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Selection of Potentially Probiotic *Lactobacillus* Strains Towards their Inhibitory Activity against Poultry Enteropathogenic Bacteria

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Abstract

Lactobacilli were isolated from chicken gastrointestinal tract and examined for their potentially probiotic properties towards their inhibitory activity against poultry enteropathogenic bacteria. Biochemical tests, ITS-PCR and cell wall protein analysis were used to characterize the *Lactobacillus* isolates. The identification of isolated *Lactobacillus* strains based on phenotypic properties was not always satisfactory. ITS-PCR together with protein profile were found to be helpful in strain identification. Lactobacilli were tested for the inhibitory activity against selected strains of poultry enteropathogenic bacteria (*Salmonella* Enteritidis, *Escherichia coli* and *Clostridium perfringens*). Examined supernatants from *Lactobacillus* broth cultures demonstrated major antimicrobial activity against *C. perfringens.* Lower antimicrobial activity were observed against *E. coli* and *Salmonella* Enteritidis. The strongest inhibition effect were obtained using supernatant of *Lactobacillus acidophilus* strain 3D. Results received from this study confirmed that identification of *Lactobacillus* spp. is often tedious. Some isolates, which are *in vitro* antagonistic against enteropathogenic bacteria may be considered as potential candidates for poultry probiotics, especially in controlling necrotic enteritis caused by *C. perfringens*.

Key words: *Lactobacillus* spp., poultry, probiotics.

Introduction

Lactobacilli are a heterogenous, non-sporing, rod-shapped and catalase-negative group of Gram-positive bacteria. They are inseparable components of natural microflora in the gastrointestinal tract of humans and animals and they have been widely used for medical and veterinary applications as well as in food fermentation processes. Bacteria of this group are commonly used as probiotics, which beneficially affect the animal host (Fuller, 1992; Gomes and Malcata, 1999).

The general concept of probiotics administration is to improve the intestinal microbial balance which is assumed to provide better protection against various diseases (Fuller, 1992). They may be also beneficial in growth promotion, better feed utilization, diseases resistance, reduction of colonization and shedding of enteropathogenic bacteria. One of the most desirable features of probiotic bacteria is their ability to synthesize antimicrobial substances such as organic acids, hydrogen peroxiode and bacteriocins (Roy *et al*., 2000). Bacteriocins are polypeptides which have bactericidal or bacteriostatic effect, usually against bacteria closely related to the producer strain. Most bacteriocins produced by lactobacilli have narrow spectrum of activity, but some of them are active also against Gram-negative bacteria (Callewaert *et al*., 1999; Cuozzo *et al*., 2000; Jack *et al*., 1995).

Bacterial strains to be used as probiotics in animal production could be therefore isolated from natural gastrointestinal microflora with the purpose of more specific application (La Ragione *et al*., 2004; Tsai *et. al*., 2005). However, testing their efficacy *in vivo* is expensive and time consuming. For that reason reliable *in vitro* methods are required for selecting new strains of probiotic bacteria to obtain profitable *in vivo* effects. Thus one of the important features of a new probiotic bacterial strains, which could be easily tested *in vitro*, is their activity against enteropathogenic bacteria (Tsai *et. al*., 2005).

Conventional phenotypical identification of lactobacilli is based on their morphology and carbohydrate fermentation profiles. However, it is not always sufficient. There are closely related strains, which can not

be easily distinguished by biochemical tests, since phenotypic properties may also depend on environmental conditions (Tynkkynen *et al*., 1999). Over the past years the significant progress has been made in the molecular taxonomy and identification of lactobacilli. Several molecular methods have been used for species identification and differentiation *i.e.*, pulse field gel electrophoresis (PFGE) (Roy *et al*., 2000; Ventura and Zink, 2002; Tynkkynen *et al*., 1999; Zapparoli *et. al*., 1998), sequencing of rRNA genes (Kullen *et al*., 2000), protein profiling (Andrighetto *et. al*., 1998; Drake *et al*., 1996; Gevers *et al*., 2001), ribotyping (Tynkkynen *et al*., 1999), conventional PCR (Guarneri *et al*., 2001; Spano *et. al*., 2002), ITS-PCR (Drake *et al*., 1996), RAPD-PCR (Daud Khaled *et al*., 1997; Roy *et al*., 2000; Tynkkynen *et al*., 1999; Zapparoli *et al*., 1998), and REP-PCR (Gevers *et al*., 2001; Ventura and Zink, 2002).

