IMMUNOLOGY, HEALTH, AND DISEASE

Screening of Lactobacillus strains of domestic goose origin against bacterial poultry pathogens for use as probiotics

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ABSTRACT Lactobacilli are natural inhabitants of human and animal mucous membranes, including the avian gastrointestinal tract. Recently, increasing attention has been given to their probiotic, health-promoting capacities, among which their antagonistic potential against pathogens plays a key role. A study was conducted to evaluate probiotic properties of Lactobacillus strains isolated from feces or cloacae of domestic geese. Among the 104 examined isolates, previously identified to the species level by whole-cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and analysis of 16S-23S regions of rDNA, dominated Lactobacillus salivarius (35%), followed by Lactobacillus johnsonii (18%) and Lactobacillus ingluviei (11%). All lactobacilli were screened for antimicrobial activity toward Salmonella Enteritidis, Escherichia coli, Clostridium perfringens, Staphylococcus aureus, Pasteurella multocida, and Riemerella anatipestifer using the agar slab method and the well diffusion method. Lactobacillus salivarius and Lactobacillus plantarum exhibited particularly strong antagonism toward all of the indicator strains. The agar slab method, the highest sensitivity to Lactobacillus was observed in R. anatipestifer and P. multocida, and the lowest in E. coli and S. aureus. The ability to produce H2O2 was exhibited by 92% of isolates, but there was no correlation between the rate of production of this reactive oxygen species and the antimicrobial activity of Lactobacillus sp. All lactobacilli showed resistance to pH 3.0 and 3.5 and to 2% bile. The data demonstrate that Lactobacillus isolates from geese may have probiotic potential in reducing bacterial infections. The antibacterial activity of the selected lactobacilli is mainly due to lactic acid production by these bacteria. The selected Lactobacillus strains that strongly inhibited the growth of pathogenic bacteria, and were also resistant to low pH and bile salts, can potentially restore the balance of intestinal microflora in geese and could offer an alternative to antibiotic therapy.

Key words: Lactobacillus, probiotic, goose, poultry, antimicrobial action

INTRODUCTION

Lactobacilli belong to the group of lactic acid bacteria (LAB) that produce lactic acid as the end product of carbohydrate fermentation. They are gram-positive rods or coccobacilli, catalase-negative, non-sporeforming, aerotolerant or anaerobic, aciduric or acidophilic, generally characterized by low G+C content (Salvetti et al., 2012). At the time of writing (May 2014), the genus Lactobacillus appears to be one of the most species-rich genera within the Firmicutes phylum, with 183 recognized species (National Center for Biotechnology Information taxonomy database). Many species of Lactobacillus are generally recognized as safe (GRAS), and they are used in manufacture of fermented food products. Lactobacilli are widely present as members of the complex microbial communities of mucous membranes, especially the gastrointestinal tract of humans and animals. They play an important role in the physiology of their host, as they maintain the balance of the intestinal microflora. Moreover, they improve digestion and assimilation of nutrients, remove toxic substances, and enhance immunity. Because of their health-promoting properties, selected lactobacilli are used as probiotics. Probiotic microorganisms gained the attention of breeders and veterinarians following the ban issued in 2006 by the European Commission on the use of antibiotics as growth stimulators in animals. This ban was prompted by the risk of creating a bacterial resistance to antibiotics as well as by the care for the human as a consumer of animals’ products and by the risk of residues, which may lead to allergic reactions and immune insufficiency (EC, 2003; Ćupić et al., 2011). Some reports have indicated that adding probiotic lactobacilli to poultry feed produced similar effects to antibiotics, manifested by...
increases in weight and better feed efficiency (Jin et al., 1998; Angelakis and Raoult, 2010), as well as resistance to pathogenic bacteria such as Salmonella sp. (Pascual et al., 1999; Van Coillie et al., 2007), Clostridium perfringens (La Ragione et al., 2004; Cao et al., 2012), Escherichia coli (Jin et al., 1996), Campylobacter sp. (Ghareeb et al., 2012), and Brachyspira pilosicoli (Mappley et al., 2013). Administration of probiotic strains is particularly recommended in chicks, which do not yet have intestinal microflora, and whenever the stability of the microflora is at risk. Lactobacilli colonize the gut a week after hatch (Mead, 1997), and lack of intestinal microbiota, as observed in newly hatched chicks, has been considered a major factor in the susceptibility of chicks to bacterial infections (Patterson and Burkholder, 2003; Tierney et al., 2004). An imbalance in the intestinal microflora in older chicks and adult birds can be caused by antibiotic administration or by stressors such as overcrowding, improper ventilation, a deficit of food or water, transport, or vaccination. When the stability is disrupted, pathogens are able to colonize the gut, leading to serious infections.

