Research article

Elimination of ochratoxin A by lactic acid bacteria strains isolated from chickens and their probiotic characteristics

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Abstract: *The aim of the study was to evaluate the ability of lactic acid bacteria strains, isolated from the gastrointestinal tract of chickens, to eliminate ochratoxin A (OTA) in vitro and to investigate whether the mechanism of OTA reduction is adsorption or hydrolysis. The probiotic characteristics of the strains, such as their growth performance and synthesis of lactic acid at 42°C, inhibition of Salmonella spp. and Escherichia spp. growth and susceptibility to antibiotics, was also evaluated. Ochratoxin A reductions in MRS broth depended on the tested strain and ranged from 1% to 29%. The level of OTA reduction was higher at 30°C than at 37 and 42°C and there were no significance differences between OTA reduction obtained with live and heat-treated bacteria cells. The evaluation of probiotic characteristics showed that Pediococcus acidilactici KKP 879 was the strain with more potential to develop a probiotic culture for chickens.*

Keywords: *lactic acid bacteria, ochratoxin A, phenylalanine, probiotics.*

Introduction

Ochratoxin A (OTA), a mycotoxin produced mainly by the fungi *Aspergillus ochraceus* and *Penicillium verrucosum*, can be found in a wide variety of food commodities and feed [1]. Though OTA amounts may be relatively low in foods, it may accumulate in the blood and organ tissues of either humans or animals that consumes contaminated food and feed (carry-over effect) [2]. Ochratoxin A damages the kidneys mainly, but at high concentrations it can also cause liver toxicity [3]. OTA poses no health hazard to ruminants since it is degraded by rumen microorganisms, but this mycotoxin may be detected in milk [4]. On the contrary, pigs and poultry are particularly sensitive to OTA [5]. When consuming feed with high levels of mycotoxins, chickens may be affected by a general

weakening of the body's resistance, increasing their susceptibility to several bacterial diseases such as salmonellosis and colibacillosis; Gumboro and Marek's disease from viral origin; and parasites like coccidiosis [6]. Intake of ochratoxin A by animals results in OTA contamination of meat and meat products. OTA has also been found in the blood, liver and kidneys of animals slaughtered [7]. For this reason there is a need to find ways to protect farm animals from the harmful effects of this toxin.

Prevention of ochratoxin A formation is often impossible so the decontamination of agricultural commodities is necessary in some cases. There are various physical and chemical methods that may be used to eliminate mycotoxins from food, but they often give unsatisfactory results. Moreover the use of various chemical compounds is sometimes impractical due to their adverse effect on the quality of agricultural raw materials [1]. For this reason, biological methods against mycotoxins are gaining attention from the research community. The biological detoxification of ochratoxin A can be carried out either *in situ* (OTA elimination from feed and food) as well as *in vivo* (decontamination takes place in the gastrointestinal tract of the animal) [8].

Many species of bacteria and yeasts are capable of degrading ochratoxin A [9-11]. It is claimed that the process of ochratoxin A degradation derives from the hydrolysis of the amide bond that links the L-β-phenylalanine molecule to the ochratoxin alfa (OTα) moiety, and that this hydrolysis is mediated by carboxypeptidase-like enzymes [12]. This process was firstly described in 1976, when testing the reduction of ochratoxin A by microorganisms isolated from the rumen of ruminants [11]. The hydrolysis of ochratoxin A by *Aspergillus niger* was further investigated [12]. In this case, the enzymatic extract from the fungus was added to an OTA solution, being observed a decrease in OTA levels and an increase of OTα presence in solution.

