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Original article

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* Enetritidis 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

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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* Enetritidis 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 10^7 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 $\log_{10}$ 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: $CaCl_2$ 0.1 mM, $MgCl_2$ 0.1 mM, NaCl 0.15 mM, NaN_3 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 OD_{600} was measured. After 2, 4, 12 and 24 h of incubation at room temperature, OD_{600} was measured again. The percent of coaggregation was calculated:

$$\% \text{ coaggregation} = (OD_1 - OD_2 / OD_1) \times 100\%$$

Where OD_1 is the initial optical density of mixture of *Lactobacillus* and pathogenic strain, OD_2 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.

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

* – 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* Enetritidis 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

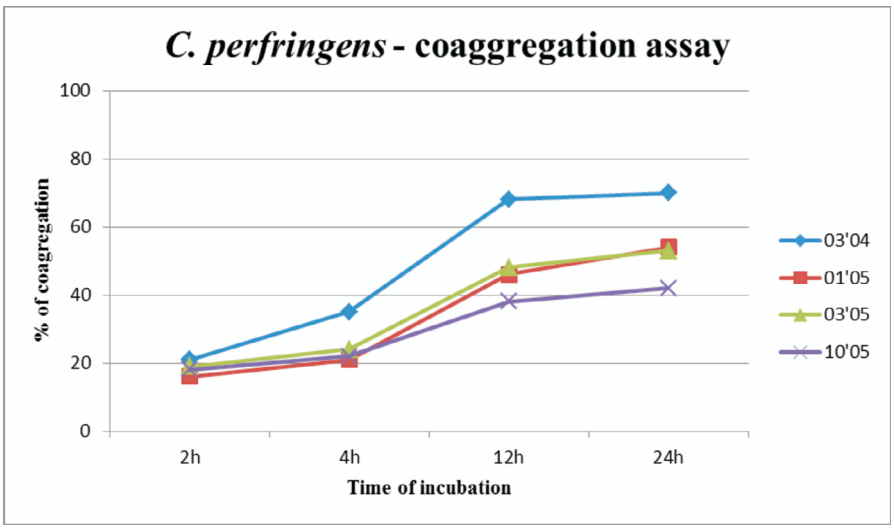

Fig. 1. Coaggregation ability of *Lactobacillus* isolates with *C. perfringens*.

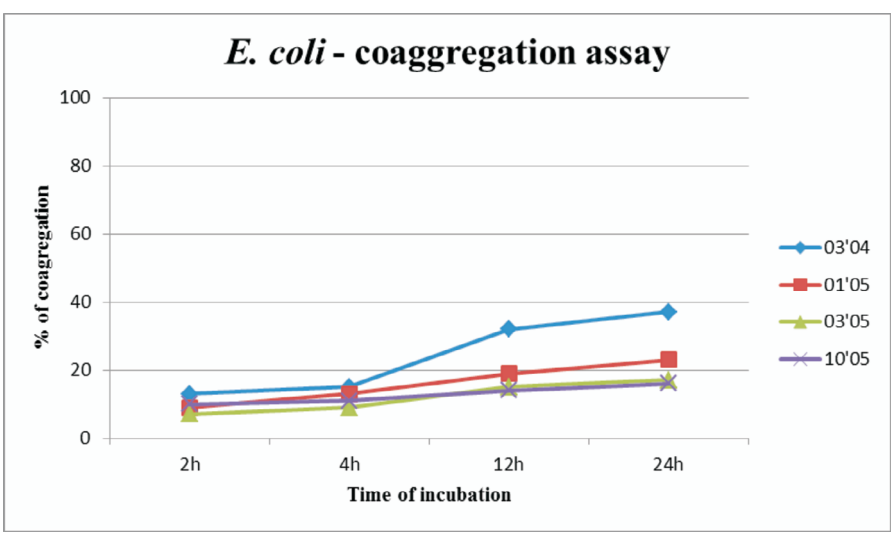

Fig. 2. Coaggregation ability of *Lactobacillus* isolates with *E. coli*.

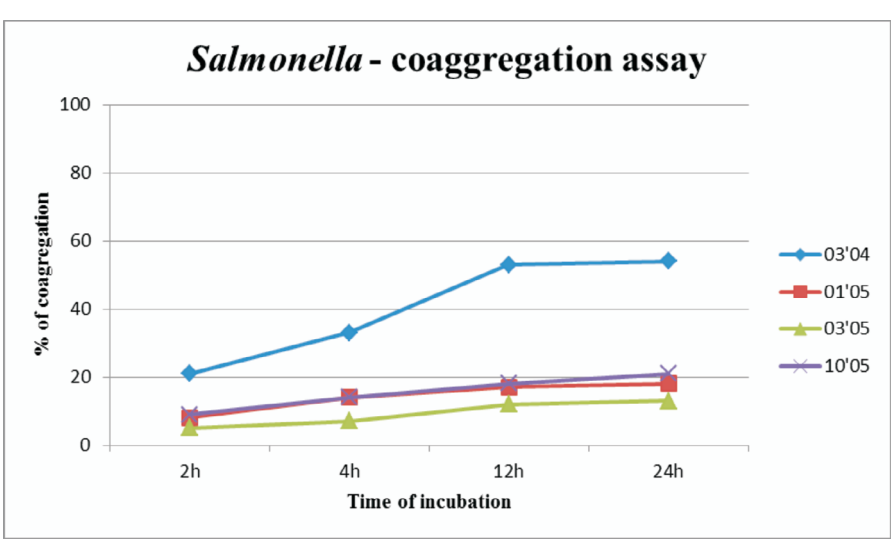

Fig. 3. Coaggregation ability of *Lactobacillus* isolates with *Salmonella* spp.

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, as compared to strains obtained from fermented food products (Ehrmann et al. 2002, Koll et al. 2008). The greater bile tolerance of particular *Lactobacillus* strains was proved to be correlated with the presence of bile salt hydrolases genes in their cells (Klaenhammer et al. 2008, Messaoudi et al. 2012b).

Coaggregation of lactobacilli with enteropathogenic bacteria is well known and has previously been reported by various authors (Garriga et al. 1998, Taheri et al. 2009). Ability to coaggregate is also a property specific to the particular genus and species. Strain specific, selective *in vitro* activity of probiotic lactobacilli against *C. perfringes* was described previously (Kizerwetter-Świda and Binek 2005). Other studies such as that of Valeriano et al. (2014) reported high coaggregation ability of lactobacilli isolated from piglet feaces with *E. coli* and *Salmonella*. It is remarkable, that some *Lactobacillus* strains demonstrate extremely wide range of antagonistic activity including also *Campylobacter jejuni*, *C. coli*, and also *Listeria* spp., and *Staphylococcus aureus* (Messaoudi et al. 2012a). The presented study showed that isolate 03'04 exhibited the highest coaggregation abilities, particularly with *C. perfringens*, as compared to the remaining three isolates.

Hydrogen peroxide is one of the metabolites that may be produced by *Lactobacillus* and other lactic acid bacteria (LAB) and may contribute to the inhibition of pathogenic bacteria. The quantity of H_2O_2 produced by different LAB varies, depending on the strain, and for some of them H_2O_2 production is not observed (Sabir et al. 2010). All four isolates tested in our study showed the ability to produce H_2O_2, which may promote their antagonistic activity against pathogenic bacteria.

All four evaluated *Lactobacillus* isolates were able to tolerate low pH and bile salt, showed good coaggregation scores with pathogenic bacteria and produce H_2O_2 production, thus 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 desirability 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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