Probiotic strains may protect animals from intestinal pathogens by several possible mechanisms, of which competitive exclusion is the most common. According to this conception, bacteria compete with each other for space and nutrients. Probiotics adhere to the intestinal wall, colonize, and multiply, thereby preventing the attachment and growth of pathogens. Moreover, Lactobacillus strains can eliminate undesirable microorganisms by producing various antimicrobial components, such as organic acids, hydrogen peroxide, carbon peroxide, diacetyl, low-molecular-weight antimicrobial substances, bacteriocins, and adhesion inhibitors, as well as by stimulating intestinal immune responses (Servin, 2004). Antimicrobial activity is a key criterion during selection of probiotic strains and is considered an important ecological factor determining the dominant bacteria in an intestinal ecosystem (Busarcevic et al., 2008).

The objective of this study was to evaluate the probiotic potential of native goose lactobacilli, expressed as the ability to suppress the growth of common pathogenic bacteria in birds, namely Salmonella enterica, E. coli, Staphylococcus aureus, C. perfringens, Pasteurella multocida, and Riemerella anatipestifer. Not only are these pathogens frequently responsible for illness in poultry, including geese, and the associated economic losses, but they can also pose a threat to consumers. We also tested the capability of strains to survive in the gastrointestinal tract by determining their tolerance to low pH and bile salts.

**MATERIALS AND METHODS**

### Bacteria and Growth Conditions

In the experiment, 104 Lactobacillus strains were used, the isolation and identification of which has been described in our previous work (Dec et al., 2014). Bacteria were obtained from the fresh feces or cloacae of 52 healthy White Koluda geese from 15 large-scale poultry farms in southeastern Poland. The age of the geese ranged from 1 d to 4 yr, but most were about 6 mo old. Bacteria were grown into MRS (DeMan, Rogosa, Sharpe) medium (BTL, Lódz, Poland) at 37°C for 24 to 48 h in 5% CO2. Catalase-negative and gram-positive rods were identified to the species level using intact-cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and analysis of polymorphic regions of rDNA located between conserved genes encoding 16S and 23S rRNA. The isolates examined belonged to 14 Lactobacillus species: L. salivarius, 37 strains; L. johnsonii, 19 strains; L. inunguiculi, 12 strains; L. agilis, 8 strains; L. plantarum, 5 strains; L. reuteri, 5 strains; L. paracasei, 5 strains; L. crispatus, 4 strains; L. amylovorus, 2 strains; L. oris, 2 strains; L. kitasatonis, 1 strain; L. mucosae, 2 strains; L. farciminis, 1 strain; and L. haimnosus, 1 strain.

The following were used as indicator strains: Salmonella Enteritidis ATCC 13311, Clostridium perfringens ATCC 13124 (toxinogenic strain), Escherichia coli ATCC 8734, Staphylococcus aureus ATCC 6538 (purchased from Argenta, Poznań, Poland), Pasteurella multocida ATCC 43137, and Riemerella anatipestifer ATCC 11845 (purchased from LGC Standards, Łomianki, Poland).

**Detection of H2O2 Production by Lactobacillus Strains**

The lactobacilli were plated on MRS supplemented with TMB (dichloride, 3,3′, 5,5′-tetramethylbenzidine) substrate (0.25 mg/mL, Sigma-Aldrich, Poznań, Poland) and horseradish peroxidase (0.01 mg/mL, Sigma-Aldrich) and grown for 48 h at 37°C, 5% CO2. Blue color in the colonies indicated H2O2 production by the bacteria. Color intensity was designated as follows: −, +, ++, and +++ (Song et al., 1999).

**Detection of Antibacterial Activity of Lactobacillus Strains—Agar Slab Method**

The Lactobacillus sp. strains grown on MRS broth were centrifuged and suspended in 0.9% NaCl so that the optical density (OD) of the suspension at 600 nm was 0.4. Plates 4 cm in diameter containing 15 mL of MRS agar were inoculated with 200 μL of lactobacilli and incubated at 37°C, 5% CO2 for 24 h. Then agar slabs 9 mm in diameter were cut and placed on agar inoculated with 0.5 mL of the target indicator strain suspended in 0.9% NaCl (OD600 = 0.1 for Salmonella sp., E. coli, and S. aureus, OD600 = 0.2 for P. multocida and R. anatipestifer, OD600 = 0.8 for C. perfringens; Strus, 1998). For initial diffusion of the substance from the agar slabs, the plates were first refrigerated for 4 h at 4°C, then kept for 20 h at 37°C, in aerobic conditions for S. enterica and E. coli, at 5% CO2 for P. multocida.
and R. anatipestifer, or in anaerobic conditions (Genbox anaer, bioMérieux, Warsaw, Poland) for C. perfringens. Salmonella enterica, E. coli, S. aureus, and C. perfringens were plated on Müller-Hinton medium, and P. multocida and R. anatipestifer on Columbia agar with 5% sheep blood. After incubation, the plates were checked for inhibition zones. The results are presented as the mean diameter of the inhibition zone ± SD for 2 independent experiments.

