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Bacteriology

Probiotic potential of *Lactobacillus* isolates of chicken origin with anti-*Campylobacter* activity

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ABSTRACT. Campylobacteriosis is currently the most frequent zoonosis in humans and the main source of infection is contaminated poultry meat. As chickens are a natural host for *Campylobacter* species, one strategy to prevent infection in humans is to eliminate these bacteria on poultry farms. A study was conducted to evaluate the probiotic potential of 46 *Lactobacillus* isolates from chickens faeces or cloacae. All lactobacilli were able to produce active compounds on solid media with antagonistic properties against *C. jejuni* and *C. coli*, with *L. salivarius* and *L. reuteri* exhibiting particularly strong antagonism. The cell-free culture supernatants had a much weaker inhibitory effect on the growth of *Campylobacter*, and the neutralization of organic acids caused them to completely lose their inhibitory properties. The ability to produce H_2O_2 was exhibited by 93% of isolates; most of isolates had a hydrophobic surface, showed excellent survival at pH 2.0 or 1.5, and displayed tolerance to bile; 50% isolates displayed the ability to biofilm formation. Determination of MICs of various antibiotics showed that as much as 80.4% of *Lactobacillus* isolates were resistant to at least one antimicrobial agent. Seven ultimately selected isolates that met all the basic criteria for probiotics may have potential application in reducing *Camylobacter* spp. in chickens and thus prevent infections in both birds and humans.

KEY WORDS: antibacterial activity, *Campylobacter,* chicken, *Lactobacillus*, probiotics

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Campylobacter are Gram-negative, microaerophilic bacteria widespread throughout the world, causing a zoonotic disease in humans known as campylobacteriosis. It has been the most commonly reported gastrointestinal bacterial infection in humans in the E.U. since 2005, and over the last decade a significant upward trend has been observed. In 2013, the number of reported confirmed cases of human campylobacteriosis was 214,779, with an E.U. notification rate of 64.8 per 100,000 population (while there were 82,694 reported cases of salmonellosis) [13]. Campylobacteriosis in humans is induced mainly by *Campylobacter jejuni* (about 90% of cases), and the remaining fraction is induced predominantly by *Campylobacter coli* [18]. The characteristic symptoms of the disease are watery-mucous diarrhoea, often with blood in the stool, nausea, vomiting, abdominal pain, and fever lasting up to seven days or longer. Although such infections are generally self-limiting, serious consequences may arise, including bacteraemia, Guillain–Barré syndrome (GBS)−an autoimmune disease affecting the peripheral nervous system, reactive arthritis, inflammatory bowel disease, and irritable bowel syndrome [15]. The current cost associated with treating acute *C. jejuni* infections and GBS is estimated to be 2.4 billion € in the E.U. and $1.2 billion per year in the U.S.A. [27]. *Campylobacter* can be transmitted human-to-human by the faecal-oral route, but zoonotic or foodborne transmission predominates. Bacteria of the genus *Campylobacter* are widespread in the environment and have been detected in various animal reservoirs, but their prevalence is particularly high in chickens. Colonization of the gastrointestinal tract by *Campylobacter* usually does not produce any disease symptoms in chickens, although some studies have reported that challenged birds may exhibit distention of the jejunum, disseminated hemorrhagic enteritis or focal hepatic necrosis [35]. Raw poultry meat is often contaminated with *Campylobacter*, and eating undercooked chicken, or ready-to-eat foods that have been in contact with raw chicken, is the most common source of infection. In 2013, the presence of *Campylobacter* was detected in 31.4% of samples of fresh broiler meat collected at slaughter, during processing and at retail facilities in various countries of the E.U., and the pathogens were found at every sampling stage [13].

Many countries are currently working to prevent foodborne campylobacteriosis, and considerable progress has been made on numerous fronts during the past ten years. In 2011, the EFSA (European Food Safety Authority) Panel on Biological Hazards issued advice on reducing *Campylobacter* in chicken meat. Recommendations include controlling *Campylobacter* in primary

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broiler production before the bacteria spread from farms to humans by various pathways and implementation of GMP-HACCP during slaughter [11]. These measures may reduce colonization of broilers with *Campylobacter* and contamination of carcasses. Quantitative risk assessment based on data from several countries has concluded that there is a linear relationship between prevalence of *Campylobacter* in broiler flocks and public health risk. *Campylobacter jejuni* colonizes chickens at densities of 10^8 colony forming units (CFU)/gram of caecal contents or higher without causing disease. These bacteria rapidly spread throughout the flock and remain present throughout the bird's lifespan. It is estimated that reducing the numbers of *Campylobacter* in the intestines at slaughter by 3 log10-units would reduce the public health risk by at least 90% [11, 27].