The objective of this study was to examine lactobacilli strains isolated from chicken gastrointestinal tract for the antagonistic activity against selected enteropothogenic bacteria. Isolates were identified using phenotyping and molecular biology methods and tested for activity against poultry enteropathogenic bacteria (*Salmonella* Enteritidis, *Escherichia coli* and *Clostridium perfringens*).

Experimental

Materials and Methods

Bacteria and growth conditions. Lactobacilli were isolated from faeces and intestines of healthy chickens. Samples were inoculated into Rogosa medium (Merck). The plates were incubated at 37°C for 48 h under anaerobic conditions generated by BBL™ GasPak system (Becton Dickinson). All isolates growing on Rogosa agar were stained by Gram method for microscopic examination and tested for catalase production using 3% H_2O_2. Carbohydrate fermentation patterns were determined using the API 50 CHL test according to the instructions of the manufacturer. The results were analyzed by MiniApi analyser (BioMérieux).

The following *Lactobacillus* spp. strains were obtained from the Collection of Industrial Microorganisms, Institute of Agriculture and Food Biotechnology in Warsaw: *L. plantarum* CIM 813, *L. brevis* CIM 369 and *L. fermentum* CIM 592.

Salmonella Enteritidis (10 strains), *Escherichia coli* (10 strains) and *Clostridium perfringens* (11 strains) have been isolated from chicken organs in Laboratory of Division of Bacteriology and Molecular Biology, Faculty of Veterinary Medicine, Warsaw Agricultural University. *Salmonella* Enteritidis, *E. coli* and *Clostridium perfringens* were grown according to standard bacteriological procedures (Malicki *et. al*., 2004). All *E. coli* isolates were haemolytic and all *C. perfringens* isolates were shown in PCR to possess genes encoding alpha toxin production, therefore they were regarded as pathogenic isolates.

Internal Transcribed Spacer PCR (ITS-PCR). For the genotypic characterization of isolated *Lactobacillus* spp. strains, ITS-PCR was applied according to the method described by Drake *et al*. (1996). ITS-PCR was carried out with primers G1 (5'-GAAGTCGTAACAAGG-3') and L1 (5'-CAAGGCATCCACCGT-3'). Genomic DNA was extracted using InstaGene™ Matrix (BioRad) from separate colonies on MRS (acc. to De Man, Rogosa and Sharpe) agar according to the manufacturer instruction. Each reaction mixture contained 0.2 mM of each dNTPs, 5 µL of PCR buffer, 1 µM of each primer, 2.5 mM $MgCl_2$, 2.5 U *Taq* DNA Polymerase and water to the volume of 50 µL. All reagents for PCR were purchased from Fermentas. The following thermal cycling conditions were used: initial denaturation for 1 min at 94°C and 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, extension for 2 min at 72°C and final extension for 7 min at 72°C. The PCR amplifications were performed with a TC-312 Thermal Cycler (Techne). Volume of 15 µL of the products was analyzed by gel electrophoresis in 2% agarose gel. The molecular size of the obtained DNA fragments was estimated by comparison with GeneRuller™ 100bp DNA Ladder Plus (Fermentas). Each ITS-PCR gel was documented by VersaDoc apparatus (BioRad).

Protein profiling. Extraction of cell wall proteins and subsequent sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed following the procedure described by Henriksson *et al*. (1995). The isolates were incubated in MRS broth at 37°C anaerobically overnight. Volume of 1 mL of culture was centrifuged at 3500 g for 10 min. The pellet was washed twice with PBS, resuspended in 1 mL lysozyme buffer (lysozyme in PBS, 60 mg/mL) and incubated at 37°C for 3 h. Then 0.5 mL of lysis buffer (2% SDS, 0.01 M Tris, 0.01 M EDTA, pH 8.0) was added and incubated at 100°C for 2 min.

Electophoresis was carried out using Mini-PROTEAN 3 Electrophoresis Cell (BioRad) on 10% polyacrylamide gels, using the Laemmli buffer system. Gels were stained with Coomasie blue.

Detection of antibacterial activity – agar spot test. Gram-positive, catalase-negative rods were tested for inhibitory activity against randomly selected *Salmonella* Enteritidis clinical isolate in agar spot test.