**Detection of Antibacterial Activity of Lactobacillus Strains—Well Diffusion Method**

The experiment was performed on Lactobacillus sp. strains that had induced growth inhibition zones with a diameter of at least 13 mm in the indicator strains in the agar slab method.

A 15-mL volume of medium obtained after a 24-h culture (37°C, 5% CO₂) of Lactobacillus sp. was lyophilized. The lyophilisate was dissolved in sterile distilled water. Each sample was divided into 2 equal volumes. In half of the samples the pH was adjusted to 6.8 to 7.0 using NaOH (to eliminate the effect of organic acids), and an equal volume of water was added to the remaining samples, with pH 4.0 to 5.0. Finally a 5-fold concentrated cell-free supernatant was obtained. The indicator strains were plated on Müller-Hinton agar or Columbia agar with 5% sheep blood, according to the protocol described above. Heated cylindrical metal wells with a diameter of 8 mm were placed on the plates and filled with 60 μL of the cell-free supernatant. After 18 h of incubation in conditions appropriate for the indicator strains (described above), the plates were checked for inhibition zones. The results are presented as the mean diameter of the inhibition zone ± SD for 2 independent experiments.

**Tolerance for Acidic pH**

Fresh broth cultures of the bacteria were centrifuged at 10,000 × g at 20°C for 5 min. Pellets were resuspended in 0.9% NaCl to obtain a final optical density of 7.0 measured at 600 nm. A 50-μL volume of the suspension was added to 500 μL of MRS broth with pH 2.0, 2.5, 3.0, or 3.5. The bacteria were incubated at low pH for 60, 90, or 120 min. Then the suspensions were centrifuged and the pellets were resuspended in fresh MRS (pH 6.8). Growth of the surviving bacteria was observed after 48 h of culture at 37°C, 5% CO₂.

**Bile Tolerance Test**

The MRS medium containing 2% bile (BTL) was inoculated with active cultures of lactobacilli. Following 24 h incubation at 37°C, 5% CO₂, the optical density of the bacterial cultures was measured at 620 nm. The control cultures were grown without oxgall. The growth of each strain was expressed as a percentage of the OD₆₂₀ value of the control samples.

**Statistical Analysis**

The mean diameters of the inhibition zones for indicator microorganisms that were determined to be sensitive to various Lactobacillus species were compared by 1-way ANOVA (with species as a categorical predictor, zone as a dependent variable and adjusted for pathogen), the Tukey honestly significant difference post hoc test, with modification for unequal N, as a correction for multiple comparisons. The normal distribution of data was examined using the Shapiro-Wilk test, and the equality of variance was tested by the Brown-Forsythe test. When there was a lack of a normal distribution, an unequal variance of data, or both, the Kruskal-Wallis ANOVA was used to analyze the differences between means. A P < 0.05 was considered statistically significant. All statistical analyses were carried out using Statistica 8.0 software (StatSoft Inc., Tulsa, OK).

**RESULTS**

**Antibacterial Activity of Lactobacillus Strains**

**Slab Method.** The diameter of the growth inhibition zones of the indicator bacteria induced by the lactobacilli ranged from 9 to 23 mm, where the diameter of the slab was 9 mm (Figure 1). A total of 102 isolates inhibited the growth of R. anatipestifer; 100, P. multocida; 84, C. perfringens; 62, Salmonella Enteritidis; 50, S. aureus; and 49, E. coli (Table 1). The antimicrobial activity of the Lactobacillus strains was correlated with their species. Particularly strong antagonism against all of the test bacteria was observed in the species L. salivarius and L. plantarum. The mean diameters of inhibitory zones for all indicator microorganisms caused by the strains of these species were ≥15 mm (Table 2, Figure 2). The mean zones of growth inhibition of each indicator microorganism caused by strains of L. salivarius and L. plantarum were significantly higher than the zones induced under the influence of antimicrobial substances produced by strains of L. johnsonii, L. inluviei, L. agilis, L. kitasatonis, L. mucosae, and L. oris (Table 2). Antimicrobial activity against all of the pathogenic strains was exhibited by 92% (34) of the L. salivarius strains and 100% (5) of the L. plantarum strains (Table 1). Strains of the species L. inluviei, L. johnsonii, L. kitasatonis, L. mucosae, L. oris, and L. agilis exhibited weak antagonistic properties; the average diameters of the growth inhibition zones of indicator bacteria caused by these species of Lactobacillus were lower than 10.6 mm (Figure 2). Statistically significant differences between the average size of the growth inhibition zones of the indicator bacteria caused by various Lactobacillus species were indicated in Table 2. Seventeen of 19 L. johnsonii strains, all L. inluviei and L. oris strains,
Table 1. Number of Lactobacillus strains exhibiting inhibitory properties toward indicator bacteria, as determined in the agar slab method; n = number of strains