One of the strategies aimed at reducing the carriage of *Campylobacter* spp. among poultry includes the use of probiotic microorganisms that compete with pathogenic bacteria for colonization of the gut. The administration of probiotics is advantageous as compared to other strategies aimed at eliminating unwanted microflora (e.g. vaccination, antibiotic treatment, or chemical disinfection), as they are easy to administer and inexpensive to produce, and because they may persist in the animal. Bacteria of the genus *Lactobacillus* are recognized candidates for probiotics. They are non-pathogenic rods that naturally inhabit the mucous membranes of humans and animals, including chickens, and play an important beneficial role in the physiology of the host by providing a protective barrier in the gut [8]. Probiotic lactobacilli may eliminate unfavorable microflora 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. These beneficial properties of lactobacilli are largely dependent on their prolonged residence in the gastrointestinal tract and are dictated by adherence to the intestinal mucosa [22].

The objective of this study was to evaluate the probiotic potential of *Lactobacillus* isolates from chickens, with particular emphasis on their ability to inhibit the growth of *C. jejuni* and *C. coli in vitro*.

MATERIALS AND METHODS

Lactobacillus isolates

A total of 46 *Lactobacillus* isolates from the fresh faeces or cloacae of 17 healthy chickens (broilers and Green-legged Partridge hens) from 5 poultry farms located in Poland were included in this study. Lactobacilli were isolated on deMan Rogosa Sharpe medium (MRS, BTL, Łódź, Poland) at 37°C for 48 hr in 5% CO_2 and identified to species level by MALDI-TOF mass spectrometry (MS) and in the case of ambiguous results, the 16S rDNA restriction analysis was additionally used, as previously described [5].

Campylobacter isolates

Campylobacter bacteria used in the experiment to evaluate the antibacterial activity of *Lactobacillus* sp. were isolated from the cloacae of 10 chickens exhibiting no disease symptoms, obtained from 3 poultry farms. Bacteria were cultured in microaerophilic atmosphere (5% O_2, 10% CO_2 and 85% N), as it was described in our previous paper [10]. *Campylobacter* isolates were identified using MALDI-TOF MS [10] and the multiplex PCR technique with species-specific primers according to protocol developed by Wieczorek and Osek [33]. Two reference *Campylobacte*r strains-*C. jejuni* ATCC 29428 and *C. coli* ATCC 43479, were used as controls.

Detection of antibacterial activity of Lactobacilli-agar slab method

The suspensions of *Lactobacillus* isolates with an optical density of 0.5 measured at 600 nm (OD_{600}=0.5, in 0.9% NaCl) were seeded onto MRS agar and incubated at 37°C in 5% CO_2 for 24 hr. Then agar slabs (9 mm in diameter) were cut and placed on Columbia agar with 5% sheep blood inoculated with 0.5 m*l* of the *Campylobacter* indicator strain (OD_{600}=0.5, in 0.9% NaCl). The plates were kept at 41.5°C in microaerophilic conditions. After 45 hr of incubation the plates were checked for inhibition zones. The experiment was performed in duplicate and the results are presented as the mean diameter of the inhibition zone ± SD [6].

Detection of antibacterial activity of Lactobacilli−well diffusion method

Lactobacillus isolates were grown in 1.2 m*l* of MRS broth for 24 hr (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 6.8–7.0 using 19 M NaOH. The *Campylobacter* isolates were inoculated on Columbia agar with 5% sheep blood 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 supernatants. After 45 hr of incubation at 41.5°C in microaerophilic conditions, the plates were checked for inhibition zones [4].

Detection of H_2O_2 production by Lactobacilli

The ability of lactobacilli to produce H_2O_2 was determined by culture them on MRS agar supplemented with TMB (3,3′,5,5′-Tetramethylbenzidine, Sigma-Aldrich, Poznań, Poland) substrate (0.25 mg/m*l*,) and horseradish peroxidase (0.01 mg/m*l*, Sigma-Aldrich, Poznań, Poland). Inoculated plates were kept for 48 hr at 37°C, 5% CO_2. Blue color of colonies indicated H_2O_2 production by the bacteria. Color intensity was designated as follows: –, +, ++, +++.

Measurement of bacterial hydrophobicity

Hydrophobicity of the bacteria was determined on the basis of microbial adhesion to hydrocarbon [4]. Isolates with hydrophobicity equal to or more than 50% were considered hydrophobic.

Tolerance for acidic pH

Lactobacillus isolates grown overnight on MRS broth were centrifuged at 10,000 × g for 5 min. Pellets were resuspended in 0.9% NaCl (OD_{600}=3.0) and 30 *µl* of the suspension was added to 470 *µl* of MRS broth with pH 1.5, 2.0 or 6.8 (positive control). The bacteria were incubated for 60, 90 or 120 min at 37°C. Then the suspensions were centrifuged and the pellets were resuspended in fresh MRS medium (pH 6.8). Growth of the surviving bacteria was observed after 48 hr of culture at 37°C, 5% CO_2.

Bile tolerance test

The tolerance of *Lactobacillus* isolates to bile salts was determined in microplate assay. MRS medium (200 *µl*) containing 2% bile (BTL, Łódź, Poland) was inoculated with 0.5 *µl* of fresh broth cultures of lactobacilli. Following 24 hr incubation at 37°C, 5% CO_2, the optical density of the bacterial cultures was measured at 620 nm. Positive controls were bacterial cultures that grew without ox gall. The growth of each strain was expressed as a percentage of the OD_{620} value of the control samples.

