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Antimicrobial activity of *Lactobacillus* strains of chicken origin against bacterial pathogens

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Summary. This study was conducted to identify and evaluate the antimicrobial activity of some *Lactobacillus* isolates of chicken origin. Among 90 isolates 14 *Lactobacillus* species were distinguished using MALDI-TOF mass spectrometry and 16S-ARDRA. The dominant species was *L. salivarius* (34.4%), followed by *L. johnsonii* (23.3%), *L. crispatus* (13.3%) and *L. reuteri* (11.1%). All lactobacilli were screened for antimicrobial activity against wild-type strains of *Salmonella enterica*, *Escherichia coli*, and *Clostridium perfringens*. Results from the agar slab method showed that all *Lactobacillus* isolates were able to produce active compounds on solid media with antagonistic properties against these pathogens. The highest sensitivity to lactobacilli was observed in *C. perfringens* strains, and the lowest in *E. coli*. *Lactobacillus salivarius* exhibited particularly strong antagonism towards all of the indicator bacteria. Strains of *L. ingluviei* and *L. johnsonii* and one strain of *L. salivarius* (10d) selectively inhibited the growth of *C. perfringens*. No antimicrobial activity of many *Lactobacillus* isolates was observed when cell-free culture supernatant was used in a well diffusion assay. All *Lactobacillus* isolates exhibited the ability to produce H_2O_2 and proved to be hydrophobic (excluding one of *L. salivarius*). [**Int Microbiol** 19(1):57-67 (2016)]

Keywords: *Lactobacillus* spp. · avian lactobacilli · antimicrobial activity · gut health · poultry pathogens

Introduction

The poultry industry is one of the fastest growing segments of the livestock sector in the world. At the same time, however, due to high production efficiency, the dietary and health needs of poultry require particular care. Among aspects that should be taken into account for optimum poultry performance, the overall health and proper functioning of the avian gastrointestinal tract (GIT) is crucial [41]. Gut health is maintained by complex mechanisms in which the commensal microflora seems to have a pivotal role. It is involved in host physiology, metabolism and absorption of nutrients, and recent studies on gut microbiota function have highlighted its importance in health and disease. The protective potency of beneficial gut microflora is of particular interest and knowledge of its composition is critical to understanding the function of members of the microbiota [37].

Enteric disorders are one of the most important problems in the poultry industry, with necrotic enteritis, salmonellosis and colibacillosis regarded as the major bacterial diseases occurring in chicken. *Clostridium perfringens*, *Salmonella* spp. and *Escherichia coli* infections range from severe acute disease to mild infections of a chronic nature. They cause substantial economic

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losses worldwide due to increased mortality rates and treatment costs, decreased rates of body weight gain, poor feed conversion efficiency, and increased risk of contamination of poultry products for human consumption. Enteric disorders can also result in reduced egg production, reductions in fertility, and low hatchability of infected eggs [27,30].

In Europe, the number of diagnosed gastrointestinal tract (GIT) infections in poultry, particularly those induced by *C. perfringens*, increased after 2006, when antibiotic growth promoters were removed from the list of allowed feed additives [6]. This has prompted the search for alternative methods for preventing intestinal infections in chickens. One strategy is based on the use of probiotics —live microorganisms which when administered in adequate amounts confer a health benefit on the host. The fundamental role of probiotics is to maintain the bacterial balance in the gut by eliminating unfavourable microflora, and antimicrobial activity is a key criterion in the selection of probiotic strains [26]. Administration of probiotic feed additives is particularly advisable in chickens whose intestinal microflora is not yet formed and whenever its stability is at risk, e.g., during antibiotic treatment, when their diet is changed, or when the birds are exposed to stress factors (e.g., overcrowding, inappropriate ventilation and temperature, insufficient water or feed). Stress lowers immune resistance and disrupts the balance of the intestinal microflora, which facilitates colonization of the GIT by pathogens, leading to the development of infections [4,26]. Hence ensuring the appropriate composition of the intestinal microflora is the best means of improving the immunity of the organism and the health of the animals.

Bacteria of the genus *Lactobacillus* are recognized candidates for probiotics. They are non-pathogenic Gram-positive rods that naturally inhabit the mucous membranes of humans and animals. In chicken, *Lactobacillus* colonization of the alimentary tract takes place soon after hatching, during food ingestion. From the crop they pass through successive parts of the chicken intestine and become important members of the microbial population [3]. Lactobacilli are lactic acid bacteria that play an important beneficial role in the physiology of their host by providing a protective barrier in the gut. In addition, they improve digestion and assimilation of nutrients, remove toxic substances, and enhance immunity [11]. The use of selected *Lactobacillus* strains as feed additives for poultry can reduce infections caused by intestinal pathogens such as *Salmonella* [34,44], *C. perfringens* [5,23], *E. coli* [18], *Campylobacter* sp. [14] and *Brachyspira pilosicoli* [28]. Administration of probiotics prevents pathogenic bacteria from colonizing the intestinal epithelium and passing into the internal organs and eggs [8]. Through elimination of unwanted microflora and other beneficial activity in the gut, selected *Lactobacillus* strains can also promote weight gain in birds [19] and increase egg production [33].

Probiotic lactobacilli may protect animals from intestinal pathogens by several possible mechanisms, including production of inhibitory substances, such as organic acids, hydrogen peroxide, bacteriocins and carbon peroxide, blocking of adhesion sites on intestinal epithelial surfaces, competition for nutrients, and stimulation of immunity [24]. These health-benefiting properties of lactobacilli are largely dependent on their prolonged residence in the GIT and are dictated by adherence to the intestinal mucosa. The adhesion mechanism involves passive forces and electrostatic and hydrophobic interaction, as well as specific binding dependent on bacterial surface adhesins [15].

The objective of this study was to identify native lactobacilli of chicken origin and evaluate their probiotic potential, expressed as in vitro ability to suppress the growth of *C. perfringens*, *S. enterica* and *E. coli*. In addition, the adhesive properties of *Lactobacillus* isolates were assessed by determination of their hydrophobicity.

