°C for 24 hours anaerobically. The resistance of LAB isolates was measured based on the level of turbidity (OD 660 nm) using a spectrophotometer. If the absorbance value (A) <0.1 then the bacterial strain is considered not resistant to NaDC, and if A ≥ 0.1 then the BAL strain is resistant to NaDC.

**Data Analysis**
The research data which is the result of optical density (OD) measurements from each test were analyzed descriptively qualitatively and presented in table or graphic form using the SPSS 25 program. Numerical data reported as mean and standard deviation. A p value of < 0.05 was considered significant.

**RESULTS**
The test results for the resistance of LAB isolates from the rumen to pH at pH 2, 3, and 4 compared to the control (pH 7.0)

### Table 1. Resistance of LAB isolates from rumen to low pH

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Control</th>
<th>pH 2</th>
<th>pH 3</th>
<th>pH 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR11</td>
<td>2.076 (+++)</td>
<td>1.892 (+++)</td>
<td>1.932 (+++)</td>
<td>2.046 (+++)</td>
</tr>
<tr>
<td>SR12</td>
<td>2.025 (+++)</td>
<td>1.939 (+++)</td>
<td>2.023 (+++)</td>
<td>2.031 (+++)</td>
</tr>
<tr>
<td>SR13</td>
<td>2.021 (+++)</td>
<td>1.988 (+++)</td>
<td>1.859 (+++)</td>
<td>1.993 (+++)</td>
</tr>
<tr>
<td>SR14</td>
<td>2.050 (+++)</td>
<td>1.898 (+++)</td>
<td>1.963 (+++)</td>
<td>1.989 (+++)</td>
</tr>
<tr>
<td>SR16</td>
<td>1.989 (+++)</td>
<td>1.886 (+++)</td>
<td>1.923 (+++)</td>
<td>1.995 (+++)</td>
</tr>
<tr>
<td>SR17</td>
<td>1.974 (+++)</td>
<td>1.857 (+++)</td>
<td>1.857 (+++)</td>
<td>1.992 (+++)</td>
</tr>
<tr>
<td>SR18</td>
<td>1.990 (+++)</td>
<td>1.876 (+++)</td>
<td>1.909 (+++)</td>
<td>1.963 (+++)</td>
</tr>
<tr>
<td>SR21</td>
<td>2.073 (+++)</td>
<td>2.007 (+++)</td>
<td>2.057 (+++)</td>
<td>2.115 (+++)</td>
</tr>
<tr>
<td>SR22</td>
<td>2.105 (+++)</td>
<td>0.717 (++)</td>
<td>1.808 (+++)</td>
<td>2.025 (+++)</td>
</tr>
<tr>
<td>SR23</td>
<td>1.994 (+++)</td>
<td>2.077 (+++)</td>
<td>1.953 (+++)</td>
<td>1.987 (+++)</td>
</tr>
</tbody>
</table>

**Note:**
- SR = Sample Rumen; Control OD value (pH 6.5); - = Absorbance value < 0.1 (no acid tolerance); + = Absorbance value 0.1-0.5 (slightly acid tolerance); ++ = Absorbance value 0.5-1.0 (acid tolerance); +++ = Absorbance value > 1.0 (high acid tolerance).
- The average value of 3 repetitions ± standard deviation of the isolate absorbance value. Different letters show a significant difference (p < 0.05) and conversely the same letters show no significant difference (p > 0.05).

**Figure 1.** Description of the pH values of LAB isolates from the rumen compared to controls.
showed that the isolates were resistant to the environment, although there was a decrease in growth rate when exposed to a lower pH as shown in Table 1 and the description of Figure 1.

The data in Table 1 which is explained descriptively in Figure 1 shows that there was a significant decrease \((p < 0.05)\) in bacterial growth between the controls (pH 6.5) compared to their growth at pH 2 and pH 3. However, bacterial growth at pH 4 seemed to return to normal and was not significantly different \((p > 0.05)\) compared to the control. Even though there was a decrease in growth at pH 2 and pH 3. An OD value > 0.1 indicated that the bacteria were resistant to low pH or had a high tolerance (+++).

Follow-up tests in the form of resistance tests for LAB isolates from the rumen of Bali cattle against bile salt were also conducted. The test done in liquid MRS media containing NaDC as a bile acid derivative at NaDC concentrations of 0 mM, 0.2 mM, 0.4 mM, and 0.6 mM showed results as shown in Table 2 and Figure 2.

Data in Table 2 and Figure 2 show that LAB isolates from the rumen could survive in NaDC with a concentration of 0.2 mM – 0.6 mM. Bacterial growth at 0 mM NaDC concentration was not significantly different \((p > 0.05)\) compared to growth at 0.2 mM and 0.4 mM concentrations. However, it was significantly different \((p > 0.05)\) from growth at 0.6 mM NaDC concentration. Even though there was a decrease in growth rate when grown at 0.2 mM and 0.4 mM NaDC concentrations, the OD value obtained was still > 0.1 indicating that the bacteria were resistant to bile salt content (NaDC) at these concentrations or had a high tolerance (+++).