Biofilm formation

Lactobacillus isolates cultured in MRS medium for 18–20 hr were diluted 1:50 in MRS broth to final volume of 200 *µl* in 96-well plates (MaxiSorb, Biokom, Janki, Poland). The bacteria were incubated 48 hr at 37°C, 5% CO_2. Adherent cells were stained with crystal violet (1% w/v) for 15 min. Unbound dye was washed off with water, and cell-bound dye was dissolved in 20% acetone in ethanol for 10 min, and the absorbance (A_{570}) was measured using a Microplate Reader 680 (Bio-Rad, Warszawa, Poland). Isolates were classified as the following four criteria: no biofilm producer (–: OD ≤ODc), weak biofilm producer (+: ODc <OD ≤2 × ODc), moderate biofilm producer (++: 2 × ODc <OD ≤4 × ODc) and strong biofilm producer (+++: 4 × ODc <OD), where the cut-off OD (ODc) was defined as the mean OD of the negative control.

Determination of minimal inhibitory concentration

Antibiotic susceptibility *Lactobacillus* isolates was determined by the broth microdilution assay using the LSM medium [7]. Inocula were prepared by suspending bacteria in 0.9% NaCl (OD_{600}=0.5). Microdilution plates were inoculated with 50 *µl* of a 1:500-diluted (in LSM broth) inoculum and 50 *µl* of the appropriate antibiotic concentration. Plates were incubated 48 hr at 37°C in 5% CO_2. The values of minimal inhibitory concentration (MIC) were read as the lowest concentration of an antimicrobial agent at which visible growth was inhibited.

Interpretation of the results for the antibiotic susceptibility of *Lactobacillus* isolates was based on the breakpoint values suggested by *EFSA's Panel* on Additives and Products or Substances used in Animal Feed (*FEEDAP*) [12] for ampicillin, tetracycline, erythromycin, streptomycin, gentamycin and chloramphenicol. For lincomycin we established cut-offs based on the distribution of MICs (bimodal/unimodal) and the breakpoint values suggested by Cauwerts *et al*. [3]−isolates were considered resistant if the lincomycin MIC was ≥32 *µ*g/m*l*.

Statistical analysis

The mean diameters of the inhibition zones for *Campylobacter* isolates determined to be sensitive to various *Lactobacillus* species were compared by one-way analysis of variance. 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, U.S.A.).

RESULTS

Identification of Lactobacillus and Campylobacter isolates

Lactobacillus bacteria were obtained from all samples, and 2–6 isolates of varying colony morphology were collected from each sample. A total of 46 isolates were classified by MALDI-TOF MS as bacteria of the genus *Lactobacillus* with a Biotyper log (score) equal to or greater than 1.70. Eight isolates, for which the two best matches with similar log (score) values (difference in log (score) <0.15) indicated different species, i.e. *L. johnsonii* and *L. gasseri*, were subjected to additional identification by restriction analysis of 16S rDNA using the *Mse*I restriction enzyme. The study showed that eight isolates pre-classified as *L. johnsonii*/*L. gasseri* belonged to *L. johnsonii* species (data not showed).

Finally, the collected *Lactobacillus* isolates were classified into seven species, i.e. *L. salivarius* (n=15), *L. johnsonii* (n=11), *L. crispatus* (n=5), *L. ingluviei* (n=5), *L. reuteri* (n=5), *L. oris* (n=2) and *L. saerimneri* (n=3).

Campylobacter bacteria were isolated from 6 out of 10 samples, one isolate from each sample. Four isolates were identified as *C. jejuni* and 2 isolates as *C. coli* by MALDI-TOF MS (BioTyper log (score) values were greater than 2.00 for all isolates). The same identification results were obtained in species-specific PCR. The electrophoretic profiles of all isolates contained 2 bands-one corresponding to 16S rDNA (860 bp), and the other corresponding to the *map*A gene (590 bp) coding for membrane-associated protein A specific for *C. jejuni* or the *ceu*E gene (460 bp) encoding an iron-chelating protein involved in siderophore transport