Materials and methods

Bacteria and growth conditions. *Lactobacillus* isolates were from the fresh faeces or cloacae of 30 healthy chickens (broilers and Green-legged Partridge hens) from eight large-scale poultry farms in Poland. The age of the birds ranged from 2 days to 7 weeks. Samples were inoculated into MRS medium (BTL, Poland) supplemented with 0.05% (w/v) cysteine hydrochloride (Sigma-Aldrich, Poland) (MRS-cys). The plates were incubated at 37 °C for 48 h in 5% CO_2. Only catalase-negative Gram-positive rods were considered as presumtively belonging to the genus *Lactobacillus* and were stored at –80 °C until further analysis.

Strains of *Salmonella enterica* subsp. *enterica* (3 strains, serovars Enteritidis, Newport and Typhimurium), *E. coli* (1 isolate) and *Clostridium perfringens* (3 isolates), used in the experiment to evaluate the antibacterial activity of *Lactobacillus* sp., were isolated from chickens (intestinal contents) with symptoms of infection affecting the digestive tract (salmonellosis, colibacteriosis and necrotic enteritis). Species identification of all pathogenic isolates was confirmed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS, Bruker, Germany) according to the procedure described below.

Identification of *Lactobacillus* strains using MALDI-TOF MS. Bacteria were identified using an UltrafleXtreme MALDI-TOF mass spectrometer (Bruker, Germany) as previously described [9]. The mass spectra were processed with the MALDI Biotyper 3.0 software package (Bruker, Germany), and the results were shown as the top 10 identification matches along with confidence scores ranging from 0.00 to 3.00. The identification result was considered reliable when at least the two best matches having a log(score) of 1.70–3.00 with the MALDI Biotyper database indicated the same species. For samples for which the top two matches indicated different

species, we took into account the first match, provided that the log(score) was ≥ 0.25 greater than the value for the second match [9]. If the scores from two independent runs were <1.7 or a sample yielded a MALDI mass spectrum with no peaks, ethanol/formic acid extraction was performed (according to the protocol recommended by the manufacturer, Bruker, Germany).

Differentiation of *L. johnsonii* and *L. gasseri* strains by 16S-ARDRA. Twenty-one isolates for which definitive species identification was not obtained using MALDI-TOF MS (*L. johnsonii*/*L. gasseri*) were identified using 16S-ARDRA with the *Mse*I restriction enzyme, as described in our previous publication [9].

Detection of antibacterial activity of *Lactobacillus* strains. Agar slab method. The *Lactobacillus* sp. isolates grown on MRS-cys broth were centrifuged and suspended in 0.9% NaCl so that the optical density of the suspension at 600 nm (OD_{600}) was 0.5. Plates 4 cm in diameter containing 15 ml MRS agar were inoculated with 200 µl of lactobacilli and incubated at 37 °C in 5% CO_2 for 24 h. Then agar slabs 9 mm in diameter were cut and placed on Müller-Hinton agar inoculated with 0.5 ml of the target indicator strain suspended in 0.9% NaCl (OD_{600} = 0.1 for *Salmonella* sp. and *E. coli*, OD_{600} = 0.8 for *C. perfringens*). For initial diffusion of the substance from the agar slabs, the plates were first refrigerated for 3 h at 4 °C and then kept for 24 h at 37 °C in aerobic conditions for *Salmonella* and *E. coli* or in anaerobic conditions for *C. perfringens*. After incubation, the plates were checked for inhibition zones. The results are presented as the mean diameter of the inhibition zone ± SD for two independent experiments.

Detection of antibacterial activity of *Lactobacillus* strains. Well diffusion method. *Lactobacillus* isolates were grown in a 1.2 ml volume of MRS-cys broth for 24 h (37 °C, 5% CO_2). The bacteria were separated from the medium by centrifugation and then each sample of medium was divided into 2 equal volumes. In half of the samples the pH was adjusted to 6.5–7.0 using NaOH (to eliminate the effect of organic acids), and an equal volume of water was added to the remaining samples, with pH 3.5–5.0.

The indicator bacteria were inoculated on Müller-Hinton agar according to the protocol described above. Cylindrical metal wells 8 mm in diameter were placed on the plates and filled with 100 µl of the cell-free supernatant. After 24 h of incubation in conditions appropriate for the indicator bacteria (described above), the plates were checked for inhibition zones. The results are presented as the mean diameter of the inhibition zone ± SD from two independent experiments.

Detection of H_2O_2 production by *Lactobacillus* isolates. The lactobacilli were plated on MRS-cys supplemented with TMB substrate (0.25 mg/ml, Sigma-Aldrich) and horseradish peroxidase (0.01 mg/ml, Sigma-Aldrich) and grown for 48 h at 37 °C, 5% CO_2. Blue colour in the colonies indicated H_2O_2 production by the bacteria. Colour intensity was designated as follows: –, +, ++, +++ [10].

Measurement of bacterial hydrophobicity. Hydrophobicity of the investigated bacteria was determined on the basis of microbial adhesion to hydrocarbon as described by Rosenberg [38]. Lactobacilli grown in MRS-cys broth were centrifugated and resuspended in 0.02 M PBS, pH 6.8, to an optical density (OD_{600}) of 0.8–1.0 (A_0), 1,7 ml xylene was added to glass test tubes containing 5 ml of bacterial suspension. The mixtures were vortexed vigorously for 90 s. After phase separation of about 15 min the optical density of the aqueous phase (A) was again measured and compared with the initial value. The percentage of cell surface hydrophobicity (%H) was calculated using the following equation: $\%H = [(A_0 - A)/A_0] \times 100$. Strains with hydrophobicity equal to or more than 50% were considered hydrophobic.

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 one-way analysis of variance (with species as a categorical predictor and zone as a dependent variable adjusted for the pathogen), with the Tukey HSD (honest significant difference) post hoc test, with modification for unequal N, as a correction for multiple comparisons. Normal distribution of data was tested using the Shapiro–Wilk test and the equality of variance was tested by the Brown–Forsythe test. Due to a lack of normal distribution and/or unequal variance of data, Kruskal–Wallis analysis of variance was used to analyse the differences between means. A level of $P < 0.05$ was considered statistically significant. All statistical analyses were carried out using Statistica 10.0 software (StatSoft, Inc., Tulsa, OK, USA).