The agar spot test was performed on MRS agar plates (diameter 90 mm) as described previously by Schillinger and Lucke (1989). Isolated lactobacilli were spot inoculated on MRS agar plates and incubated at 37°C for 24 h under anaerobic atmosphere. The plates were then overlaid with 7 mL of soft agar (0.75% agar) containing 10^7 cells of *Salmonella* Enteritidis per mL and incubated aerobically at 37°C for additional 24 h. Formation of clear zones of growth inhibition around *Lactobacillus* colonies and their diameters were recorded. Inhibition was scored as positive if a diameter of clear zone around the colony was 5 mm or larger. Only lactobacilli, which showed inhibitory activity against *Salmonella* Enteritidis in agar spot test were selected for further analysis.

Detection of antibacterial activity – agar well diffusion assay. The inhibitory activity of culture supernatants of selected *Lactobacillus* strains were tested against *Salmonella* Enteritidis, *E. coli* and *C. perfringens* by the agar well diffusion assay following the procedure of Schillinger and Lucke (1989).

Selected *Lactobacillus* strains were grown in MRS broth for 24 h at 37°C under anaerobic conditions. Cells were removed by centrifugation (4000 g for 30 min at 4°C), the pH of the supernatant was adjusted to 6.0 with 10 M NaOH and supernatant was filtered through 0.45 µm-pore-size membrane (Millipore). The culture supernatants were concentrated five times using rotary

evaporation according to Strompfová *et. al*. (2003) and Zhu *et. al*. (2000). Portions of 35 mL of Mueller-Hinton agar (BioMérieux) or TSN agar (BioMérieux) were autoclaved and cooled to about 48°C and then 100 µL of overnight cultures of eneropathogenic bacteria containing approximately 2×10^7 cells per mL were added. The inoculated medium was then poured into plates (diameter 90 mm) and wells 6 mm in diameter were cut. Aliquots (100 mL) of supernatants from different *Lactobacillus* isolates were dispensed into the wells and plates were incubated overnight at 37°C with appropriate atmosphere. The diameter of clear zones of growth inhibition around each well was measured, inhibition zones of 8 mm or more were scored as positive.

Results

Bacteriological examination of chicken faeces and intestinal content provided to obtained sixteen isolates of Gram-positive, catalase-negative rods, which phenotypically correspond to the genus of *Lactobacillus*.

According to the carbohydrate fermentation pattern analyzed with MiniApi system three strains were recognized as *L. salivarius* (9m, R1, 1a), three as *L. brevis* (E, 1/12, K1), two as *L. acidophilus* (3D, K2), one as *L. fermentum* (K13), one as *L. plantarum* (1/2s), four as *Leuconostoc lactis* (1D, 6d, K16, 2m) alternatively *L. salivarius*, one as *Lactococcus lactis* (Goł) alternatively *L. brevis* and one as *Lactococcus raffinolactis* (2/s) alternatively *L. plantarum* (Table I).

Table I
Identification of examined strains with API 50 CHL, ITS-PCR profile similarity and protein clusters

Strain	Identification with API 50 CHL		ITS-PCR profile similarity	Protein cluster
	Identification /Other possibility	Percentage of identification		
1D	*Leuconostoc lactis* / *L. salivarius*	99.5	*	A
3D	*Lactobacillus acidophilus*	99.9	*	A
6d	*Leuconostoc lactis* / *L. salivarius*	99.5	*	A
9m	*Lactobacillus salivarius*	98.9	*	A
R1	*Lactobacillus salivarius*	99.9	*	A
K13	*Lactobacillus fermentum*	97.3	*	A
K16	*Leuconostoc lactis* / *L. salivarius*	96.9	*	A
E	*Lactobacillus brevis 3*	98.8	Similar to *L. brevis* CIM 369	B
1/12	*Lactobacillus brevis 3*	98.5	Similar to *L. brevis* CIM 369	B
2m	*Leuconostoc lactis* / *L. salivarius*	99.5	*	B
″s	*Lactobacillus plantarum 1*	98.6	Similar to *L. plantarum* CIM 813	C
1a	*Lactobacillus salivarius*	98.9	*	D
2/s	*Lactococcus raffinolactis* / *L. plantarum 1*	82.3	**	D
Goł	*Lactococcus lactis* / *L. brevis 1*	84.2	**	D
K1	*Lactobacillus brevis 1*	49.7	unique	E
K2	*Lactobacillus acidophilus 1*	51.0	unique	F