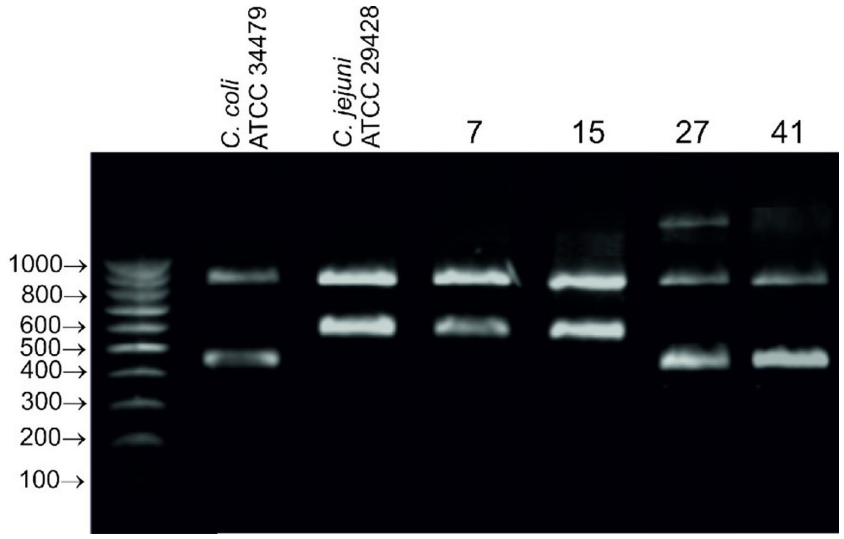

Fig. 1. Agarose gel (2%) showing the amplicon patterns obtained in multiplex PCR for reference and wild isolates (7, 15, 27, 41) of *C. jejuni* and *C. coli*; profiles contained the following amplicons: 16S rDNA 860 bp, *map*A 590 bp, *ceu*E 460 bp.

specific for *C. coli* (Fig. 1). Two isolates of *C. jejuni* (7, 15) and two isolates of *C. coli* (27, 41) with different colony morphology were selected for further analysis.

Agar slab method

All *Lactobacillus* isolates tested showed an inhibitory effect towards *Campylobacter* (Supplementary Fig. 1). The diameter of the growth inhibition zones of the test bacteria induced by the lactobacilli ranged from 11.0 ± 0.0 to 26.5 ± 0.7 mm, where the diameter of the slab was 9 mm. The antimicrobial activity exhibited by lactobacilli was correlated with their species. The strongest inhibition of *Campylobacter* growth was exhibited by the isolates of *L. salivarius* (mean inhibitory zone 21.0 ± 2.0 mm) and *L. reuteri* (20.3 ± 2.3 mm). Isolates of the species *L. johnsonii*, *L. saerimneri* and *L. oris* exhibited weak antagonistic properties (≤15.9 ± 1.9 mm) (Fig. 2, Supplementary Table 1). However, individual isolates of some *Lactobacillus* species showed large differences in anti-*Camylobacter* activity. Particularly large heterogenity of the size of inhibitory zones was observed for isolates of the species *L. crispatus* and *L. ingluviei* (Table 1).

C. jejuni isolates tended to be more susceptible (mean inhibition zone 18.3 ± 4.3 mm) than *C. coli* ones (16.7 ± 3.7 mm) to the antagonistic substances produced by lactobacilli, but the differences were not statistically significant (Table 1, Supplementary Fig. 2).

The *Lactobacillus* isolates which showed the strongest antagonism towards the *Campylobacter* bacteria were chosen. Among the 10 selected isolates, which caused inhibition zones of ≥22 mm in the case of *C. jejuni* and ≥20 mm for *C. coli*, 7 belonged to the species *L. salivarius* (4a, 9b, 24a, 24b, 27e, 37b, 48a), one to the species *L. crispatus* (49a), one to the species *L. reuteri* (14a) and one to the species *L. ingluviei* (28c) (Table 1).

Well diffusion method

The pH of the cell free media obtained from the 24 hr culture of *Lactobacillus* isolates ranged from 3.0 to 4.5. The inhibition of growth of *Campylobacter* bacteria by native cell-free broth was much weaker than the inhibition observed in the agar slab method. The size of the inhibition zones caused by native acidified supernatants was up to 16.6 ± 0.5 mm for *C. jejuni* and 16.5 ± 0.7 mm for *C. coli*, where the well diameter was 8 mm. Cell-free supernatants with neutralized acids (pH 6.5–7.0) did not exhibit antagonistic activity towards the indicator bacteria (data not showed). 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

Among 46 *Lactobacillus* isolates tested, 43 (93%) produced H_2O_2. The highest rate of production (+++) was observed in 22 isolates, belonging mainly to the species *L. johnsonii*, *L. ingluviei*, *L. saerimnerii* and *L. oris.* Moderate hydrogen peroxide production (++) was noted in 11 isolates of different species, and the group with the lowest H_2O_2 production (+) comprised 10 isolates, including 8 isolates of *L. salivarius*, one isolate of *L. crispatus* and one of *L. reuteri* (Table 1, Supplementary Fig. 3).

Hydrophobicity

Forty-five out of 46 *Lactobacillus* isolates showed high affinity towards xylene. Their percentage of hydrophobicity (% H) was ≥50, and therefore these isolates were considered hydrophobic. Only one isolate (*L. salivarius* 60b) proved to be hydrophilic, as its % H was 10. For 69.6% *Lactobacillus* isolates the % H was as high as 87–100%, and for 30.4% it ranged between 50 and 80% (Table 1).

