Assessment of potentially probiotic properties of Lactobacillus strains isolated from chickens

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Abstract

This study was performed in order to isolate lactobacilli from chicken droppings and to select strains with the most promising probiotic properties. Lactobacillus strains were isolated from a flock of healthy laying hens. The first selection criterion was the ability to inhibit the growth of Salmonella Enteritidis. Then the tolerance to low pH and bile salt, the ability to coaggregate with pathogenic bacteria and hydrogen peroxide production were evaluated. Four isolates showing the best antagonistic activity against Salmonella Enteritidis were selected for further research. All isolates tested tolerated low pH and bile salt, likewise all produced hydrogen peroxide. They efficiently coaggregated with C. perfringens and relatively less with E. coli. Isolate 03'04 displayed above-average results in all criteria, thus it is considered as a potential probiotic for chickens, and will be further evaluated for health promoting effect in animals. The results presented in this study confirm the strain specific probiotic properties and prove the probiotic potential of isolate 03'04. Strong antagonistic properties against C. perfringens exhibited by certain Lactobacillus strains indicate the possibility to use them as a component of probiotic supplement in necrotic enteritis of poultry.

Key words: Lactobacillus, probiotic, chickens, coaggregation, E. coli, C. perfringens, Salmonella Enteritidis

Introduction

Probiotics have a number of beneficial health effects in the host intestinal microbiota (Saarela et al. 2000, Walter 2008). They have been commonly used in humans as well as in animal production. Presently, the popular approach is that probiotic bacterial strains should be individually tailored for each animal species because probiotic properties are strain specific (Ehrmann et al. 2002). Bacteria belonging to different lactic acid bacteria (LAB) are available as probiotic supplements, but lactobacilli are the most commonly used. Different properties contribute to the positive effects of probiotics on health, including interactions between intestinal bacteria and effects on the host. Beneficial effects are associated with production of lactic acid, acetic acid and other organic acids, hydrogen peroxide, competition for nutrients, ability to reduce adherence and colonization of pathogenic bacteria in gastrointestinal tract and production of inhibitory proteins called bacteriocins (Jin et al. 1996, Lima et al. 2007, Rehman et al. 2007, Walter 2008). Another mechanism by which probiotics exert their beneficial effect is the stimulation of the intestinal mucosal
immune response and regulation of the inflammation processes by changing expression levels of the cytokines (Cao et al. 2012, Messaoudi et al. 2012b).

The search for a new probiotic strains is driven by the growing demand for reducing the antimicrobials use in food-production animals. There are many different probiotic products designated for chickens, however some of them may be not fully effective, most likely because of lack of the proper studies on the probiotic properties of bacterial strains included in these formulations. The selection criteria for a new probiotic strains include a series of in vitro and in vivo experiments. One of the desirable attribute is the ability of lactobacilli to coaggregate with pathogenic bacteria, which supports the activity of inhibitory agents secreted by lactobacilli (Ehrmann et al. 2002, Klaenhammer et al. 2008). Other important functional properties, which provide survival in the digestive system are the tolerance to gastric acid and bile salt (Garriga et al. 1998, Klaenhammer et al. 2008, Walter 2008).

This study was planned as a preliminary step for selection of potentially probiotic lactobacilli for further in vivo experiments. Hence, in this research Lactobacillus spp. were isolated from chicken GIT, isolates showing antagonistic activity against Salmonella Enteritidis were selected for further evaluation. Lactobacilli were investigated for low pH tolerance, bile salt tolerance, the ability to coaggregate with pathogenic bacteria and to produce hydrogen peroxide.

**Materials and Methods**

**Bacterial isolates and growth conditions**

Lactobacillus spp. have been isolated from fresh droppings of healthy laying hens (Withe Leghorn, 40 weeks of age) kept on litter. All birds were provided with free access to clean water and a standard feed. Bacteria were grown on De Man-Rogosa-Sharpe (MRS) agar (Oxoid) in anaerobic conditions at 37°C for 48 h. The isolates were identified based on Gram’s stain morphology, catalase reaction, and biochemical properties tested in API 50CHL (bioMerieux). Pathogenic bacteria: *Escherichia coli*, *Salmonella Enteritidis* and *Clostridium perfringens* have been isolated from chicken internal organs in the Microbiological Diagnostic Laboratory, Faculty of Veterinary Medicine, Warsaw Agriculture University.