Results

Identification of Lactobacillus isolates. *Lactobacillus* bacteria were isolated from all samples tested, and 2–7 strains of varying colony morphology were isolated from each sample. A total of 90 isolates was identified as bacteria of the genus *Lactobacillus* using MALDI-TOF mass spectrometry. For 32 (35.5%) of the strains the log(score) was 1.70–1.99, for 50 (55.5%) strains it was 2.00–2.29, and for 8 (8.8%) it was 2.30–3.00. For 69 (76.7%) strains either at least the two best matches in Biotyper indicated the same species or the difference between the first and second best matches indicating different species was greater than 0.25. Identification of these isolates was considered to be reliable. For 21 strains (23.3%) the first and second best matches indicated different species, and the differences between their log(score) values were less than 0.25. For these isolates the best match indicated *L. johnsonii* and the second best match indicated *L. gasseri*.

The 90 isolates identified belonged to 8 *Lactobacillus* species: *L. salivarius* 31 strains (34.4%), *L. johnsonii/L. gasseri*, 21 (23.3%), *L. crispatus* 12 (13.3%), *L. reuteri* 10 (11.1%), *L. ingluviei* 8 (8.9%), *L. agilis* 3 (3.3%), *L. saerimneri* 3 (3.3%) and *L. oris* 2 strains (2.2%).

Differentiation of *L. johnsonii/L. gasseri* strains by 16S-ARDRA. Analysis of the electrophoretic profiles obtained by digestion of 16S amplicon with *Mse*I showed that all the strains previously identified in MALDI-TOF MA as *L. johnsonii*/*L. gasseri* belonged to the species *L. johnsonii*. The electrophoretic profiles of these wild isolates and the reference strain *L. johnsonii* LMG 9436 contained five bands of molecular size 940, 256, 145, 130 and 90 bp. The electrophoretic profile of the reference strain *L. gasseri* ATCC 19992 differed from the profiles of the remaining strains and comprised 5 restriction fragments of 680, 450, 237, 130 and 90 bp (Fig. 1).

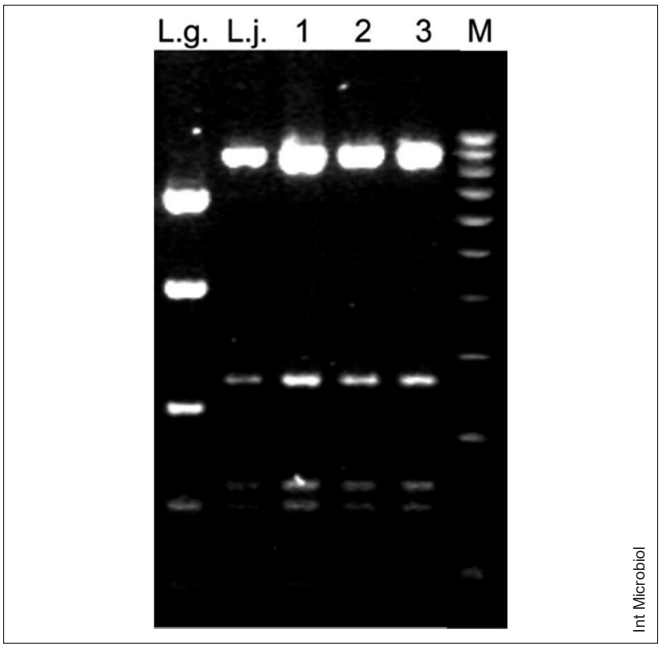

Fig. 1. Agarose gel image of 16S amplicons digested with *Mse*I. Lines: L.g. – *Lactobacillus gasseri* ATCC 19992; L.j. – *L. johnsonii* LMG 9436; 1–3 – examples of wild *Lactobacillus* strains identified in MALDI-TOF MS as *L johnsonii* /*L.gasseri*.

Agar slab method. The diameter of the growth inhibition zones of the test bacteria induced by the lactobacilli ranged from 9 ± 0.0 to 27 ± 2.83 mm, where the diameter of the slab was 9 mm (Fig. 2). Individual indicator strains exhibited varied susceptibility to the lactobacilli. All 90 *Lactobacillus* isolates inhibited the growth of all three strains of *C. perfringens*. The *Salmonella* Typhimurium ST strain was inhibited by 60 (33.3%) strains of *Lactobacillus*, *Salmonella* Typhimurium A by 58 (64.4%), *Salmonella* Enteritidis by 48 (53.3%), *Salmonella* Newport by 47 (52.2%), and *E. coli* by 34 (37.7%) lactobacilli. The largest mean inhibition zones (16.7–17.3 mm) were observed in the case of *C. perfringens* isolates, and ANOVA of the mean diameters showed that the *C. perfringens* strains were more sensitive ($P < 0.05$) than other indicator bacteria to the antagonistic substances produced by lactobacilli (Fig. 3, Table 1). More detailed analysis showed that the average inhibition zones of *C. perfringens* strains were significantly higher ($P < 0.05$) than the zones obtained for *Salmonella* (all serovars) and *E. coli* due to the antagonistic effect of *L. salivarius*, *L. ingluviei*, *L. johnsoni, L. crispatus and L. reuteri* (Table 1). The *E. coli*, *Salmonella* Enteritidis and *Salmonella* Newport strains were found to be the least susceptible to *Lactobacillus* activity. The average inhibition zone of these pathogens (10.3±1.8 – 11.0±2.1 mm) caused by the lactobacilli (when all lactobacilli were considered as one group) differed ($P < 0.05$) from the average zone of inhibition obtained for *Salmonella* Typhimurium and *C. perfringens*. Moderately large zones were observed for *Salmonella* Typhimurium strains (12.7 ± 3.6 – 12.8 ± 3.5 mm) (Table 1, Fig. 3).