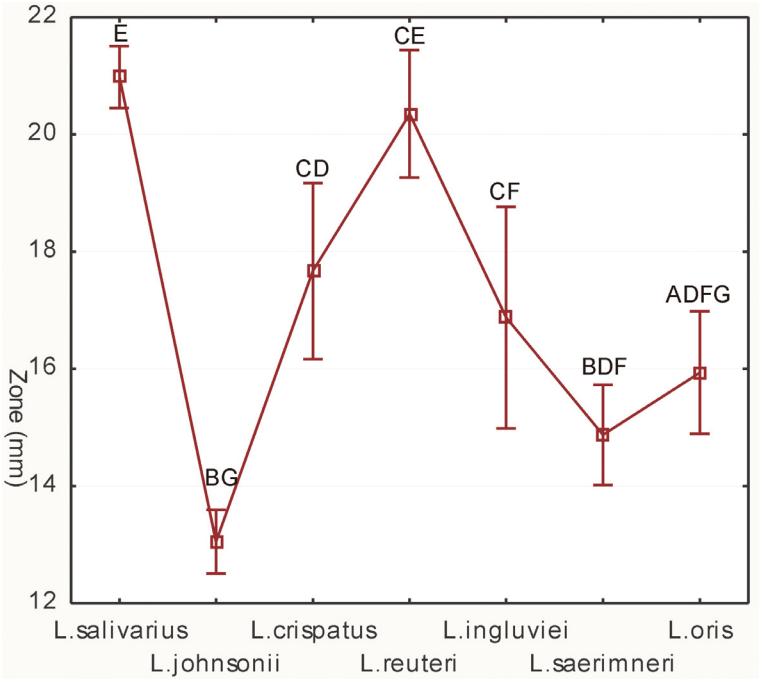

Fig. 2. Effect of isolates of various *Lactobacillus* species on the growth of indicator bacteria, as determined by the agar slab method. Different capital letters (A–G) indicate significant differences ($P<0.05$) between mean diameters of growth inhibition zones caused by individual *Lactobacillus* species; vertical bars denote 0.95 CI.

Resistance to low pH

Lactobacillus isolates showed excellent survival rates at *pH* values as low as 1.5 and 2.0. Forty-four out of 46 *Lactobacillus* isolates were able to survive pH 1.5 for 1 hr and 39 isolates survived as long as 3 hr at this pH. At pH 2.0, all *Lactobacillus* isolates survived 2-hr incubation, and only one (*L. ingluviei* 9e) was unable to survive 3 hr (Table 1).

Resistance to bile

Forty-one (89%) out of 46 *Lactobacillus* isolates tested were able to survive for 24 hr in the presence of 2% bile. Moreover, 13 isolates of various species demonstrated growth (8.9–90.5%) in the MRS broth containing 2% bile. The highest resistance to bile was demonstrated by 2 isolates of *L. oris*, whose growth intensity in the medium supplemented with bile salts was similar (~90%) to that of in a medium without bile (100%) (Table 1).

In vitro biofilm formation

Among the 46 isolates tested, 23 showed biofilm formation. Three isolates (*L. salivarius* 4a and 60d and *L. reuteri* 45b) were classified as strong biofilm-producers (+++), 4 isolates (*L. johnsonii* 7d, *L. reuteri* 4c and 14a and *L. oris* 50c) as moderate biofilm-producers (++) and 16 isolates of different species showed week ability (+) to biofilm formation. None of the isolates of *L. crispatus* and *L. saerimneri* exhibited the ability to biofilm formation (Table 1).

Antibiotic susceptibility

According to established criteria, 37 isolates (80.4%) were resistant to at least one antimicrobial agent, and 18 (39.1%) isolates showed multidrug resistance (resistance to 3 or more antibiotic groups). Only 9 (19.5%) *Lactobacillus* isolates, which were susceptible to all antibiotics tested, met the criteria for bacterial feed additives (*L. salivarius* 9b and 60d, *L. johnsonii* 4b, 8f, 9d, *L. ingluviei* 9e and 43d, *L. crispatus* 49b and *L. oris* 50c) (Table 2).

DISCUSSION

In the present study we demonstrated the probiotic potential of *Lactobacillus* isolates from chickens. *In vitro* tests showed that most of the lactobacilli were able to inhibit the growth of *Campylobacter* and survive the transit through the stomach and duodenum. Additionally, the high hydrophobicity of the isolates may indicate their ability to adhere to the mucosa, enabling them to colonize the intestine.

The use of the agar slab method allowed to shown that lactobacilli have an inhibitory effect on the growth of *Campylobacter*, which is the result of the production of certain antimicrobial substances on the solid medium. The strongest antagonism towards pathogenic bacteria was exhibited by *L. salivarius* and L. reuteri, as well as single isolates of *L. ingluviei* and *L. crispatus*.