<table>
<thead>
<tr>
<th>Item</th>
<th>Salmonella Enteritidis</th>
<th>Escherichia coli</th>
<th>Staphylococcus aureus</th>
<th>Pasteurella multocida</th>
<th>Riemerella anatipestifer</th>
<th>Clostridium perfringens</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of Lactobacillus strains exhibiting antagonism toward indicator bacteria</td>
<td>62/37</td>
<td>49/37</td>
<td>50/37</td>
<td>100/37</td>
<td>102/37</td>
<td>84/37</td>
</tr>
<tr>
<td>Lactobacillus salivarius (n = 37)</td>
<td>37/37</td>
<td>37/37</td>
<td>36/37</td>
<td>36/37</td>
<td>37/37</td>
<td>34/37</td>
</tr>
<tr>
<td>Lactobacillus johnsonii (n = 19)</td>
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<td>0/19</td>
<td>0/19</td>
<td>19/19</td>
<td>19/19</td>
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<td>0/12</td>
<td>0/12</td>
<td>11/12</td>
<td>10/12</td>
<td>11/12</td>
</tr>
<tr>
<td>Lactobacillus agilis (n = 8)</td>
<td>1/8</td>
<td>1/8</td>
<td>1/8</td>
<td>7/8</td>
<td>8/8</td>
<td>4/8</td>
</tr>
<tr>
<td>Lactobacillus plantarum (n = 5)</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>Lactobacillus reuteri (n = 5)</td>
<td>3/5</td>
<td>0/5</td>
<td>1/5</td>
<td>5/5</td>
<td>5/5</td>
<td>4/5</td>
</tr>
<tr>
<td>Lactobacillus paracasei (n = 5)</td>
<td>5/5</td>
<td>0/5</td>
<td>1/5</td>
<td>5/5</td>
<td>5/5</td>
<td>3/5</td>
</tr>
<tr>
<td>Lactobacillus crispatus (n = 4)</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td>Lactobacillus amylovorus (n = 2)</td>
<td>2/2</td>
<td>1/2</td>
<td>1/2</td>
<td>2/2</td>
<td>2/2</td>
<td>2/2</td>
</tr>
<tr>
<td>Lactobacillus kitasatonis (n = 1)</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>1/1</td>
<td>1/1</td>
<td>0/1</td>
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<tr>
<td>Lactobacillus oris (n = 2)</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>2/2</td>
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<tr>
<td>Lactobacillus mucosae (n = 2)</td>
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<td>0/2</td>
<td>0/2</td>
<td>2/2</td>
<td>2/2</td>
<td>2/2</td>
</tr>
<tr>
<td>Lactobacillus farciminis (n = 1)</td>
<td>1/1</td>
<td>1/1</td>
<td>1/1</td>
<td>1/1</td>
<td>1/1</td>
<td>1/1</td>
</tr>
<tr>
<td>Lactobacillus rhamnosus (n = 1)</td>
<td>1/1</td>
<td>0/1</td>
<td>0/1</td>
<td>1/1</td>
<td>1/1</td>
<td>1/1</td>
</tr>
</tbody>
</table>

The ANOVA of the mean diameters demonstrated that the P. multocida and R. anatipester were more sensitive (P < 0.05) than other indicator microorganisms to the antagonistic substances produced by lactobacilli (Figure 3). The average diameter of inhibition zones for P. multocida was 15.2 mm, and for R. anatipester, 16.0 mm. The E. coli and S. aureus strains were found to be the least susceptible to Lactobacillus activity. The average zone of inhibition (9.5 and 9.6 mm) of these indicator strains caused by all examined lactobacilli differed (P < 0.05) from the average zone of inhibition obtained for other indicator bacteria.

Moderately large zones were observed for Salmonella Enteritidis and C. perfringens (Figure 3, Table 2).