The ability to reduce the contamination of mycotoxins was also detected among lactic acid bacteria (LAB), a group of microorganisms commonly used in the production of fermented food and feed. LAB are able to detoxify some mycotoxins such as OTA [13]. The problem associated with the reduction of OTA by LAB has been the object of frequent interest. Many factors that influence the antimycotoxin activity of lactic acid bacteria have already been examined. For example, Fuchs et al. [9] examined the process of OTA elimination by 30 LAB strains belonging to 20 species. Among them, *Lactobacillus acidophilus* VM20 removed OTA from the culture medium by 97%. Piotrowska and Żakowska [14] came to the conclusion that the ability to eliminate ochratoxin A depends on the individual characteristics of each strain. Fuchs et al. [9] observed that OTA reduction depends on the number of bacterial cells in the medium since it was significantly lower when the number of bacterial cells were lower than 8 $CFU \cdot mL^{-1}$.

It is generally accepted that the mechanism involved in OTA elimination by lactic acid bacteria is adsorption to the cells walls [15]. However, detoxification activity was studied among two strains of *Lactobacillus spp.* in wine, in which the bacteria were incubated in the presence of ochratoxin A. The amount of OTA decreased and the presence of OTα form in wine was detected [16].

Given the collected literature data it can be assumed that lactic acid bacteria are of particular interest because of their beneficial effects for humans and animals (as probiotics) and their extensive use in the food and feed industry. The use of probiotic LAB strains which degrade ochratoxin A into nontoxic metabolites could be an useful biological method for OTA decontamination *in vivo*.

The aim of this research was to study the ability of lactic acid bacteria isolated from chickens to eliminate OTA by degradation to phenylalanine. As an additional goal probiotic characteristics of isolated bacteria were evaluated to explore the possibility of using the strains as probiotic cultures for chickens feeding.

Experimental

Materials

Six strains of lactic acid bacteria were used throughout this study. The strains were previously isolated from gastrointestinal track of chickens bred in organic farms. Criterion for the selection of strains were their resistance to low pH (1.5-2.5) and to the presence of bile salts in the environment.

Identification of the strains were performed by analysis of 16S rRNA sequence. The sequences obtained were compared with those available in GenBank database. The strains belonged to the following species: *Lactobacillus reuteri* (D2), *Lactobacillus agilis* (D5), *Lactobacillus plantarum* (D6), *Lactobacillus salivarius* (D8), *Pediococcus acidilactici* (KKP 879) and *Lactobacillus plantarum* (KKP 825). The strains are deposited in Culture Collection of Industrial Micro-organisms sited in Wacław Dąbrowski Institute of Agricultural and Food Biotechnology in Warsaw, Poland. The Collection has the status of International Depository Organ, entitled to receipt microbial strains being the subjects of patent applications. The Collection is a member of World Federation of Culture Collections as well as European Culture Collections' Organization.

Methods

Removal assay and determination of OTA by HPLC and ELISA

The ability to eliminate ochratoxin A by bacterial strains depending on the temperature was verified. Bacteria were incubated in tubes containing 10 mL of MRS medium. Ochratoxin A (Supelco) was added to the medium so that a concentration of 180 $\mu g \cdot L^{-1}$ was achieved. Overnight culture of the strains was added to each tube, so that the initial number of bacteria in medium was log 9 $CFU \cdot mL^{-1}$. Bacteria were incubated at 30, 37 and 42°C. Experiment was

carried out in triplicate. A control sample was prepared with the medium containing OTA, but without bacteria. After 24 hours of incubation the quantitative analysis of OTA was assessed in the fermented medium after centrifugation at 15000 *g* for 15 min. at 4°C to remove bacteria cells. The AgraQuant® Ochratoxin Test Kit (Romer Labs®, USA) was used according to manufacturer's instruction. The decrease in OTA in the medium in relation to the concentration in control sample was expressed as removal percentage.