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Table 1. Anti-*Campylobacter* activity, tolerance to low pH and bile, hydrophobicity, production of H_2O_2 and biofilm formation of *Lactobacillus* isolates

Species	Strain	Results of agar slab method		Ability to survive at low pH				Ability to surviving in MRS + 2% bile	Ability to growth on MRS + 2% bile (%)	Ability to produce H_2O_2	Hydrophobicity (%)	Biofilm formation	Resistance to any antibiotic
		Average inhibition zone of *C. jejuni* (mm)	Average inhibition zone of *C. coli* (mm)	pH 1.5		pH 2.0							
				1 hr	3 hr	1 hr	3 hr						
L. salivarius	4a	22.5 ± 2.1	21.0 ± 0.9	+	+	+	+	+	no	–	100	+++	+
	6a	20.7 ± 1.1	18.5 ± 0.7	+	+	+	+	–	no	+	60	–	+
	6b	21.7 ± 0.35	20.5 ± 1.4	–	–	+	+	+	no	+	100	+	+
	8b	19.0 ± 0.0	18.5 ± 0.7	+	+	+	+	+	no	+	67	–	+
	9b	22.2 ± 0.35	20.2 ± 1.1	+	+	+	+	+	no	++	60	–	–
	10a	21.5 ± 2.1	20.0 ± 0.0	+	+	+	+	+	no	+	100	–	+
	10d	18.2 ± 0.35	18.7 ± 1.1	+	+	+	+	+	no	+	100	–	+
	24a	22.0 ± 0.0	20.0 ± 1.4	+	+	+	+	+	no	+	80	+	+
	24b	22.5 ± 0.7	20.5 ± 0.7	+	+	+	+	+	no	+++	100	–	+
	27e	25.0 ± 0.0	23.5 ± 2.1	+	+	+	+	+	48.3	++	90	+	+
	37b	23.5 ± 0.7	20.0 ± 0.0	+	+	+	+	+	6.5	+	100	+	+
	40a	23.0 ± 0.0	18.5 ± 0.7	+	+	+	+	+	55.3	+	55	–	+
	48a	23.7 ± 0.35	20.5 ± 0.7	+	+	+	+	+	no	–	100	+	+
	60b	21 ± 1.4	19.2 ± 1.1	+	+	+	+	+	no	–	10	+	+
	60d	23.5 ± 0.0	19.0 ± 0.0	+	+	+	+	+	no	++	100	+++	–
L. johnsonii	4b	12.5 ± 0.7	13.5 ± 0.7	+	+	+	+	–	no	+++	100	+	–
	7d	18.5 ± 0.7	16.5 ± 2.1	+	+	+	+	+	28.0	+++	87	++	+
	8f	14.5 ± 0.7	13.5 ± 0.7	+	+	+	+	+	no	+++	100	+	–
	9d	12.2 ± 0.35	12.5 ± 0.7	+	+	+	+	–	no	+++	50	+	–
	13b	12.0 ± 0.0	12.0 ± 0.0	+	+	+	+	+	no	+++	70	+	+
	17c	12.5 ± 0.7	12.5 ± 0.7	+	+	+	+	+	no	+++	80	+	+
	25b	11.7 ± 0.35	11.7 ± 0.3	+	+	+	+	+	no	+++	60	–	+
	32a2	14.0 ± 1.4	13.4 ± 0.9	+	+	+	+	+	no	+++	100	–	+
	37c	11.5 ± 0.7	12.0 ± 0.0	+	+	+	+	+	no	+++	70	–	+
	40b	11.5 ± 0.7	11.5 ± 0.7	+	+	+	+	+	no	+++	87	+	+
	44c	14.0 ± 0.0	13.0 ± 0.0	+	+	+	+	+	no	+++	100	+	+
L. crispatus	1b	15.0 ± 1.4	15.0 ± 0.7	+	–	+	+	+	23.0	++	100	–	+
	17b	18.2 ± 0.35	15.0 ± 1.4	+	+	+	+	+	8.9	++	80	–	+
	44b	16.0 ± 0.0	17.0 ± 1.4	+	+	+	+	+	no	++	100	–	+
	49a	22.2 ± 0.9	22.0 ± 0.8	+	+	+	+	+	no	+	100	–	+
	49b	14.0 ± 1.4	13.2 ± 1.1	+	+	+	+	+	14.9	+++	73	–	–
L. reuteri	4c	22.0 ± 0.0	19.0 ± 1.4	+	+	+	+	+	54.6	++	100	++	+
	12d	19.5 ± 0.7	18.0 ± 1.4	+	+	+	+	+	28.6	+++	73	+	+
	14a	24.5 ± 0.7	22.0 ± 1.4	+	–	+	+	+	69.0	+	89	++	+
	43a	19.7 ± 2.5	17.2 ± 0.3	+	+	+	+	+	16.0	++	90	–	+
	45b	22.0 ± 0.0	19.5 ± 0.7	+	+	+	+	+	26.4	++	100	+++	+
L. ingluviei	9e	14.25 ± 0.5	12.4 ± 0.5	+	–	+	–	+	no	+++	100	–	–
	14e	12.0 ± 0.0	12.0 ± 1.4	+	+	+	+	+	no	++	100	–	+
	18b	14.0 ± 0.0	12.0 ± 0.0	+	–	+	+	+	no	+++	100	+	+
	28c	23.7 ± 0.3	21.7 ± 1.2	+	+	+	+	+	no	+++	95	–	+
	43d	22.7 ± 0.3	19.25 ± 0.3	+	+	+	+	+	no	+++	100	–	–
L. saerimneri	7b	16.5 ± 0.7	15.5 ± 0.0	+	–	+	+	+	no	+++	100	–	+
	24c	14.2 ± 1.1	14.0 ± 1.4	–	–	+	+	–	no	+++	100	–	+
	44d	15.7 ± 0.3	13.2 ± 1.1	+	+	+	+	–	no	+++	100	–	+
L. oris	40g	16.7 ± 0.5	13.7 ± 1.5	+	+	+	+	+	90.5	+++	91	+	+
	50c	17.9 ± 0.6	15.4 ± 1.9	+	+	+	+	+	88.4	+++	100	++	–
Mean:		18.3 ± 4.3	16.7 ± 3.7										

Grey highlights indicate strains that meet all basic criteria for probiotics.