**Well Diffusion Method.** The experiment was conducted on 72 strains, which induced growth inhibition zones ≥13 mm for at least one indicator microorganism in the agar slab method. The pH of the 5-fold concentrated supernatant obtained from the culture of L. reuteri, L. crispatus, and L. johnsonii, L. ingluviei, and L. oris strains ranged from 4.5 to 5.1, whereas in the case of the remaining strains (L. salivarius, L. plantarum, L. agilis, L. paracasei, L. amylovorus, and L. farciminis), the pH was 4.0 to 4.7. The acidified cell-free supernatant induced growth inhibition zones from 8 to 19 mm in diameter in the indicator bacteria, where the well diameter was 8 mm. The supernatants of the pH range of 4.0 to 4.7 caused larger mean zones of inhibition (≥10 mm) than the supernatants at pH 4.5 to 5.1 (<10 mm; Figure 4), but in the case of most Lactobacillus species, these differences were statistically insignificant. Cell-free supernatants with neutralized acids (pH 6.8–7.0) did not exhibit antagonistic activity toward the indicator strains, with the exception of the 3 supernatants from the cultures of L. salivarius 55, L. salivarius 70, and L. plantarum 22, which exhibited a slight inhibitory effect (mean diameter of growth inhibition zone ≤11 mm) toward R. anatipester and P. multocida (Figures 4 and 5). Mean diameters of inhibitory zones for indicator microorganisms cause by the cell-free supernatants obtained from the culture of different Lactobacillus species are shown in Figure 4. Significant differences were observed between sizes of zones of inhibition caused by native and neutralized supernatants of most Lactobacillus species and the correlation between the average zone of inhibition and the species of Lactobacillus (Figure 4). The biggest mean growth inhibitory zones (≥11.9 mm) for indicator bacteria were caused by native cell-free supernatant obtained from the culture of L. plantarum and L. salivarius (Figure 4), but there

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**Figure 1.** Antagonistic activity of Lactobacillus sp. against indicator bacteria with the agar slab method. A) Salmonella Enteritidis, B) Clostridium perfringens, C) Pasteurella multocida, D) Staphylococcus aureus. Color version available in the online PDF.
Table 2. ANOVA of the growth inhibition of indicator microorganisms by the *Lactobacillus* isolates, as determined by the agar slab method

<table>
<thead>
<tr>
<th>Item</th>
<th>Indicator strain</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Salmonella</em> Enteritidis</td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus salivarius</em> (n = 37)</td>
<td>14.6 ± 2.2b,A</td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus johnsonii</em> (n = 19)</td>
<td>9.0 ± 0.2a-A</td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus ruminis</em> (n = 12)</td>
<td>9.0 ± 0.0a-A</td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus agilis</em> (n = 8)</td>
<td>9.2 ± 0.6a-A</td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus plantarum</em> (n = 5)</td>
<td>15.0 ± 1.0a,A</td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus reuteri</em> (n = 5)</td>
<td>9.5 ± 1.0a,AB</td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus paracasei</em> (n = 5)</td>
<td>11.6 ± 1.6ab,A</td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus crispatus</em> (n = 4)</td>
<td>12.1 ± 1.7ab,A</td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus anginosus</em> (n = 2)</td>
<td>11.5 ± 1.0ab,A</td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus kotsasorum</em> (n = 1)</td>
<td>9.0 ± 0.0a-A</td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus oris</em> (n = 2)</td>
<td>9.0 ± 0.0a-A</td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus mucosae</em> (n = 2)</td>
<td>9.2 ± 0.5a-A</td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus farciminis</em> (n = 1)</td>
<td>12.0 ± 1.4ab,A</td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus</em> (n = 1)</td>
<td>12.0 ± 1.4ab,A</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>11.7 ± 3.0a</td>
<td></td>
</tr>
</tbody>
</table>

*Different superscripted lowercase letters indicate differences between mean values in columns (comparison of the sensitivity of the indicator microorganism to the antagonistic substances produced by the strains of each *Lactobacillus* species); P < 0.05.

A–CDifferent superscripted capital letters indicate differences between mean values in rows (comparison of the inhibitory effect of the antagonistic substances on each indicator organism); P < 0.05.

*The results are presented as mean diameter of the growth inhibition zone (mm) for 2 independent experiments; the diameter of the agar slab was 9 mm.

*n* = number of strains.
were no significant differences between these species and most remaining *Lactobacillus* species.

The highest susceptibility to the antagonistic activity of an acidic environment (native cell-free supernatants) was exhibited by *R. anatipestifer*, as its mean inhibitory zones differed significantly from the mean zones for *E. coli*, *S. aureus*, and *C. perfringens* (Figure 5). The growth of *R. anatipestifer* was inhibited by 96% of the media with acidic pH. The maximum resistance to the antagonistic activity of the acidified supernatants from cultures of the *Lactobacillus* sp. strains was noted for *E. coli* and *C. perfringens* whose mean inhibitory zones were significantly less than zones for *S. enterica*, *P. multocida*, and *R. anatipestifer* (Figure 5). Only 58% of the acidic supernatants used in the experiment inhibited the growth of these indicator strains.