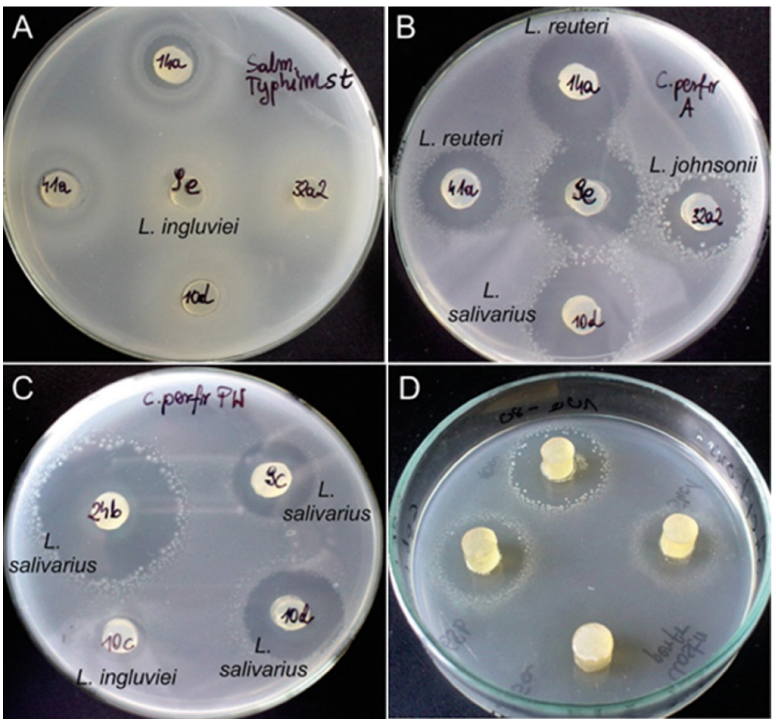

Fig. 2. Anatagonistic activity of *Lactobacillus* sp. against indicator bacteria in the agar slab method. (**A**) *Salmonella* Typhimurium ST. (**B**) *Clostridium perfringens A*. (**C**) *C. perfringens* PW1. (**D**) *C. perfringens* L3.

Lctobacillus salivarius exerted particularly strong antagonism against all of the pathogens, as the mean growth inhibition zone diameter for the indicator strains (when all the indicator strains were considered as one group) induced by isolates of this species was 16.0 ± 3.5 mm and differed significantly ($P < 0.05$) from the mean growth inhibition zones caused by the remaining *Lactobacillus* species (≤ 13.3 ± 3.8 mm) (Table 1, Fig. 4). Such a remarkable potent antimicrobial activity of *L. salivarius* strains was also observed when the sensitivity of each indicator microorganism was considered individually (Table 1). Strains of the species *L. oris*, *L. johnsonii*, *L. saerimneri* and *L. ingluviei* exhibited weak antagonistic properties. The average diameters of the growth inhibition zones of the pathogenic bacteria (considered as one group) induced by these species of *Lactobacillus* were ≤ 12.0 ± 4.3 mm (Table 1, Fig. 4). However, the assays in which each indicator strain was considered individually showed that the activity of *Lactobacillus* species varied depending on the species of an indicator bacterium. The isolates of *L. ingluviei* and *L. johnsonii* were ineffective towards *Salmonella* and *E. coli*, but they selectively inhibited (14.7 ± 2.7–17.0 ± 3.0 mm) the growth of *C. perfringens* (Figs. 5C and 5D).

Based on the experiment, the strains with the strongest antagonism towards the indicator strains were chosen. Among 15 selected isolates, 13 belonged to the species *L. salivarius* and 2 to the species *L. ingluviei* (Table 2). Particularly noteworthy is the strong selective inhibitory activity of the strains *L. salivarius* 10d and *L. ingluviei* 9a and 18b against growth of *C. perfringens*. Average diameters for the inhibition of the growth of *C. perfringens* caused by these strains ranged from 20.0 ± 0.9 mm (9e) to 21.2 ± 2.2 mm (10d), while the growth of other indicator strains was inhibited only slightly (isolate10d inhibited the growth of *Salmonella*, up to 10.2 ± 1.3 mm) or not all (9e, 18b).

Well diffusion method. The pH of the supernatant obtained from the 24 h culture of *Lactobacillus* strains ranged from 3.0 to 4.5. The inhibition of growth of pathogenic bacteria by na-

Table 1. ANOVA of the growth inhibition of indicator microorganisms by *Lactobacillus* isolates, as determined by the agar slab method[1]