In order to evaluate the ability of viable and dead bacterial cells to eliminate OTA a further experiment was performed (in triplicate). The incubation of the strains was carried out in tubes containing 10 mL of medium without protein addition: 20 $g \cdot L^{1}$ glucose, 2 $g \cdot L^{-1}$ potassium phosphate, 2 $g \cdot L^{-1}$ ammonium citrate, 5 $g \cdot L^{-1}$ sodium acetate, 0.6 $g \cdot L^{-1}$ magnesium sulphate, 0.2 $g \cdot L^{-1}$ manganese sulfate. Initial pH was 6.7. To each tube 5 μg of vitamin B_{12} and 200 $\mu g \cdot L^{-1}$ of OTA (Supelco) was added. Bacteria were incubated in MRS medium overnight and then harvested by centrifugation (15000 *g*, 15 min., 4°C). The supernatants were discarded and the pellets resuspended in 10 mL physiological saline. The washing step was repeated three times in order to remove culture medium residues. Subsequently, the pellets were resuspended in physiological saline to obtain the final concentration and then added to tubes with OTA, so that the initial number of bacteria was log 9 $CFU \cdot mL^{-1}$. Dead bacteria were obtained by boiling the final saline solution of bacteria in a water bath. Liveliness of heat-treated bacteria was confirmed by their inoculation on MRS agar. Alive and heat-treated bacteria were incubated in medium with OTA at 30°C. Then, the quantitative analysis of ochratoxin A was assessed in the fermented medium after centrifugation (15000 *g*, 15 min., 4°C). Before OTA analysis, samples were applied to OchraTest™ immunoaffinity columns (Vicam, USA). The columns were washed with a solution containing sodium chloride (2.5%) and sodium hydrogen carbonate (0.5%), followed by water. OTA was eluted with methanol and quantified by HPLC method with fluorescence detection. An C-18 Nucleosil column (Supelco) and phosphate buffer (43 mM)-acetonitrile-isopropanol eluent (55:19:22 v/v) was used for the chromatographic separation. Samples (10 μL) were injected, eluted with a flow rate of 0.5 $mL \cdot min^{-1}$ and detected with a fluorescence detector (Schimazu) at λex = 330 and λem = 460 nm. Additionally analysis of phenylalanine in the medium after live bacteria incubation was performed.

Phenylalanine determination

Qualitative analysis of phenylalanine in fermented medium was assessed using Phenylalanine Assay Kit (fluorometric test, Sigma-Aldrich) according to manufacturer's instruction. Fluorescence of samples was compared with the fluorescence of control samples containing: pure medium, medium with OTA but without bacteria and samples containing medium with bacteria but without OTA addition.

Evaluation of bacteria resistance to antimicrobials

The minimum inhibitory concentration (MIC) of the antimicrobials expressed as $mg \cdot L^{-1}$ was determined. All antibiotics originated from Sigma-Aldrich. LSM broth microdilution method according to [17] were used. Briefly, serial twofold dilutions of antibiotic stock solutions were prepared and inoculated into wells of microtiter plate containing LSM broth. No antibiotic was added as antibiotic-free grown control of strain. No strain was added to check sterility.

Bacteria inoculum was prepared by diluting 18 h cultures of strain to the final concentration of inoculum log 5 $CFU \cdot mL^{-1}$. After a 24 and 48 hours incubation of plates under aerobic condition at 35ºC, MIC values were determined as the lowest concentration of an antibiotic able to inhibit visible growth of bacteria. Susceptibility of strains was established in accordance to the breakpoints proposed by the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) [17].

Determination of bacteria ability to inhibit the growth of pathogenic bacteria

Evaluation of the antimicrobial activity of the tested LAB strains was performed according to modified method [18]. After 24 h of incubation of LAB at 42°C in liquid MRS medium, bacteria were centrifuged (15000 *g*, 15 min, 4°C). Then 10 µL of supernatant was spotted onto solidified medium having the consistency of soft agar, previously inoculated with the indicator bacterial strain in the number of log 6 $CFU \cdot mL^{-1}$. The indicator bacteria strains were isolated from sick chicken broilers and obtained from National Veterinary Research Institute in Puławy (Poland). Plates were incubated for 18-20 hours at 42°C and then the pathogen reduction was assessed by measuring the indicator strain growth inhibition zone.