**Production of H₂O₂**

Of the 104 *Lactobacillus* sp. strains examined, 97 (93.3%) produced H₂O₂. Bacterial colonies grown on MRS agar supplemented with TMB and horseradish peroxidase varied in the intensity of color. In some strains, such as *L. plantarum* and *L. salivarius*, the

![Figure 2](https://academic.oup.com/ps/article-abstract/93/10/2464/1533926)  
**Figure 2.** The effect of strains of different species of *Lactobacillus* on the growth of indicator strains, as determined by the agar slab method. The results are presented as mean diameter of the growth inhibition zone (mm) of all indicator bacteria for 2 independent experiments; the diameter of the agar slab was 9 mm; the vertical bars denote 0.95 CI. Color version available in the online PDF.

![Figure 3](https://academic.oup.com/ps/article-abstract/93/10/2464/1533926)  
**Figure 3.** The susceptibility of the indicator strains to *Lactobacillus* species, as determined by the agar slab method. The results are presented as mean diameter of the growth inhibition zone (mm) for 2 independent experiments; the diameter of the agar slab was 9 mm; the vertical bars denote 0.95 CI. Different capital letters (A–C) indicate significant differences (*P* < 0.05) between mean diameter of growth inhibition zones caused by all *Lactobacillus* strains. Color version available in the online PDF.

![Figure 4](https://academic.oup.com/ps/article-abstract/93/10/2464/1533926)  
**Figure 4.** The effect of strains of different species of *Lactobacillus* on the growth of indicator bacteria, as determined by the well diffusion method with using native and neutralized cell-free supernatant obtained from the *Lactobacillus* strain culture. The results are presented as mean diameter of the growth inhibition zone (mm) for 2 independent experiments; the diameter of the metal well was 8 mm; the vertical bars denote 0.95 CI. *Significant differences (*P* < 0.5) between the mean size of the inhibition zones caused by native and neutralized supernatants (Kruskal-Wallis 1-way ANOVA). Color version available in the online PDF.

![Figure 5](https://academic.oup.com/ps/article-abstract/93/10/2464/1533926)  
**Figure 5.** The susceptibility of the indicator strains to *Lactobacillus* species, as determined by the well diffusion method. The results are presented as mean diameter of the growth inhibition zone (mm) for 2 independent experiments; the diameter of the metal well was 8 mm; the vertical bars denote 0.95 CI. Different lowercase letters (a–g) indicate significant differences (*P* < 0.05) between mean diameter of growth inhibition zones caused by all *Lactobacillus* strains. Color version available in the online PDF.
blue color appeared only around the bacterial colonies, whereas in others (e.g., *L. johnsonii*, *L. inerubiei*, and *L. agilis*) entire cells were blue. In some *L. salivarius* strains, only the middle of the colony was yellow.

The highest rate of production (+++) was observed in 25 isolates belonging mainly to the species *L. agilis*, *L. johnsonii*, and *L. inerubiei*. Moderate H$_2$O$_2$ production (+) was noted in 26 strains, most of which were of the species *L. salivarius*. The group with the lowest H$_2$O$_2$ production (+) contained 46 strains, predominantly of the species *L. salivarius*. The 7 isolates that did not exhibit the ability to produce H$_2$O$_2$ belonged to the species *L. paracasei* (5), *L. salivarius* (2), and *L. plantarum* (1).

**Resistance to Low pH**

All 104 *Lactobacillus* strains were able to survive pH 3.0 and 3.5 for 2 h. At pH 2.5, 61 strains survived 1 h of incubation, 32 survived 1.5 h, and only 26 survived 2 h. In a medium with pH 2.0, 10 strains survived 1 h of incubation. These were strains of the species *L. reuteri* (53b1, 54b, 67a, 69b), *L. salivarius* (G2K, 19a, 56a, 65a), and *L. johnsonii* (G1jc, 29b1). Only 5 isolates, belonging to *L. salivarius* (19a, 56a) and *L. reuteri* (53b1, 67a, and 69b), survived 1.5 h of incubation in pH 2.0. Considerable resistance to low pH was observed in strains of *L. reuteri*, whereas the highest sensitivity to an acidified environment was noted for *L. agilis*. None of the 8 *L. agilis* isolates survived incubation in pH 2.5 and 2.0.

**Resistance to Bile**

All 104 *Lactobacillus* strains tested were able to survive for 24 h in the presence of 2% bile. Moreover, 94 isolates demonstrated growth in the MRS medium containing 2% bile. High resistance to bile salts was observed in 24 strains, whose growth, evaluated based on optical density, varied from 50 to 100% with respect to the control. This group included 7 strains of *L. salivarius*, all strains of *L. plantarum* (5) and *L. reuteri* (5), and 1 strain each from the species *L. johnsonii*, *L. inerubiei*, *L. crispatus*, *L. amylovorus*, *L. oris*, and *L. farcininis*.