	Salmonella Newport	*Salmonella* Enteritidis	*Salmonella* Typhimurium ST	*Salmonella* Typhimurium A	*E. coli* D7	*C. perfringens* A	*C. perfringens* L3	*C. perfringens* PW1	Mean
L. salivarius (n=31)	$13.2 \pm 1.2^{a,A}$	$13.1 \pm 1.8^{a,A}$	$16.3 \pm 2.4^{a,C}$	16.4 ± 1.9^{aC}	$12.1 \pm 1.6^{a,A}$	$18.8 \pm 2.5^{a,B}$	$19.0 \pm 2.4^{a,B}$	$19.6 \pm 2.5^{a,B}$	16.0 ± 3.5^{a}
L. johnsonii (n=21)	$9.0 \pm 0.0^{b,A}$	$9.0 \pm 0.0^{bc,A}$	$9.1 \pm 0.4^{b,A}$	$9.0 \pm 0.1^{c,A}$	$9.0 \pm 0.0^{b,A}$	$14.7 \pm 2.4^{b,B}$	$14.8 \pm 2.6^{b,B}$	$15.4 \pm 2.3^{b,B}$	11.3 ± 3.3^{b}
L. crispatus (n=12)	$11.2 \pm 2.1^{c,A}$	$10.7 \pm 2.0^{c,A}$	$12.0 \pm 3.3^{b,A}$	$11.7 \pm 2.4^{bd,A}$	$9.9 \pm 1.4^{b,A}$	$16.4 \pm 2.2^{b,B}$	$17.3 \pm 3.5^{ab,B}$	$17.0 \pm 2.7^{b,B}$	13.3 ± 3.8^{c}
L. reuteri (n=10)	$9.9 \pm 1.3^{bc,AD}$	$9.8 \pm 1.3^{b,AE}$	$11.8 \pm 1.6^{b,CDE}$	$12.0 \pm 1.9^{b,CD}$	$9.1 \pm 0.4^{b,A}$	$15.7 \pm 2.6^{b,B}$	$15.0 \pm 3.4^{b,BC}$	$16.1 \pm 2.2^{b,B}$	12.4 ± 3.3^{cd}
L. ingluviei (n=8)	$9.2 \pm 0.9^{bc,A}$	$9.0 \pm 0.0^{b,A}$	$9.4 \pm 1.4^{bc,A}$	$9.4 \pm 1.5^{sd,A}$	$9.1 \pm 0.3^{b,A}$	$16.3 \pm 3.1^{ab,B}$	$17.0 \pm 3.0^{ab,B}$	$16.5 \pm 3.6^{b,B}$	12.0 ± 4.2^{bd}
L. agilis (n=3)	$9.7 \pm 1.2^{bc,AB}$	$9.7 \pm 1.2^{ac,AB}$	$11.9 \pm 1.3^{acb,ABC}$	$11.8 \pm 1.6^{abc,ABDE}$	$9.2 \pm 0.4^{b,A}$	$15.6 \pm 1.2^{ab,CDE}$	$14.4 \pm 0.8^{b,BE}$	$16.2 \pm 0.8^{a,CE}$	12.3 ± 2.8^{bcd}
L. saerimmeri (n=3)	$9.0 \pm 0.0^{bc,A}$	$9.0 \pm 0.0^{b,A}$	$9.2 \pm 0.4^{bc,A}$	$10.3 \pm 1.7^{bc,AC}$	$9.0 \pm 0.0^{b,A}$	$14.6 \pm 2.4^{b,AC}$	$14.6 \pm 2.4^{b,AC}$	$15.2 \pm 1.1^{b,BC}$	11.3 ± 3.0^{bd}
L. oris (n=2)	$9.4 \pm 0.5^{abc,AB}$	$9.7 \pm 0.9^{ac,AB}$	$10.7 \pm 0.5^{acb,AB}$	$10.9 \pm 0.8^{abc,AB}$	$9.0 \pm 0.0^{b,B}$	$11.9 \pm 0.8^{b,A}$	$11.0 \pm 0.8^{b,AB}$	$11.1 \pm 1.9^{b,AB}$	10.5 ± 1.2^{bd}
Mean	11.0 ± 2.1^{A}	10.9 ± 2.3^{A}	12.7 ± 3.6^{B}	12.8 ± 3.5^{B}	10.3 ± 1.8^{A}	16.7 ± 3.0^{C}	16.8 ± 3.4^{C}	17.3 ± 3.2^{C}	

1The results are presented as mean diameter of the growth inhibition zone (mm) for two independent experiments; the diameter of the agar slab was 9 mm.
2n = number of strains.
a–d Different superscript lower-case letters indicate differences between mean values in columns (comparison of the sensitivity of the indicator microorganism to the antagonistic substances produced by strains of each *Lactobacillus* species); $P < 0.05$.
A–E Different superscript capital letters indicate differences between mean values in rows (comparison of the inhibitory effect of various *Lactobacillus* species on each strain of indicator bacteria); $P < 0.05$.

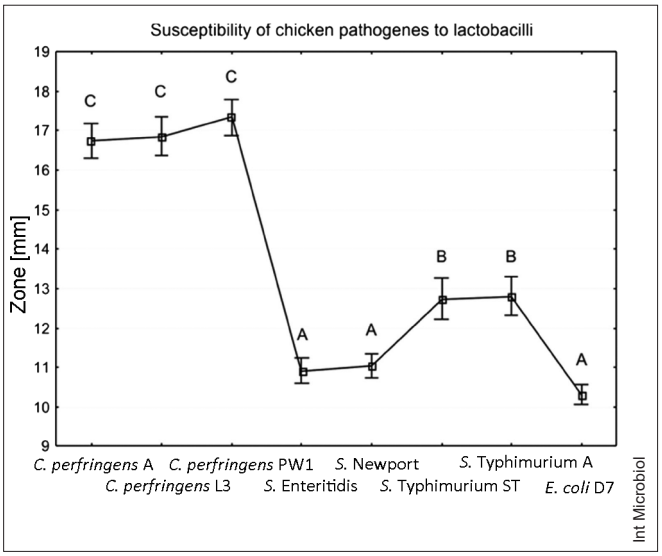

Fig. 3. The susceptibility of the indicator strains to *Lactobacillus* species, as determined by the agar slab method. The results are presented as mean diameter of the growth inhibition zone (mm) for two independent experiments; the diameter of the agar slab was 9 mm; Different capital letters (A–C) indicate significant differences ($P < 0.05$) between mean size of growth inhibition zones of pathogenic bacteria caused by *Lactobacillus* isolates (when all *Lactobacillus* strains where considered as one group); the vertical bars denote 0.95 CI.

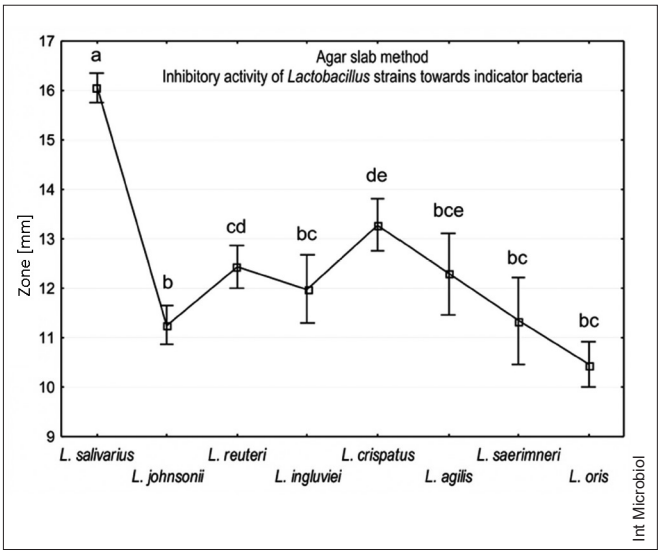

Fig. 4. The effect of strains of different *Lactobacillus* species on the growth of indicator strains, as determined by the agar slab method. The results are presented as mean diameter of the growth inhibition zone (mm) of all indicator bacteria for two independent experiments; the diameter of the agar slab was 9 mm. Different small letters (a-e) indicate significant differences ($P < 0.05$) between mean diameter of growth inhibition zones caused by various *Lactobacillus* species; the vertical bars denote 0.95 CI.

tive cell-free broth was generally very weak or absent, even in the case of many *Lactobacillus* strains that showed a strong inhibitory effect in the agar slab method. The size of the inhibition zones caused by native acidified supernatants was up to 14.5 ± 0.7 mm, where the well diameter was 8 mm.