Additional features of LAB

The growth of tested strains in MRS medium at 30, 37 and 42°C was evaluated. Ten mL of MRS liquid medium was inoculated with 24 h-old cultures of tested strains in the ration 9:1. After 24 h of incubation the number of bacteria was evaluated by incorporation on MRS agar. Colony formic units were counted after 72 h of incubation at 30°C.

Additionally, contents of D(-) and L(+) lactic acid forms were analyzed in the medium after 24 h of incubation at 42°C. Bacteria were centrifuged (15000 *g*, 15 min, 4°C) and the lactic acid was evaluated in supernatant using enzymatic method (r-Biopharm UV test, product number 11112821035).

Statistical analysis

In order to study the effect of temperature and liveliness of LAB cells on the reduction of ochratoxin A, an analysis of variance to data obtained was performed. In the case of significance of the effects, a post hoc analysis (Tukey test) was performed to compare mean values. For all analyzes the level of significance was 0.05. Analyses were performed using Statistica 12.

Results and Discussion

The first experiment was performed to check if normal body temperature of chicken (42ºC) influenced the ability of bacteria to eliminate OTA. The results of OTA reduction depending on the temperature of incubation are shown in Fig. 1.

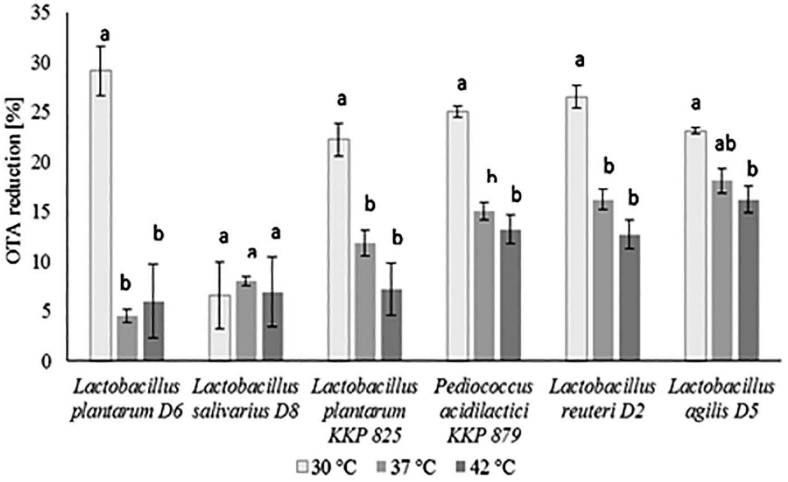

Figure 1. OTA reduction by lactic acid bacteria strains depending on the temperature, mean values and standard deviations. a, b, ab – mean values for selected strain differ significantly, $p \leq 0.05$

Decrease of OTA in the medium was significantly higher after incubation at 30°C compared to the OTA reduction at 37 and 42°C. *Lactobacillus salivarius* D8 was the exception, OTA removal by this strain did not differ significantly over the temperature range tested. The average OTA reduction obtained with the strains at 30°C ranged between 6.6% (*Lactobacillus salivarius* D8) and 29.1% (*Lactobacillus plantarum* D6).

Temperature is an essential factor that modulate LAB growth and significantly affects the amounts of antifungal metabolites produced. Influence of temperature on OTA reduction by LAB have not been examined so far. The effect of temperature (range 4-37°C) on OTA binding to yeast cell walls was investigated by Ringot et al. [19]. In their studies the effect of ochratoxin A adsorption decreased with increasing temperature and the adsorption isotherms indicated the exothermic nature of the process. The decrease of OTA elimination with increasing incubation temperature may indirectly indicate the physical nature of OTA removal from medium by tested strains This was probably the phenomenon of adsorption to cell walls structures, which is widely recognized as the mechanism of mycotoxins removal by LAB [20]. This is a fast process, but unfortunately partially reversible. For that reason potential future application of LAB strains to reduce OTA rely on the stability of complex mycotoxin-bacteria.