* similarity between nine isolated strains (2m, 6d, 9m, R1, 1D, 1a, 3D and non scheduled in Fig. 1 K13 and K16) in ITS-PCR profile
** similarity between two isolated strains (2/s and non scheduled in Fig. 1, Goł) in ITS-PCR profile

The application of ITS-PCR allows to obtain bands from about 300 bp to 1500 bp (Fig. 1). Products below 300 bp were obtained from all isolates. Strain 1/2s showed very similar ITS-PCR pattern to *L. plantarum* CIM 813. Four additional bands, which have been observed for 1/2s were from 900 bp to above 1500 bp in size. Two isolates 1/12 and E produced similar ITS-PCR profiles to *L. brevis* CIM 369. Additional band, about 350 bp was seen only in *L. brevis* CIM 369. Other profiles did not correspond with strains from the Collection of Industrial Microorganisms, Institute of Agriculture and Food Biotechnology, used in this study. Nine strains (2m, 6d, 9m, R1, K13, K16, 1D, 1a, 3D and non scheduled in Fig. 1 K13 and K16) had very similar ITS-PCR profiles comprised of three or four bands and additional fourth band was detected only in two (1a, 3D) isolates. Two strains 2/s and non scheduled in Fig. 1 Goł produced the same ITS-PCR pattern and two others (K1, K2) showed a unique ITS-PCR patterns (data not shown).

Based on their cell wall protein profiles all examined strains were divided into six clusters. Representative cell wall protein profiles are shown in Fig. 2. Strains and protein profiles are presented in Table I. Each

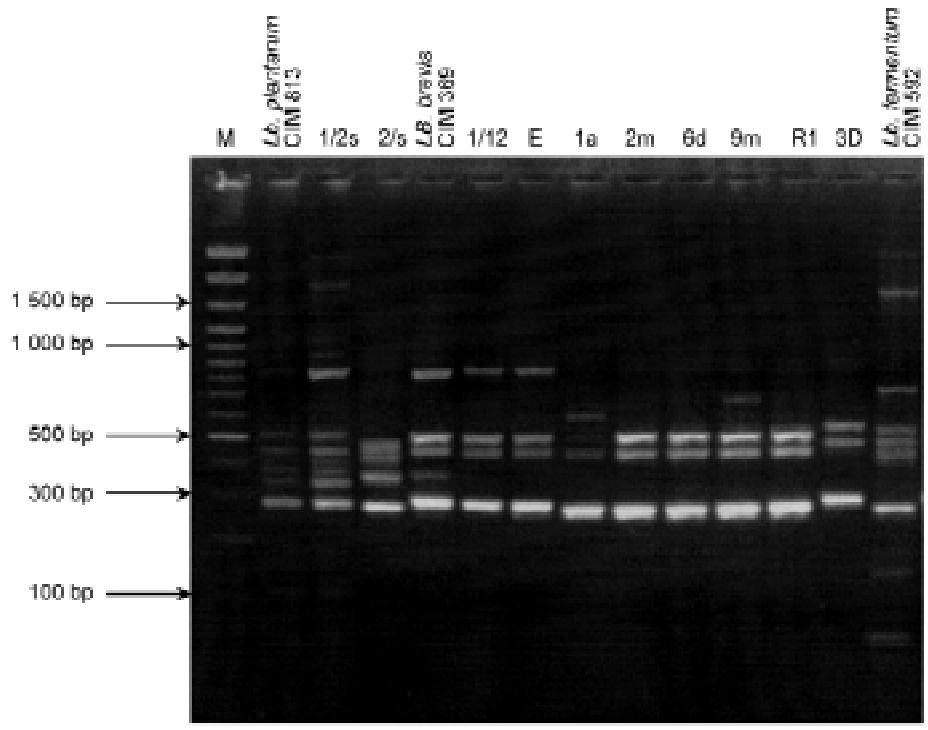

Fig. 1. Representative ITS-PCR products of isolated strains and reference *Lactobacillus* spp.

protein profile was represented by several bands, but some of them were cluster typical. Seven strains were included into cluster A (1D, 3D, 6d, 9m, R1, K13, K16) characterized by presence of distinct bands at approximately 25, 30, 37 and 48 kDa. Three strains were included into cluster B (E, 1/12, 2m) together with *L. brevis* CIM 369. *L. brevis* CIM 369 produced species specific bands at 38 kDa, which were identified also