The highest susceptibility to the antagonistic activity of an acidic environment was exhibited by the *C. perfringens* strains, as their growth was inhibited by 90–92% (depending on the *C. perfringens* strain) of the media with acidic pH. The inhibition zones for these indicator strains ranged from 8.00 ± 0.0 to 14.5 ± 0.7 mm, and the ANOVA of the mean diameters showed that the *C. perfringens* strains were more sensitive ($P < 0.05$) than the other indicator microorganisms to the antagonistic substances present in the cell-free *Lactobacillus* media (Fig. 6). In the case of *Salmonella* strains (all serovars), very small inhibition zones of up to 9.0 ± 0.0 mm were observed, and inhibition was caused only by 8–12% (depending on the indicator strain) of unneutralized cell-free supernatants. None of the native media was able to inhibit the growth of the *E. coli* indicator strain.

Cell-free supernatants with neutralized acids (pH 6.5–7.0) did not exhibit antagonistic activity towards the indicator strains, with the exception of ten supernatants (from the cultures of different *Lactobacillus* species) which exhibited a slight inhibitory effect towards *C. perfringens* (∅ 8.5–11 mm). Statistical analysis (Kruskal-Wallis test) showed no significant difference between the inhibitory effect of the cell-free culture supernatants of different species of *Lactobacillus.*

Production of H_2O_2. All *Lactobacillus* strains tested produced H_2O_2. The highest rate of production (+++) was observed in 59 isolates, including all strains belonging to the species *L. johnsonii*, *L. ingluviei*, *L. agilis*, *L. saerimnerii* and *L. oris* and some strains of *L. salivaius*, *L. crispatus* and *L. reuteri*. Moderate hydrogen peroxide production (++) was noted in 14 strains, most of which were of the species *L. salivarius.* The group with the lowest H_2O_2 production (+) comprised 17 strains, including 15 strains of *L. salivarius* and 2 strains of *L. crispatus*.

The bacteria capable of producing H_2O_2, grown on MRS medium supplemented with TMB and horseradish peroxidase, varied not only in colour intensity, but also in the manner in which the colonies were coloured. In some strains of *L. salivarius* the blue colour appeared only on the periphery of the bacterial colonies, while in other lactobacilli, e.g., *L. johnsonii*, *L. ingluviei* and *L. agilis*, entire colonies were blue. In some *L. salivarius* strains only the middle of the colony and their periphery was blue, resembling an eye.

Table 2. *Lactobacillus* strains inducing the largest inhibition zones of pathogenic indicator bacteria in the agar slab method

Pathogen	Size of inhibition zone	*Lactobacillus* isolates
Clostridium perfringens (A, L3, PW1)	≥20 mm	*L. salivarius*: 6a, 8b, 10d, 17a, 23a, 24b, 27e, 40a *L. ingluviei*: 9e, 18b
Salmonella Typhimurium (ST, A)	≥18 mm	*L. salivarius*: 5a, 21b, 22a, 24b, 30b
Salmonella Enteritidis and Newport	≥14 mm	*L. salivarius*: 6b, 17a, 21a, 24b, 27e, 40a
Escherichia coli (D7)	≥14 mm	*L. salivarius*: 6b, 17a, 27e

Hydrophobicity. The %H of all lactobacilli tested, except one strain of *L. salivarius* (50d), was ≥50%, and therefore these isolates were considered hydrophobic. The vast majority of the strains tested showed high affinity towards xylene - for 65.5% *Lactobacillus* isolates the %H was 90–100% (Table 3). The strains of *L. johnsonii* showed relatively low hydrophobicity compared to the other lactobacilli tested; only 38.1% of isolates displayed hydrophobicity at 90–100% and as many as 33.3% exhibited %H in the range of 50–69%.

Discussion

In the present work, we successfully identified chicken lactobacilli to the species level using MALDI-TOF mass spectrometry, and for some strains additionally by 16S-ARDRA. The reliability and effectiveness of these methods in typing lactobacilli has been confirmed in our previous research [9]. The *Lactobacillus* species identified in this study from broiler chickens and laying hens raised in Poland are similar to those identified from broiler chickens around the world, supporting the notion that these species are autochthonous inhabitants within the chicken GIT. The occurrence of *L. salivarius*, *L. crispatus*, *L. johnsonii* and *L. reuteri* in the GIT of broilers have been also observed by Wang [47] and Vidanarachchi et al. [45]. Some other reports have pointed out the predominance of *Lactobacillus crispatus, L. reuteri and L. salivarius,* but not L. johnsonii among intestinal chicken lactobacilli [3,7,16,17]. The occurrence of *L. ingluviei*, *L. agilis* and *L. sarimneri* strains in the chicken GIT has been also described [31,44]. Differences in the frequency of isolation of some *Lactobacillus* species from the chicken GIT reported by various authors may be the result of different breeding conditions, the diet of the birds, and the procedures for isolating and identifying bacteria.