Weak binding interactions may cause that specific LAB strains could be of little use for a biological method of ochratoxin A decontamination in gastrointestinal tract. Nevertheless, there are a few scientific reports in which authors described the ability of some lactic acid bacteria strains to enzymatic degradation of OTA [13, 16].

No significant differences in OTA reduction were obtained in case of live and heat-treated bacteria incubation ($p \geq 0{,}05$), (Fig. 2).

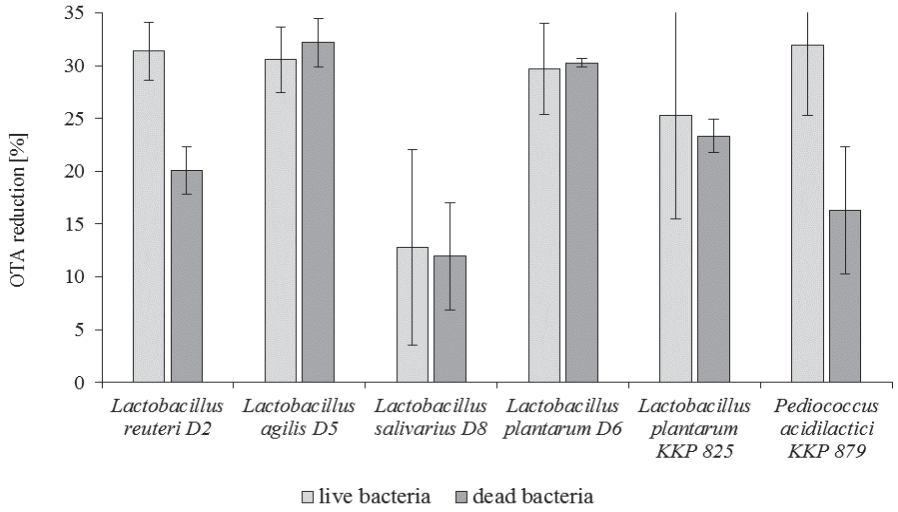

Figure 2. OTA removal by alive and heat-treated lactic acid bacteria strains, mean values and standard deviations

The greatest difference between OTA reduction by live or dead bacteria was observed in the case of *Pediococcus acidilactici* KKP 879 incubation. Live bacteria removed OTA by 31.9% and dead by 16.3%, but differences were not statistically significant (Fig. 2). However, after the incubation of *Lactobacillus reuteri* D2 and *Pediococcus acidilactici* KKP 879 in the medium with ochratoxin A, the presence of phenylalanine was detected. The presence of phenylalanine in the medium after incubation of *Lactobacillus reuteri* D2 and *Pediococcus acidilactici* KKP 879 may indicate the ability of these strains to degrade enzymatically OTA or is resulting from cells lysis. For the recognition of the mechanism of OTA elimination and to fully prove whether it is biodegradation or not, it is necessary to determine the presence of OTalfa in medium.

In order to investigate the mechanism of ochratoxin A decontamination many researches have already studied the differences in the level of ochratoxin A reduction by LAB depending on cell viability. Fuchs et al. [9] observed that the viability of the cells has an important role in the process of OTA removing from the solutions. Thermally inactivated cells of *Lactobacillus acidophilus* removed OTA slightly compared with live bacteria cells. In contrast Turbic et al. [21] observed a significantly lower rate of OTA elimination by viable LAB cells then

by acid- and heat-treated cells. It is proved that the high temperature leads to a denaturation of proteins, formation of Maillard reaction products and to generate pores in the cell wall structure. While acid causes the release of monomers from proteins and cell wall polysaccharides. Both methods of bacteria treatment increase the number of OTA 'binding sites' on the surface of the cell wall which results in increasing the effect of ochratoxin A adsorption [22, 3].

The results of antimicrobial susceptibility of studied strains are listed in Table 1.