### Table 2. Resistance of LAB isolates from the rumen to bile salts / Sodium Deoxycholate (NaDC)

<table>
<thead>
<tr>
<th>Isolates</th>
<th>LAB growth indicator (Optical Density (OD) pada 660 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NaDC 0 mM</td>
</tr>
<tr>
<td>SR11</td>
<td>2.005 (+++)</td>
</tr>
<tr>
<td>SR12</td>
<td>1.858 (+++)</td>
</tr>
<tr>
<td>SR13</td>
<td>1.957 (+++)</td>
</tr>
<tr>
<td>SR14</td>
<td>1.977 (+++)</td>
</tr>
<tr>
<td>SR15</td>
<td>1.845 (+++)</td>
</tr>
<tr>
<td>SR16</td>
<td>1.825 (+++)</td>
</tr>
<tr>
<td>SR17</td>
<td>1.966 (+++)</td>
</tr>
<tr>
<td>SR18</td>
<td>1.941 (+++)</td>
</tr>
<tr>
<td>SR19</td>
<td>1.914 (+++)</td>
</tr>
<tr>
<td>SR20</td>
<td>1.819 (+++)</td>
</tr>
</tbody>
</table>

**Average (SD)**

|         | 1.911 (± 0.069)ab | 1.956 (± 0.081)b | 1.857 (± 0.126)b | 1.952(± 0.056)b |

Note: SD = Standard Deviation; SR = Sample Rumen; - = Absorbance value < 0.1 (no bile salt tolerance); + = Absorbance value 0.1-0.5 (slightly bile salt tolerance); ++ = Absorbance value 0.5-1.0 (bile salt tolerance); +++ = Absorbance value > 1.0 (highly bile salt tolerance).

*) The average value of 3 repetitions ± standard deviation of the isolate absorbance value.

**DISCUSSION**

Data in Table 1 and Figure 1 show that LAB isolates from the rumen had good growth in the range of pH 2 - pH 4 after incubation for 3 hours, according to the time needed for food to pass through the stomach. The high tolerance at low pH of isolates from the rumen is in line with research conducted by Sukrama et al., and slightly different from the results of research by Sujaya et al, who found no isolates that survived to grow at pH 2 of 17 isolates grown tested. The stomach is the first barrier that must be passed before bacteria enter the intestine (bile salts). Generally, most of the microbes will die in the stomach which has environmental conditions with a low pH. These results indicated that LAB isolates from the rumen of Bali cattle fulfilled one of the requirements as a probiotic, namely being resistant to low pH. The low acid tolerance of bacteria is not only important for resisting gastric acid stress but is also a prerequisite for its use when applied as a food additive and allows the strain to survive long periods of time under high-acid food conditions without reducing growth of bacteria. The declining growth of several isolates after exposure to low pH refers to the theory stated by Yang et al who said that the effect of excessive acidification on the cell wall will cause the destruction of bacterial cell membranes. This phenomenon is caused by several important components such
as magnesium, potassium, and fat, going out of cells and they will cause the lysis of bacteria. Furthermore, tolerance to bile salts is a prerequisite for the colonization and metabolic activity of bacteria in the small intestine of the host. This will help lactic acid bacteria to reach the small intestine and large intestine and contribute to balancing the intestinal microflora.

The results of isolates testing at various concentrations of bile salts (NaDC) also showed their resistance to survival up to 0.6 mM NaDC concentration. The results obtained are in accordance with research conducted by Thamacharoensuk et al. who tested the resistance of lactic acid bacteria isolates in media containing bile salts with different concentrations, and it was seen that there was an increase in microbial cell death when the concentration of bile salts was increased. The high tolerance for NaDC from isolates from the rumen is in line with research conducted by Sukrama et al. According to Smet et al., some LAB has bile salt hydrolase (BSH) enzymes with activity to hydrolyze bile salts. This enzyme is able to change the physical and chemical abilities possessed by bile salts so that they are not toxic to LAB. This is what makes it possible to cause BAL to be resistant to bile salt conditions. But the higher the concentration of bile salts, the number of dead microbial cells will also increase. This is due to increased intracellular enzyme activity from bacteria (β-galactosidase) against bile salts, thereby increasing the permeability of cell membranes. Increased cell permeability will result in a lot of intracellular material coming out of the cell. If this continues, it will cause bacterial lysis. Taking into account the resistance of the isolates to continue growing at low pH and high concentrations of deoxycholic acid (NaDC), LAB isolates from the rumen of Bali cattle have the potential to be developed as superior probiotics. Bacteria would contact at pH values ranging from 2.0 to 8.0 in the gastrointestinal tract if consumed. Thus, probiotic cultures must survive in an environment with gastric and bile acids when viable cells go through the gastrointestinal tract. Resisting at pH 3 for 2 h and growing in the medium containing 1.000 ppm of bile acids are considered as standards for acid and bile tolerance of probiotic culture. Moreover, with the situation of increasing patterns of agent resistance to antibiotics, probiotics are specifically selected based on their ability to not contribute to the spread of antibiotic resistance and are not carry transferable antibiotic resistance also. Like the results of previous studies, these strains from the rumen have the potential to be developed as a bio preservative, although they must be tested further.

CONCLUSION
LAB isolates from the rumen of Bali cattle have the potential to be developed into new probiotic candidates because they show tolerance to low pH (survival at pH 2) and relatively high NaDC concentrations (0.6 mM concentration).

CONFLICT OF INTEREST
The authors declare no conflict of interest regarding the publication of this article

ETHICAL CONSIDERATION
This study has been approved by The Faculty of Veterinary Animal Ethics Committees Universitas Udayana.

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AUTHOR CONTRIBUTIONS
All authors have contributed to all processes in this research including preparation, data gathering and analysis, drafting, and approval for publication of this manuscript.

REFERENCES