The results of the agar slab method showed that *Lactobacillus* bacteria originating in chickens have growth-inhibiting properties for bacterial poultry pathogens and that this antagonistic effect depends on the type of pathogen and is due to the production of antimicrobial substances by lactobacilli. Our findings are in agreement with those of Kizerwetter-Swida and Binek [21] who observed a greater antibacterial in vitro effect of chicken lactobacilli against *C. perfringens* than against *E. coli* and *Salmonella* Enteritidis. Other authors

Table 3. Percentage hydrophobicity of *Lactobacillus* strains

%H / species	90–100%	70–89%	50–69%	<50%
L. salivarius (n = 31)	17 (54.8%)	7 (22.6%)	6 (19.3%)	1 (3.2%)
L. johnsonii (n = 21)	8 (38.1%)	6 (28.6%)	7 (33.3%)	–
L. crispatus (n = 12)	11 (91.7%)	1 (8.3%)	–	–
L. reuteri (n = 10)	7 (70.0%)	3 (30.0%)	–	–
L. ingluviei (n = 8)	8 (100%)	–	–	–
L. agilis (n = 3)	3 (100%)	–	–	–
L. saerimneri (n = 3)	3 (100%)	–	–	–
L. oris (n = 2)	2 (100%)	–	–	–
Total (n = 90)	59 (65.5%)	17 (18.9%)	13 (14.4%)	1 (1.1%)

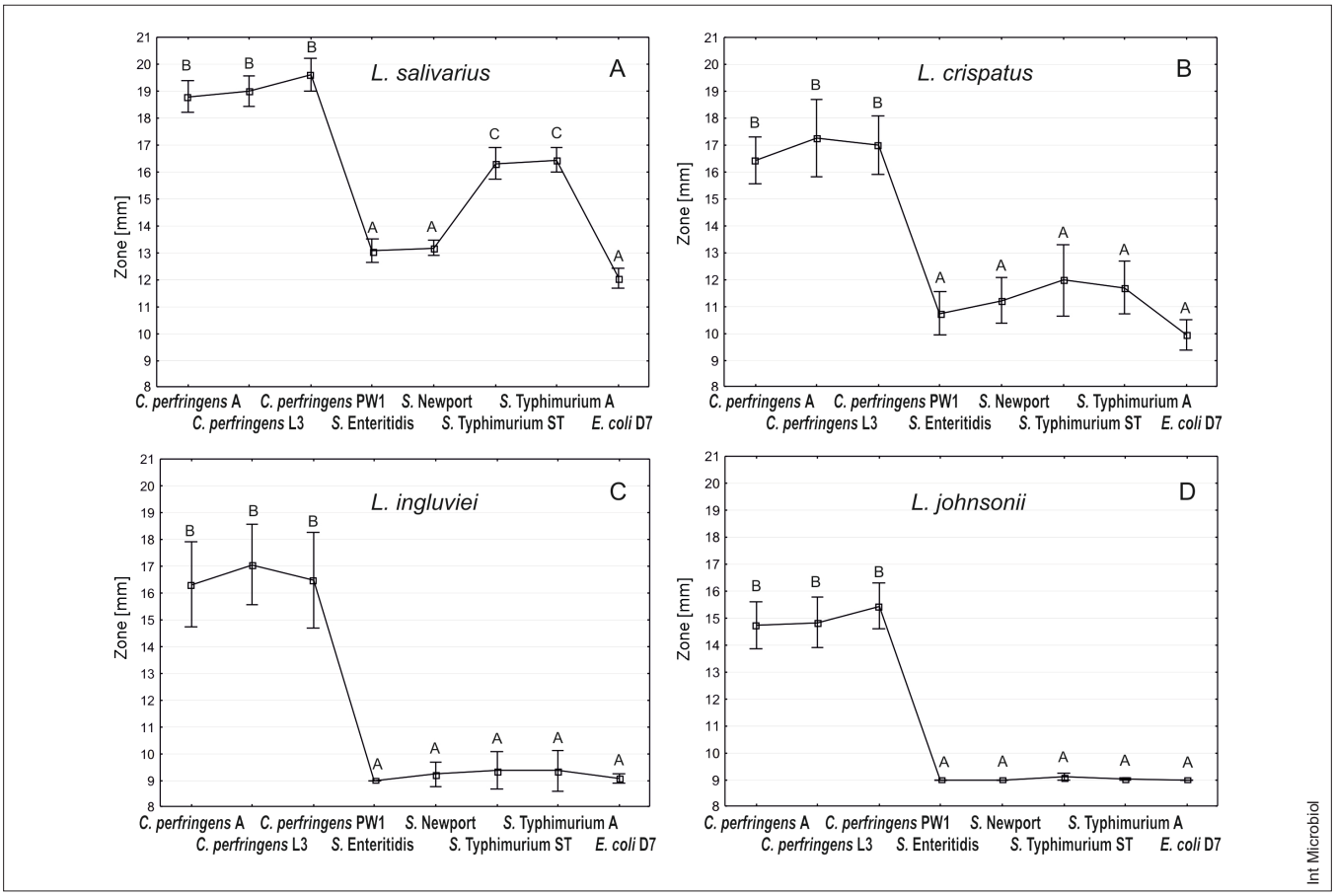

Fig. 5. The susceptibility of the indicator strains to selected *Lactobacillus* species, as determined by the agar slab method. Different capital letters (A–C) indicate significant differences ($P < 0.05$) between mean diameter of growth inhibition zones caused by the strains of *L. salivarius* (**A**), *L. crispatus* (**B**), *L. ingluviei* (**C**) and *L. johnsonii* (**D**). Different capital letters (A–C) indicate significant differences ($P < 0.05$) between mean size of growth inhibition zones of pathogenic bacteria caused by the strains of individual *Lactobacillus* species; the vertical bars denote 0.95 CI.

[18,43] showed that chicken lactobacilli were more effective in inhibiting the growth of *Salmonella* than *E. coli*, but contrary to our findings they did not observe greater sensitivity of *Salmonella* Typhimurium compared to *Salmonella* Enteritidis.

Antimicrobial in vitro activity of *L. salivarius* strains and some other *Lactobacillus* species isolated from chickens against *Salmonella*, *E. coli* and *C. perfringens* has also been observed by many other authors [2,25,32,42,48], and some of them [13] concluded that organic acids produceb by lactobacilli are resposible for inhibityory effect. It was also shown that *Lactobacillus* strains of chicken origin exert a protective effect in vivo, especially *L. salivarius* against *Salmonella* in chickens [20,34,35,40]. Kizerwetter-Świda and Binek [20] and La Ragione et al. [23] demonstrated antimicrobial effect of selected strains of *L. salivarius* and *L. johnsonii* against *C. perfringens* in chickens, but there are no reports of anti-clostridial activity of *L. ingluviei* strains.