Table 1. The minimum inhibitory concentration MIC ($mg \cdot L^{-1}$) of antibiotics for the tested strains

Susceptibility to antimicrobials	*L. reuteri* D2	*L. agilis* D5	*L. plantarum* D6	*L. salivarius* D8	*L. plantarum* KKP 825	*P. acidilactici* KKP 879
ampicillin	0.5	0.25	2	**8**	**4**	2
vancomycin	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
gentamicin	0.125	4	2	8	8	8
kanamycin	0.25	32	32	**128**	64	64
streptomycin	4	4	n.r.	**128**	n.r.	64
erythromycin	0.125	0.015	0.125	0.125	0.25	0.125
clindamycin	0.015	0.06	0.5	0.03	1	0.06
tetracycline	1	0.25	8	**16**	16	8
chloramph.	1	1	2	4	8	2

n.r. – not required according to [17].

All MIC values were situated in the tested concentration range. Strains *Lb. reuteri* D2, *Lb. agilis* D5, *Lb. plantarum* D6 and *P. acidilactici* KKP 879 were susceptible to all antibiotics as the microbiological cut-off values were below the proposed by the FEEDAP Panel breakpoints. Strain *Lb. plantarum* KKP 825 was resistant the antibiotic ampicillin and strain *Lb. salivarius* D8 was found resistant to 4 antibiotics: ampicillin, tetracycline and two aminoglycosides. These strains could be suspected of possessing acquired resistance to drug.

The development of resistance amongst bacteria to antimicrobials remains a serious concern. For this reason, microorganisms used as food and feed additives should not transfer antimicrobial resistance genes to gut bacterial population. The European Commission (advised by the European Food Safety Authority EFSA) has requested that antibiotic resistance should be investigated before LAB application in animal feeds in order not to pose a potential risk for transfer resistance genes to pathogenic bacteria (European Parliament and Council Regulation EC 429/2008).

Resistance to antibiotics may be inherent to a bacterial species (intrinsic resistance, natural resistance) and is typical of all the strains of that species. Antibiotic resistance of LAB is species-dependent, so that the application of resistant strains as probiotics may not be possible [24].

The strain *Lactobacillus rhamnosus* KKP 825 (not shown in Fig. 3) demonstrated very high antimicrobial activity against all indicator strains, it inhibited the growth of the pathogenic strains completely. *Pediococcus acidilactici* KKP 879 did not inhibit the growth of *Salmonella* DO and *Salmonella* CO strain (Fig. 3).