**Agar spot test – detection of antibacterial activity**

Lactobacilli were tested for inhibitory activity against randomly selected clinical Salmonella Enteritidis isolate. The agar spot test was performed according to the method described by Schillinger and Lucke (1989). Briefly, lactobacilli were spot inoculated on MRS agar and incubated at 37°C for 48 h in anaerobic conditions. Then, the plates were overlaid with 7 ml of soft agar (0.75% agar) containing 10⁷ CFU/ml of Salmonella Enteritidis and incubated aerobically at 37°C for 24h. The diameters of growth inhibition zones around lactobacilli were recorded.

**Acid and bile tolerance**

Acid and bile tolerance were examined according to the method described by Anderson et al. (2010) with some modifications. Each Lactobacillus isolate was grown overnight at 37°C in MRS broth. To determine acid tolerance the pH of each MRS broth was adjusted with HCl to 2.5 and then lactobacilli were incubated for 4h at 37°C under anaerobic conditions. The number of viable cells was established using quantitative cultures on MRS agar before and after the incubation, in triplicate. Bile salts tolerance was verified by incubation of each isolate in MRS broth containing 0.5% (w/v) of bile salt (Sigma). After 4 h of incubation at 37°C under anaerobic conditions, viable cells were counted as described above. The survival rate was calculated as log₁₀ values of CFU/ml.

**Coaggregation experiments**

Coaggregation experiments were performed as described by Juárez Tomás et al. (2005). Overnight broth cultures of lactobacilli and pathogenic bacteria (*E. coli*, *Salmonella Enteritidis* or *C. perfringens*) were centrifuged, washed twice in coaggregation buffer of the following composition: CaCl₂ 0.1 mM, MgCl₂ 0.1 mM, NaCl 0.15 mM, NaN₃ 3.1 mM in 1 mM Tris buffer, pH 7.0, and resuspended in this buffer. The volume of 2 ml of each Lactobacillus suspension was mixed with 2 ml of pathogenic bacteria and the OD₆₀₀ was measured. After 2, 4, 12 and 24 h of incubation at room temperature, OD₆₀₀ was measured again. The percent of coaggregation was calculated:

\[
\% \text{ coaggregation} = \left( \frac{\text{OD}_1 - \text{OD}_2}{\text{OD}_1} \right) \times 100\%
\]

Where OD₁ is the initial optical density of mixture of Lactobacillus and pathogenic strain, OD₂ is the optical density after 2 h, 4 h, 12 h, and 24 h of incubation.
Table 1. The tolerance of isolated lactobacilli to low pH and bile salt.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Initial log_{10} CFU/ml*</th>
<th>pH 2.5 Log_{10} CFU/ml after 4 h</th>
<th>% of viability</th>
<th>0.5% of bile salt Log_{10} CFU/ml after 4 h</th>
<th>% of viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>03'04</td>
<td>8.10 ± 0.09</td>
<td>7.89 ± 0.11</td>
<td>97%</td>
<td>7.65 ± 0.19</td>
<td>94%</td>
</tr>
<tr>
<td>01'05</td>
<td>7.69 ± 0.12</td>
<td>7.30 ± 0.17</td>
<td>95%</td>
<td>7.04 ± 0.21</td>
<td>92%</td>
</tr>
<tr>
<td>03'05</td>
<td>8.42 ± 0.37</td>
<td>7.97 ± 0.21</td>
<td>95%</td>
<td>7.43 ± 0.24</td>
<td>88%</td>
</tr>
<tr>
<td>10'05</td>
<td>8.08 ± 0.19</td>
<td>7.63 ± 0.18</td>
<td>94%</td>
<td>7.41 ± 0.18</td>
<td>92%</td>
</tr>
</tbody>
</table>

* – Values are the mean ±SD from three independent experiments

Determination of hydrogen peroxide production

*Lactobacillus* isolates were tested for hydrogen peroxide production (H_2O_2) by the qualitative plate method, using horseradish peroxide incorporated in 3,3',5,5'-tetramethylbenzidine (TMB, Sigma) MRS agar medium (Eschenbach et al. 1989). Lactobacilli were cultured on MRS agar containing 1mM TMB and 2U/ml horseradish peroxide (Sigma). Plates were incubated in anaerobic conditions at 37°C for 48 h. Colonies of H_2O_2-producing strains turned blue after exposure to air for 30 min.