Table II
Inhibitory activity of examined *Lactobacillus* spp. strains obtained in agar spot test against *Salmonella* Enteritidis

Strain	Diameter of growth inhibition zone
1D	6 mm
3D	15 mm
6d	10 mm
9m	5 mm
R1	10 mm
K13	6 mm
K16	10 mm
E	5 mm

Strain	Diameter of growth inhibition zone
1/12	6 mm
2m	10 mm
½ s	10 mm
1a	15 mm
2/s	10 mm
Goł	15 mm
K1	8 mm
K2	10 mm

Table III
Inhibitory activity of *Lactobacillus* spp. culture supernatants against selected intestinal pathogens

Tested strains	Number of tested strains	Concentrated culture supernatant of *Lactobacillus* strains									
		3D		1/2s		1a		R1		Goł	
		Inhibition zone in mm									
		8–10	>10	8–10	>10	8–10	>10	8–10	>10	8–10	>10
Clostridium perfringens	11	3	8	9	2	7	4	4[*1]	1	10	1
E. coli	10	1[*2]	5	6[*2]	0	6[*2]	0	6[*2]	0	4[*2]	2
Salmonella Enteritidis	10	1[*3]	4	1[*4]	0	3[*1]	1	1[*4]	0	1[*4]	0

[*1] – 6 strains non inhibited; [*2] – 4 strains non inhibited; [*3] – 5 strains non inhibited; [*4] – 9 strains non inhibited