Table 2. Distribution of MICs of antibiotics tested among *Lactobacillus* species of chicken origin

	Strain	AMP	TET	ERY	LIN	STR	GEN	CHL
L. salivarius	4a	2	8	0.5	256	>512	8	4
	6a	2	128	0.5	2	64	8	4
	6b	0.5	128	0.5	2	64	8	4
	8b	1	64	0.5	4	64	8	8
	9b	1	2	0.5	≤2	64	8	4
	10a	0.5	256	>64	>1,024	128	16	4
	10d	1	256	>64	1,024	64	8	8
	24a	1	512	>64	1,024	64	8	8
	24b	4	512	>64	1,024	>1,024	256	8
	27e	8	256	>64	128	128	512	8
	37b	2	256	0.5	32	64	8	4
	40a	2	256	0.5	4	64	8	4
	48a	1	2	≤0.125	4	128	16	2
	60b	1	1	0.5	8	128	4	8
	60d	1	≤1	0.25	4	64	8	4
L. johnsonii	4b	2	≤1	≤0.125	4	4	8	4
	7d	2	≤1	≤0.125	4	32	8	2
	8f	2	2	≤0.125	4	4	4	4
	9d	1	≤1	≤0.125	8	≤2	8	2
	13b	1	64	>64	1,024	32	4	4
	17c	0.5	64	>64	512	32	8	1
	25b	2	256	>64	128	16	8	4
	32a2	1	4	>64	1,024	≤2	8	4
	37c	>64	128	≤0.125	8	≤2	4	4
	40b	>64	256	>64	512	4	8	4
	44c	1	128	0.25	256	4	4	8
L. crispatus	1b	1	2	≤0.125	8	8	64	4
	3b	1	64	≤0.125	64	16	4	2
	17b	4	256	>64	512	≤2	4	2
	44b	4	64	≤0.125	≤2	≤2	2	2
	49a	0.5	≤1	0.25	8	64	32	4
	49b	0.5	1	≤0.125	8	4	8	4
L. reuteri	4c	8	16	1	256	64	8	4
	12d	1	256	>64	512	16	≤1	4
	14a	2	256	≤0.125	128	128	4	4
	43a	2	512	>64	128	8	≤1	16
	45b	1	256	0.25	64	128	≤1	4
L. ingluviei	9e	0.5	8	0.25	<0.5	64	8	4
	14e	2	512	≤0.125	32	64	4	4
	18b	1	256	>64	512	64	8	4
	28c	0.5	512	0.25	64	64	4	4
	43d	0.5	≤1	≤0.125	≤2	64	8	4
L. saerimneri	7b	0.5	≤1	≤0.125	≤2	32	16	2
	24c	1	128	≤0.125	32	64	32	2
	44d	1	64	>64	128	128	32	4
L. oris	40g	>64	256	>64	512	128	8	4
	50c	0.5	1	≤0.125	8	64	8	4

Grey highlights indicate resistance; ampicillin (AMP), tetracycline (TET), erythromycin (ERY), lincomycin (LIN), streptomycin (STR), gentamicin (GEN) and chloramphenicol (CHL).

Antimicrobial *in vitro* activity of *Lactobacillus* bacteria from chickens against Campylobacter has been also observed by other authors, and similar to our studies, the most active isolates were generally *L. salivarius* and *L. reuteri* [23, 25, 36].

To determine the mechanism of the antimicrobial activity of *Lactobacillus* bacteria, we analyzed the activity of cell-free broth and the ability of the isolates to synthesize hydrogen peroxide. The results of the well diffusion method indicated that the reduced pH of the supernatant (due to lactic acid) play a key role in inhibiting pathogenic bacteria. The antimicrobial activity of organic acids produced by lactobacilli against *Campylobacter* has been observed by several authors. Neal-McKinney *et al*. [26] showed that lactic acid disrupts the membrane of *C. jejuni* and is responsible for inhibiting the growth of these bacteria *in vitro* and for reducing intestinal colonization in chickens. Antagonistic anti-*Campylobacter* activity of lactic and acetic acid produced by