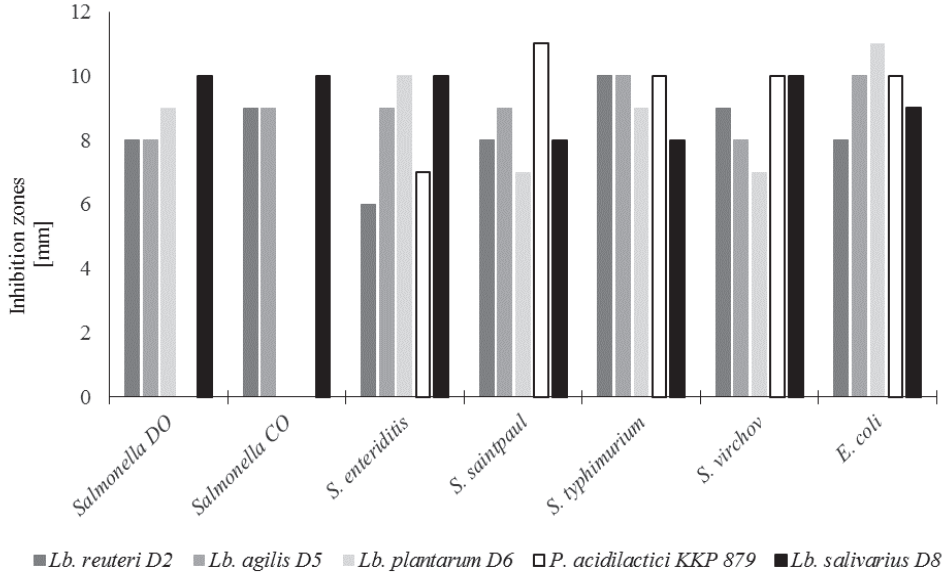

Figure 3. Inhibition of bacteria growth by lactic acid bacteria strains

One of the most important group of bacteria inhabiting the ileum and caeca during the early life of the chicks is *Enterobacteria*. Direct-fed microbials are known to benefit the host animal by improving its intestinal microflora balance [25]. The results of presented analysis are supported by those of Olnood et. al [26] who demonstrated that *Lactobacillus spp*. exert a direct influence on enterobacterial population. The study of Olnood et al. [25] showed that the number of *Enterobacteria* decreased in the caeca and ileum significantly when chicks were fed with probiotics.

Tested LAB strains were characterized for having an effective growth at different temperatures, nevertheless in most cases their growth was significantly lower at 42°C (temperature of body of birds) than at 30 or 37°C. Only *Pediococcus acidilactici* KKP 879 was characterized by having the same growth at studied range of temperatures. All strains produced larger amount of L(+) lactic acid than D(-) form (except of *Pediococcus acidilactici* KKP 879) at the temperature of incubation 42°C (Table 2).

Table 2. Additional features of lactic acid bacteria strains

	L. reuteri D2	*L. agilis* D5	*L. plantarum* D6	*L. salivarius* D8	*L. plantarum* KKP 825	*P. acidilactici* KKP 879
			Growth [log 10 CFU·mL^{-1}]			
30°C	9.43^{a}	9.96^{a}	9.79^{a}	9.15^{a}	9.30^{a}	9.69^{a}
37°C	9.47^{a}	8.00^{b}	8.60^{b}	9.79^{a}	9.49^{a}	9.49^{a}
42°C	7.95^{b}	8.60^{bc}	7.30^{c}	8.00^{b}	8.95^{ab}	9.56^{a}
			Lactic acid production at 42°C [g·L^{-1}]			
D(-)	1.07	1.36	1.69	2.73	0.3	6.08
L(+)	5.48	11.56	7.00	11.08	8.8	6.23

a, b, c, bc, ab – mean values (in columns) for a given strain differ at significance level $\alpha = 0.05$.

The criteria for selection of probiotic strains are safety, functionality and technological relevance [27]. The measure of functionality of LAB strains intended for chickens is, among others, the ability to effectively grow and maintain its metabolic activity at the place of destination, the gastrointestinal tract. As it has been reported in many studies lactic acid is one of the most important metabolites of LAB because of their antimicrobial activity. L(+) form of lactic acid is absorbed by organism very fast and fully metabolized so that is consider as an energetic substrate. D(-) form of lactic acid is absorbed much slower, thus its main issue is to lower pH in digestive tract [26]. Ability to synthesis D(-) lactic acid is an evaluation criteria used in probiotics strains selection. Inhibition properties of D(-) lactic acid is one of the mechanism of *Enterobacteria* decreasing in the gastrointestinal tract. It is proved that *Enterobacteria* are more susceptible to short chain fatty acids than lactobacilli [25].

Conclusion

Selecting probiotic microorganisms, that have beneficial effect in the gastrointestinal tract of broiler chickens, requires an extensive search for the best candidate which will perform desire activity under practical conditions. The evaluation of probiotic characteristics showed that *Pediococcus acidilactici* KKP 879 was the strain with more potential to develop as probiotic culture for chickens. Moreover, using this strain as probiotic culture for chickens could be beneficial for animals constantly exposed to ochratoxin A by lowering the content of the toxin in the gastrointestinal tract. However, further studies are needed to investigate whether the elimination of OTA occurs only through adsorption to cell walls or due to the enzymatic hydrolysis. Ultimately, *in vitro* studies must be verified by nutritional experiments on chickens concerning the real effect of LAB on ochratoxin A decontamination. So-called 'efficiency' studies are mandatory in the process of registration of new bacteria strains as feed additives in Register of Feed Additives pursuant to Regulation (EC) No 1831/2003.

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