Results

In total 62 isolates were obtained from chicken droppings. All these bacteria were presumptively identified as lactobacilli, because of the ability to grow on MRS agar, Gram positive, rod shape cell morphology and the negative results in catalase test. Four isolates showing the widest inhibition zones 10 mm (isolate designated as 1'05), 11 mm (10'05), 14 mm (03'04), and 15 mm (3'05) obtained in agar spot test with *Salmonella* Enteritidis were selected for further evaluations. These isolates, in accordance to API50CH, were identified as *L. salivarius* (03'04, 01'05 and 10'05) and *L. brevis* (03'05).

The results of acid and bile tolerance are shown in Table 1. All isolates were able to tolerate pH 2.5 with the loss of viability from 97% to 94%. Slightly lower tolerance was observed to 0.5% of bile salt, however the loss of viability was within a range from 94% to 88%. The isolate 03'04 has showed a clearly greater resistance to low pH and bile salt.

Figures 1-3 present the results of coaggregation of evaluated *Lactobacillus* isolates with pathogenic bacteria. In general, all *Lactobacillus* isolates shown higher ability to coaggregate with *C. perfringens* comparing to other pathogens. The percentage of coaggregation with *C. perfringens* observed after 24 hours for isolate 03'04 was 71%, and from 43% to 52% for the remaining three isolates. The percentage of coaggregation with *E. coli* and *Salmonella* displayed by isolate 03'04 was 37% and 53% respectively. These values for the other isolates ranged from 17% to 23% for *E. coli* and from 12% to 21% for *Salmonella*.

All *Lactobacillus* isolates tested has been classified as hydrogen peroxide producers, as confirmed by observing the dark blue colonies after exposure to air.

Discussion

In 2002 the FAO/WHO Working Group developed new guidelines for the introduction of a new probiotics microorganisms. The special attention was put on safety assessment and effectiveness. The guidelines contain the following recommendations: *in vitro* tests to evaluate probiotic potential and *in vivo* clinical on animals or humans (FAO/WHO, 2002). The use of *in vitro* test as selection criteria is unavoidable and contributes to reducing the number of strains for *in vivo* testing (Taheri et al. 2009). The isolation of various *Lactobacillus* species including *L. salivarius* and *L. brevis* from chicken GIT has been reported previously, which is in line with identification of isolates described in the present study (Kizerwetter-Świda and Binek 2009, Bujnakova et al. 2014).

One of the crucial properties of probiotic lactobacilli is the ability to survive in the low pH of the stomach and in the high concentration of bile salt of the upper GIT. The capability to tolerate acid and bile is considered as good indicators for the viability in the GIT, thus these characteristics are often assessed in preliminary examination of potentially probiotic strains (Bull et al. 2013).

In our experiment four *Lactobacillus* isolates showing the best ability to inhibit the growth of *Salmonella* Enteritidis, were selected for further evaluations. Isolate 03'04 showed significantly higher tolerance to low pH and bile salt, with the viability 97% at pH 2.5 and 94% in 0.5% of bile salt. However, all other isolates showed good tolerance to these conditions and their viability ranged from 94% to 95% in low pH, and from 88% to 92 in bile salt. Obtained
Fig. 1. Coaggregation ability of *Lactobacillus* isolates with *C. perfringens*.

Fig. 2. Coaggregation ability of *Lactobacillus* isolates with *E. coli*.

Fig. 3. Coaggregation ability of *Lactobacillus* isolates with *Salmonella* spp.
results confirm that the acid and bile tolerance is specific only for some *Lactobacillus* isolates (Ehrmann et al. 2002, Taheri at al. 2009). Survival of different lactobacilli was dependent on the time and the pH or bile salts concentration used in individual studies. Some isolates of chicken origin exhibited even 100% survival rate at pH of 2.0, while others showed no viability at those conditions (Garriaga et al. 1998, Lee et al. 2008, Bujnakova et al. 2014). Hashemi et al. (2014) described significantly lower viability of lactobacilli after 2 h of incubation at the pH 2 or in 0.3% of bile salt, but all evaluated bacteria were obtained from traditional kurdish cheese. It is well documented that lactobacilli isolated from GIT are able to tolerate low pH and bile salt, and they could be considered as a components of probiotics supplement for poultry.

Isolate 03’04 showing above-average results in all investigated attributes seems particularly promising candidate for *in vivo* experiments. These results strongly suggest the desireability of *in vitro* assessment of a new potentially probiotic bacteria, what is expected to contribute to the development of more effective probiotic supplements for poultry.

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**References**


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