heterofermentative *L. pentosus* CWBI B78 has been demonstrated by Dubois-Dauphin *et al*. [9], while Bratz *et al.* [2] showed that anti-*Campylobacter* activity of cell-free supernatants of *L. fermentum* ATCC 1493, *L. johnsonii* BFE 663 and *L. paracasei* IMT 22353 was pH-dependent (pH<4.3). Similar findings were reported by Wang *et al.* [32], who demonstrated that the cell-free supernatant of selected lactic acid bacteria (LAB) characterized by the highest bactericidal capacity contained a high concentration of organic acid, and their inhibition effects against *C. jejuni* were pH sensitive. Several other authors have demonstrated that lactic acid may be effective in eliminating contamination of poultry meat by *Campylobacter.* Van Netten *et al.* [31] reported that decontamination with 1% lactic acid at pH 3.0 for at least 30 sec. was effective for *C. jejuni*, and Rasschaert *et al.* [28] showed that immersing the carcasses in a 1.5% lactic acid solution was far more effective in reducing the number of *Campylobacter* bacteria than spraying them with this acid. Protective effects of *Lactobacillus* strains of avian origin, especially *L. salivarius*, against *C. jejuni* in chickens have also been observed in experiments using animal models [17, 20, 24, 34].

Elimination of *Campylobacter* by probiotic LAB strains depends not only on production of antimicrobial substances, but also on the ability of these bacteria to adhere to the intestinal epithelium. The adhesion mechanism involves passive forces and electrostatic and hydrophobic interaction, as well as specific binding dependent on bacterial surface adhesins [19]. Our research showed that most of the *Lactobacillu*s isolates were characterized by high hydrophobicity and several researchers have reported a positive correlation between hydrophobicity of *Lactobacillus* strains and their adhesion to epithelial cells [1, 14, 21, 30]. It has been demonstrated [16] that colonization of the caecum in chickens by *Campylobacter* sp. was reduced by adhesion of human probiotic strains to mucous in the chicken gut. Similar conclusions can be drawn from a study by Wang *et al.* [32], who showed *in vitro* that adhesion of *Campylobacter* to intestinal cells (HT-29) was inhibited by four selected *L. plantarum* and *L. casei* strains. The hydrophobicity of these isolates ranged from 38% to 56%, and *L. casei* ZL4, characterized by the highest hydrophobicity, displayed excellent ability to inhibit *C. jejuni* invasion in exclusion assays.

The results of the present study showed that *Lactobacillus* sp. isolates originating in chickens produce H_2O_2. However, production of this reactive oxygen species was not clearly correlated with the antimicrobial activity of lactobacilli observed in the agar slab method. All isolates of *L. johnsonii*, *L. ingluviei* and *L. oris* exhibited strong production of hydrogen peroxide, but their inhibition of the growth of *C. jejuni* and *C. coli* was generally weak. Moreover, some of the *L. salivarius* isolates that most strongly inhibited the growth of pathogens were marked as '+' or '–' in terms of H_2O_2 production. The lack of relationship between antimicrobial activity of poultry lactobacilli and the intensity of H_2O_2 production, were also observed in our previous research [4, 6].

Biofilm formation is an important feature for LAB enabling them to resist environmental conditions, leading to the successful colonization and maintenance of their population while displacing unfavorable microflora. A fundamental characteristic of biofilms is the production of an extracellular polysaccharide matrix, which provides protection against antibiotics and enzymes and supports the generation of a microenvironment for the metabolic interaction of the population [29]. We have shown that some lactobacilli have the ability to biofilm formation, but it should be taken into account that this activity depends on many factors, including environmental parameters such as texture of surface (rough or smooth), hydrophobicity, pH, nutrient concentration and temperature, and thus it may differ *in vivo* [29].

Despite the anti-*Campylobacter* activity and properties determining survival in the intestine, the majority of the *Lactobacillus* isolates tested cannot be directly used as feed additives due to their antibiotic resistances. According to the EFSA's FEEDAP Panel, strains carrying acquired resistance should not be used as feed additives unless it can be demonstrated that the resistance is a result of chromosomal mutation(s). Only 9 isolates, for which the MIC did not exceed the established breakpoints, can be used without reservation as feed additives. Unfortunately, 2 (*L. johnsoni* 4b and 9d) of these 9 antibiotic susceptible isolates were sensitive to bile salts. Therefore, 7 isolates, i.e. *L. salivarius* 9b and 60d, *L. johnsonii* 8f, *L. crispatus* 49b, *L. ingluviei* 9e and 43d, *L. oris* 50c, met all the basic criteria for probiotics and were finally selected. Among these isolates, *L. salivarius* 9b and 60d displayed strong antagonism against *Campylobacter,* and 5 isolates (8f, 49b, 9e, 43d and 50c) of different species exhibited high resistance to low pH and bile as well as high hydrophobicity, which may enable the elimination of *Campylobacter* through stronger adhesion of these isolates to the intestinal mucosa. Moreover the isolates 60d, 50c and 8f displayed the ability to biofilm formation.

In summary, our research showed that seven finally selected *Lactobacillus* isolates may have potential application in reducing *Camylobacter* spp. in chickens and thus prevent infections in both birds and humans. Prior to commercialization, the efficiency as well as the safety of these isolates should be confirmed on animal models. In the case of the *Lactobacillus* isolates that has exceeded the MIC breakpoints, but strongly inhibit the growth of *Camylobacter* spp., more precise genetic testing can be conducted to determine whether the resistance to a given antibiotic is a caused by a genomic mutation or to exogenous DNA.

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