The results of the well diffusion method indicated that the reduced pH of the supernatant (probably due to lactic acid) might play a role in inhibiting pathogenic bacteria. However we were unable to clearly identify which substances produced by lactobacilli growing on agar inhibited the growth of pathogenic bacteria. This was because the antagonistic activity of the native cell-free broth was very weak or absent in the case of most *Lactobacillus* strains, including those that showed a strong inhibitory effect in the agar slab method. This phenomenon, also observed by some other authors [25,36], can be explained by the fact that the release of antimicrobial molecules by lactobacilli is influenced by culture conditions, cell density and population kinetics [1]. Moreover, *Lactobacillus* bacteria grown on agar medium are able to synthesize bacteriocins in significantly greater amounts than in a liquid culture [39]. In the case of *C. perfringens*, which was grown in anaerobic conditions, it should be taken into account that after

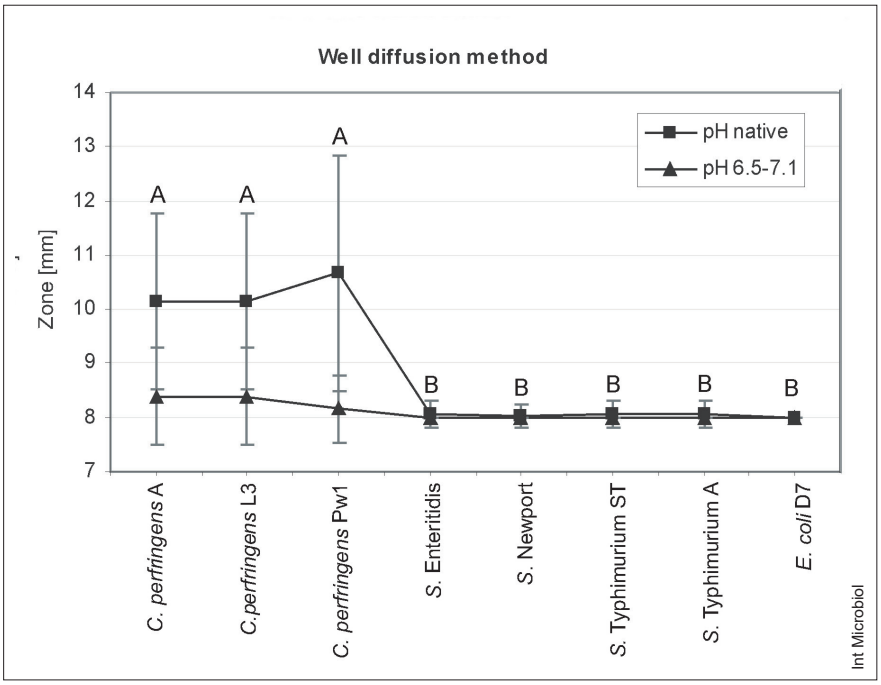

Fig. 6. The susceptibility of the pathogenic bacteria to *Lactobacillus* species, as determined by the well diffusion method. The results are presented as mean diameter of the growth inhibition zone (mm) for two independent experiments (the media obtained after cultivation of all *Lactobacillus* strains were considered as one group); the diameter of the metal well was 8 mm; the vertical bars denote 0.95 confidence intervals. Different capital letters (A-B) means significant differences ($P < 0.05$) between mean diameter of growth inhibition zones caused by native cell free-media.

the antagonistic bacteria were transferred onto the agar slab they were able to grow simultaneously with the indicator bacteria. Anaerobic culture conditions may have stimulated production of bacteriocins or other antibacterial substances by lactobacilli.

The results of the present study showed that *Lactobacillus* sp. strains originating in chicken produce H_2O_2. However, production of this reactive oxygen species was not clearly correlated with the antimicrobial activity of lactobacilli observed in the slab method. All strains of *L. johnsonii, L. ingluviei* and *L. oris* exhibited strong production of hydrogen peroxide, but they were generally inactive towards *Salmonella* and *E. coli*. Moreover, some *L. salivarius* strains that most strongly inhibited the growth of pathogens exhibited intermediate (++) or even weak (+) H_2O_2 production. Correlations between *Lactobacillus* species and the ability to produce H_2O_2 and a lack of relationship between antimicrobial activity of lactobacilli and the intensity of H_2O_2 production were also observed in our previous work on goose lactobacilli [10]. The ability of chicken intestinal lactobacilli to produce H_2O_2 was also reported by Heravi et al. [17] and Mota et al. [29], but contrary to our findings, these authors recognised *L. salivarius* strains as the best producers of H_2O_2, while the isolates of *L. johnsonii* were considered weak producers (+) or H_2O_2-negative (–).

Hydrophobicity of bacteria is dependent on cell surface components and generally reflects the adhesive ability of bacteria. Several researchers have reported a high degree of correlation between hydrophobicity of *Lactobacillus* strains and their adhesion to epithelial cells [12,22,46]. The results of our study showing high hydrophobicity of lactobacilli tested are in line with data obtained by Heravi et al. [17], who reported that the adhesion of 8 chicken strains of *L. salivarius, L. crispatus, L. johnsonii* and *L. reuteri* to xylene ranged from 78.2% to 93.2%. Mota et al. [29] found that almost 80% of chicken intestinal lactobacilli had hydrophobic surfaces (H > 50%).

In summary, gut health challenges are currently the most important issue for poultry production. Knowledge of the composition of the intestinal microflora is critical for understanding the contribution of microbiota members to the well-being of the avian host and for selection of probiotics. The results presented here demonstrate that *Lactobacillus* isolates from chickens may have probiotic potential in reducing intestinal infections. The study made it possible to select strains of *Lactobacillus* characterized by antagonistic properties towards bacterial pathogens resulting from the production of growth inhibitory compounds and adhesive properties. They can be considered for use as prophylactic agents or as an alternative to antibiotic therapy for infections with *Salmonella*, *E. coli* or *C. perfringens* in chickens.

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