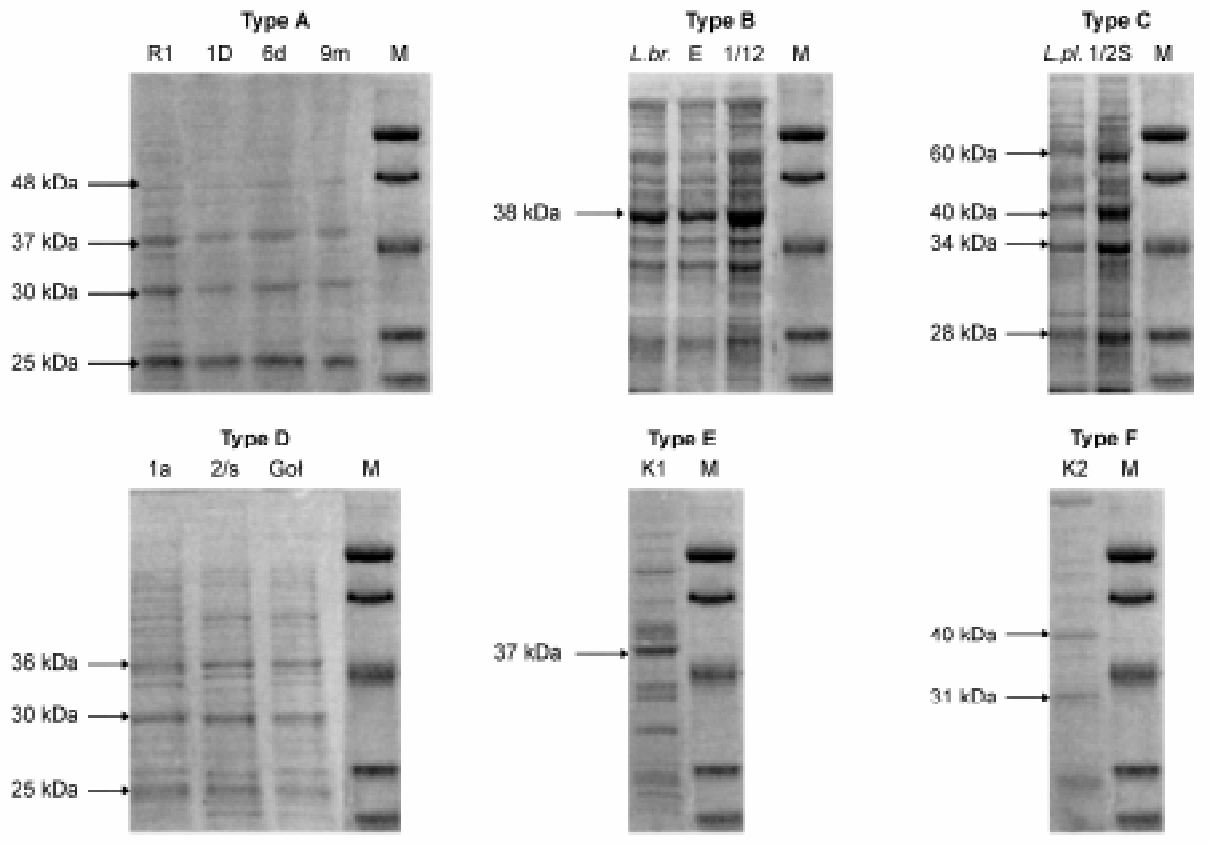

Fig. 2. Types of protein profiles for representative *Lactobacillus* strains
M – protein weight marker (77.0 kDa; 50.0 kDa; 34.3 kDa; 28.8 kDa; 20.7 kDa); L. br. – *L. brevis* CIM 369; L. pl. – *L. plantarum* CIM 813.

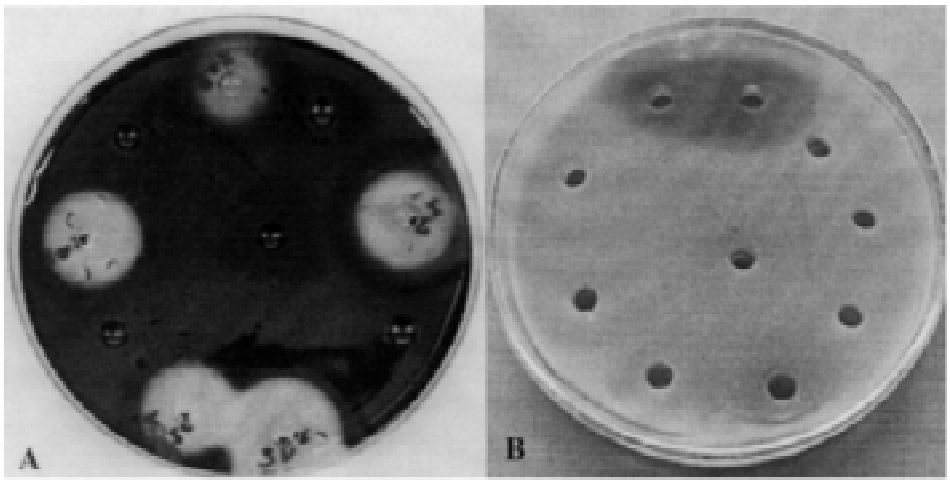

Fig. 3. Agar well diffusion assay: A – *C. perfringens*; B – *Salmonella* Enteritidis

in three isolates from cluster B. Only one strain was included into cluster C (1/2s), which revealed very similar protein profile as *L. plantarum* CIM 813. Cluster D was characterized by three distinct bands at 25, 30 and 36 kDa, contained three isolates (1a, 2/s, Goł). Two isolates (K1, K2) produced a unique protein profiles and were included in separate clusters: E and F.

Results obtained from agar spot test are presented in Table II and from agar well diffusion assay are presented in Table III. Examined supernatants demonstrated the strongest antimicrobial activity in agar well diffusion assay against *C. perfringens.* Lower activity were observed against *E. coli* and *Salmonella* Enteritidis. Culture supernatant from strain 3D showed the best inhibition properties and the diameter of zones of growth inhibition for two *C. perfringens* isolates were larger then 15 mm.

Discussion

Identification of sixteen isolated strains based on API 50 CHL system revealed that ten were *Lactobacillus* spp., four were *Leuconostoc* spp. and two were *Lactococcus* spp. Other properties such as cell morphology, protein profile and ITS-PCR pattern suggested that all isolates belong to the genus *Lactobacillus*. This finding confirmed that sugar fermentation profile should not be used for reliable identification of lactobacilli (Nigatu *et. al*., 2000; Reinheimer *et. al*., 1995). Percentage of identification (%id) obtained from MiniApi computer system was 96.9 or higher, with exception of two strains recognized as *Lactococcus* (2/s, Goł) with %id 82.3 and 84.2 respectively. Two other strains recognized as *L. brevis* and *L. acidophilus* (K1, K2) with %id respectively 49.7 and 51.0, were classified as separate clusters E and F, based on their protein profile. However, even satisfactory %id obtained for *Leuconostoc* might not be reliable because examined strains showed other properties common with genus *Lactobacillus*.