**DISCUSSION**

The aim of the study was to assess the antimicrobial activity of *Lactobacillus* strains of goose origin against poultry pathogens, including *S. Enteritidis*, *E. coli*, *S. aureus*, *C. perfringens*, *P. multocida*, and *R. anatipestifer*, and evaluation of resistance of these bacteria to low pH and bile salts. The study showed that *Lactobacillus* bacteria originating in geese have growth-inhibiting properties for poultry pathogenic bacteria. The antimicrobial activity of the lactobacilli was generally correlated with their species. The strong antagonism was observed in strains of *L. salivarius* and *L. plantarum*.

Among the pathogens tested, the highest (*P < 0.5*) susceptibility to *Lactobacillus* activity in the agar slab method was noted for *R. anatipestifer* and *P. multocida*, and the lowest in *E. coli* and *S. aureus*. The results obtained using the well diffusion method showed that the antibacterial activity of a substantial majority of the selected *Lactobacillus* bacteria was due to production of organic acids by these bacteria.

The antimicrobial activity of lactobacilli isolated from birds, including *L. salivarius* and *L. plantarum*, has also been observed by other authors. Several studies have demonstrated the inhibitory effect of chicken lactobacilli on the growth of *Salmonella*, enteropathogenic bacteria that cause significant losses in poultry farming. Lima et al. (2007) showed an antagonistic effect of chicken intestinal strains of *L. salivarius*, *L. reuteri*, and *Lactobacillus* sp. on the growth of *Salmonella Enteritidis*, *Salmonella Typhimurium*, and *Salmonella Pullorum*. Yamazaki et al. (2012) selected LAB strains from chicks and laying hens that were able to inhibit the growth of *S. Enteritidis* and *S. Typhimurium* in the spot-on-lawn method. *Lactobacillus salivarius, L. plantarum, L. rhamnosus*, and *L. reuteri* exhibited powerful inhibitory effects against both *Salmonella* strains. These observations are partially divergent with our results, as our isolates of *Lactobacillus reuteri* showed a statistically weaker inhibition of growth of indicator bacteria, including *Salmonella Enteritidis*, when compared with the *L. salivarius* strains or *L. plantarum*. Nouri et al. (2010) demonstrated that *Lactobacillus* sp. isolated from the digestive tract of chickens exhibit antagonistic activity toward *E. coli* and *Salmonella Enteritidis*. The 5 selected strains inducing the strongest growth inhibition in these pathogens belonged to the species *L. salivarius* (4 strains) and *L. crispatus* (1 strain).

Antimicrobial protective activity of selected *L. salivarius* against *Salmonella Enteritidis* was also observed in experiments in vivo. A single dose of the probiotic strain *L. salivarius* 3d (Kizerwetter-Świda and Binek, 2009) or *L. salivarius* CTC2197 (Pascal et al., 1999) reduced the number of pathogenic bacteria in the intestines of chicken or led to their complete disappearance, respectively. Strain *L. salivarius* 3d also showed a protective effect against the infection of chickens with *C. perfringens*. The strong antibacterial activity of *L. plantarum* observed in our study has also been demonstrated by other authors. Bednarski and Kuczkowski (2006) in the agar slab method showed the strong inhibitory effect of *L. plantarum* toward *Salmonella Enteritidis* and *E. coli* originating in poultry. The antimicrobial activity of *L. plantarum* (Lp1, Lp2, Lp3) was also demonstrated by Fazeli et al. (2009). The strains examined, which were isolated from fermented olives, inhibited the growth of *Salmonella Typhimurium* (PTCC1639) in the in vitro experiments.

The results of our study showing that the organic acids are responsible for the antimicrobial activity of lactobacilli are consistent with the observations of Gargiga et al. (1998). These authors observed antimicrobial
activity of cell-free native supernatants obtained from the culture of chicken LAB toward bacterial poultry pathogens (i.e., *Salmonella* Enteritidis and *E. coli*), and as in our study, no inhibition was detected after the fluid was neutralized. The strong antimicrobial activity of lactic acid produced by lactobacilli from commercial preparations was also confirmed by Makras et al. (2006) in vitro using the indicator strain *Salmonella Typhimurium* SL1344.