Analysis of cell wall proteins has been used to differentiate numerous strains of lactobacilli. In particular Drake *et. al*. (1996) reported that extraction of cell wall proteins followed by SDS-polyacrylamide gel electrophoresis (PAGE) has been proved to be a reliable and rapid method for strains characterization. SDS-PAGE of cell wall proteins and ITS-PCR were found to be useful as complementary methods for strain identification (Gatti *et. al*., 1997; Du Toit *et.al*., 2003; Henriksson *et. al*., 1995; Mukai *et. al.* 2003).

Protein cluster A contained seven isolates recognized with API 50 CH system as *L. salivarius* (2), *L. fermentum* (1), *L. acidophilus* (1), and also as *Leuconostoc lactis* (3). What is interesting, three isolates identified as *Leuconostoc lactis*, each with different carbohydrate fermentation pattern, were alternatively recognized as *L. salivarius*. Two other strains from cluster A identified phenotypically as *L. fermentum* (K13) and *L. acidophilus* (3D) produced protein profile characteristic for cluster A, therefore their phenotypical identification was not reliable.

Two isolates from cluster B (E, 1/12) produced identical protein profile as *L. brevis* CIM 369, thus confirming their identification. Another strain 2m from cluster B recognized as *Leuconostoc lactis* might also belonging to *L. brevis*, because it produced protein bands specific for this species. In one strain from cluster C (1/2s) phenotypical identification as *L. plantarum* was confirmed by protein profile. Cluster D contained three isolates (1a, 2/s, Goł) recognized as *L. salivarius*, *Lactococcus raffinolactis* and *Lactococcus lactis.* Results of phenotypical identification seems to be not reliable, because for *Lactococcus* %id was not satisfactory, as was mentioned above.

Results confirmed that identification of *Lactobacillus* spp. is tedious. Particularly *L. acidophilus* is difficult to identify, since *L. acidophilus* group representing six species was described (Johnson *et. al*., 1980). Gatti *et. al*. (1997) described different proteins specific for this genus (20, 31 and 55 kDa).

Results obtained in this study proved the antimicrobial activity of the culture supernatants from isolated *Lactobacillus* strains. The activity might be due to antimicrobial substances, probably proteinaceous molecules, which are produced into the culture broth. Further studies focused on the nature of those antimicrobial substances produced by isolated *Lactobacillus* strains are in progress and will render more information about their characteristic.

Bacteriocins produced by *Lactobacillus* spp. are mainly antagonistic against other members within this genus and against other Gram-positive bacteria (Sablon *et. al*., 2000). Previous studies on bacteriocin producing lactobacilli were conducted predominantly using another *Lactobacillus* spp. as an indicator strains (Callewaert *et al*., 1999; Cuozzo *et al*., 2000). The aim of our study was to isolate lactobacilli strongly antagonistic against enteropathogenic poultry microorganisms including *C. perfringens* and Gram negative bacteria *i.e.*, *Salmonella* Enteritidis and *E. coli*. Those pathogens are frequently associated with poultry diseases and is of great significance in human health protection. Our objective was to isolate *Lactobacillus* spp. with desirable *in vitro* properties, which can be further analyzed and eventually used for developing a new probiotic for poultry.

Strain 3D revealed inhibitory activity against *C. perfringens* and some *E. coli* and *Salmonella* Enteritidis and will be used in future *in vivo* studies in chickens. Demonstrated antibacterial activity may enable to use them in prevention or even therapy of necrotic enteritis caused by *C. perfringens*. Specific antagonistic activity of *L. johnsonii* FI9785 against *C. perfringens* in poultry was previously reported by La Ragione *et al*. (2004). They showed that single dose of *L. johnsonii* FI9785 suppressed *in vivo* colonization and persistance of *C. perfringens*, reduced *E. coli* colonization, but there were no significant effects against *Salmonella* Enteritidis colonization.

The use of antibiotics in animal production should be seriously considered due to increased bacterial resistance and antibiotic residues in animal products. Particular attention was given to the subject when use

of certain antibiotics was banned by the Council of the European Commission (1998). As a result, alternative methods such as probiotics have been widely investigated. Presented results indicate that potentially probiotic lactobacilli may be isolated from natural gut microflora of poultry.

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