As demonstrated in our study, the size of inhibition zones depends not only on the sensitivity of the target strain to the antimicrobial compounds produced by the lactobacilli but also on the method used for detection. In the agar slab method, more *Lactobacillus* strains showed inhibitory activity and larger inhibition zones were obtained than in the well diffusion assay. Other authors (Lima et al., 2007; Polak-Berecka et al., 2009) have also obtained a stronger inhibitory effect toward bacterial pathogens using live cultures of *Lactobacillus* sp. than using cell-free media. This can be explained by the fact that *Lactobacillus* bacteria grown on agar medium are able to synthesize other inhibitory substances, known as bacteriocins, in significantly greater amounts than in a liquid culture (Sip, 1999). Production and release of antimicrobial molecules (bacteriocins, *H*₂*O*₂) by lactobacilli are known to vary with factors such as cell density, population kinetics, and culture conditions (Aasen et al., 2000; Jaroni and Brashears, 2000). Such differences could account for the failure to detect inhibition zones in well diffusion tests. In the case of *C. perfringens*, which was grown in anaerobic conditions, it should be taken into account that after the antagonistic bacteria were transferred on the agar slab, they were able to grow simultaneously with the indicator bacteria.

To determine the mechanism of the antimicrobial activity of *Lactobacillus* bacteria, we analyzed the ability of the isolates to synthesize *H*₂*O*₂. This metabolite exhibits varying inhibitory properties toward different microorganisms. Its effectiveness is due to the generation of hydroxyl radicals, which are highly active and toxic for the cell (Kraszewska et al., 2005). The results of the present study showed that most *Lactobacillus* sp. strains originating in geese produce *H*₂*O*₂. However, production of this reactive oxygen species was not correlated with the antimicrobial activity of lactobacilli observed in the slab method and the well diffusion method. Most strains that strongly inhibited the growth of poultry pathogens exhibited intermediate (+++) or weak (++) *H*₂*O*₂ production. Strong production of *H*₂*O*₂ was observed mainly in strains of *L. johnsonii*, *L. infantum*, and *L. agilis*. Other authors have also observed the ability of intestinal bacilli to produce *H*₂*O*₂. Heravi et al. (2011) found that *H*₂*O*₂ was produced by all *Lactobacillus* strains (except one *L. reuteri* strain) isolated from the digestive tract of broilers. Based on the intensity of the blue color in the colonies (plate method), they found, in partial contrast to our results, that the best producers of *H*₂*O*₂ were 2 strains of *L. salivarius* and one of *L. crispatus*. Kraszewska et al. (2005) demonstrated that strains of *L. plantarum* are incapable of producing *H*₂*O*₂, whereas in our study the isolates of *L. plantarum* obtained from geese exhibited moderate or low *H*₂*O*₂ production. Only one strain of this species showed no ability to produce *H*₂*O*₂. Differences in the results obtained may be due to different incubation temperatures (e.g., 28°C in the case of the study by Kraszewska et al., 2005). Various factors can influence production of *H*₂*O*₂ by LAB and its activity, including culture temperature, oxygenation, the composition of the medium, and even the conditions in which the bacteria were previously stored (Jaroni and Brashears, 2000).

Apart from antimicrobial properties, another important criterion for selecting probiotic strains is resistance to the low *pH* in the stomach. The *pH* of the gastric contents in geese varies from 3.5 to 5.0 depending on time postfeeding (Clemens et al., 1975). All of the *Lactobacillus* strains tested were resistant to *pH* 3.5 and 3.0. Based on the ability to survive in an environment with *pH* 2.0, selected strains of the species *L. salivarius*, *L. reuteri*, and *L. johnsonii* were identified as the most acid resistant. The results obtained in our study are consistent with those of experiments conducted by other authors. According to Ehrmann et al. (2002), strains of the genus *Lactobacillus* isolated from ducks are able to survive 4-h incubation in *pH* 3.0 and 2.0, and a few (*L. salivarius* TMW 1.992, *L. animalis* TMW 1.972) can even survive for 1 h in *pH* 1.0. On the other hand, in a study by Hutari et al. (2011), aimed at selecting probiotic strains isolated from chickens, only a few *Lactobacillus* strains (*L. salivarius* and *L. fermentum*) were able to survive 3-h incubation in *pH* 2.5.

The study made it possible to select strains of *Lactobacillus* sp. characterized by antagonistic properties toward bacterial pathogens as well as resistance to low *pH* and bile salts. These strains can potentially restore the balance of intestinal microflora in geese and can be considered for use as prophylactic agents or as an alternative to antibiotic therapy for infections by *Salmonella* Enteritidis, *E. coli*, *S. aureus*, *C. perfringens*, *P. multocida*, or *R. anatipestifer*. The obtained results are the basis for further more detailed studies that are required for the selection of probiotic strains. It should be noted that the results of laboratory tests do not necessarily correlate directly with in vivo activity, and the effectiveness of selected *Lactobacillus* strains must be verified in animal models.

### REFERENCES


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