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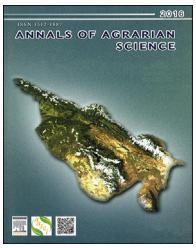

Antimicrobial susceptibility and antibiotic resistance profiles of cultivable lactic acid bacteria from intestinal tract of domestic chickens collected in Adjara

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ABSTRACT

Elaboration and introduction of safe, effective probiotic preparations as alternatives to antibiotics are being actively conducted throughout the world. 66 LAB isolates were isolated from ileal, cecal and rectal samples collected from domestic chickens collected in different districts of Adjara, Georgia. Their resistance to 17 antibiotics and antibacterial activity were studied using the agar diffusion method. Among the isolates, widespread resistance was found to metronidazole and nystatin, sensitivity – to ampicillin, tylosin, rifampicin and bacitracin. Most of isolates have intermediate susceptibility to the majority of the antibiotics. 3 LAB isolates were selected by antibacterial action against the several bacterial indicator strains that makes them effective remedy to control antibiotic-independent pathogen through competitive exclusion and promotion of good protective microbiota and perspective probiotic additives for chicken food. Future investigations, proving the safety of the strains and their antimicrobial compounds will enable to apply *in vivo* probiotic properties on poultry production.

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Introduction

Modern intensive industry of poultry farming often applies antibiotics and other chemical preparations for diseases prevention, maintenance and enhancement of productivity in poultry. These compounds having pernicious influence on not only pathogenic organisms, but also on normal microflora, significantly destroy microbial balance in intestines of young poultry and often cause disbacteriosis and reduce immunobiological characteristics of host organism. Frequent and nonsystematic application of antibiotics promotes formation of microbial forms resistant to these preparations and makes non-effective treatment and preventive measures.

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Table 1 – CFU of bacteria of chicken intestinal tract different segments on MRS agar, M17 agar and bifidobacteria selective agar.

Sample#	District, village	Segment of Intestine	Log10 CFU/g on different nutrient media		
			MRS agar	M17 agar	Bifidobacteria selective agar
1–1	Khulo, Ghorjomi	Ileum	7.39	6.88	7.21
1–2		Cecum	10.65	10.74	11.2
1–3		Rectum	8.44	9.6	8.14
2–1	Khelvachauri, Akhalsopheli	Ileum	3.56	3.55	4.1
2–2		Cecum	10.55	10.04	10.62
2–3		Rectum	3.73	3.66	3.96
3–1	Keda, Zendidi	Ileum	4.04	3.91	4.19
3–2		Cecum	6.88	6.72	6.9
3–3		Rectum	7.25	5.88	6.19
4–1	Shuakhevi, Dabadzveli	Ileum	3.64	3.78	3.7
4–2		Cecum	7.97	7.26	7.83
4–3		Rectum	5.61	5.87	5.98

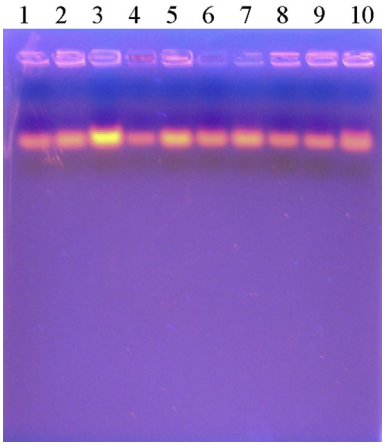

Fig. 1 – Electrophoregram of PCR-products (primers: 27F-1492R)) of certain LAB strains DNA isolated from intestinal samples of chicken: 1 – LC 2; 2 – LC LC 10; 3 – LC 18; 4 – LC 21; 5 – LC 26; 6 – LC 33; 7 – LC 37; 8 – LC 40; №9 – LC 49; 10 – LC 50.

Consequently, elaboration and introduction of safe, effective probiotic preparations as alternatives to antibiotics are being actively conducted throughout the world. Being created antimicrobial compounds, energy-dependent fat acids and chemically modified bile acids, bacteria of intestines form local ambient unfavorable for development of pathogenic microorganisms [1]. Probiotics have received increasing attention as an alternative to in-feed antibiotics and for improving productivity in the poultry industry [2].

Despite the efforts of veterinary service on conducting massive vaccination of animals and poultry against intestine infections, improvement of schemes form application of known and searching of novel antibiotics, morbidity and murrain of youngsters caused by disease of gastro-intestinal tract, remain high [3].

The aim of this work was to study the antimicrobial susceptibility and antibiotic resistance profiles of lactic acid bacteria, isolated from intestines of chicken from different districts of Adjara (Georgia).

Table 2 – Antibiotic susceptibility for LAB isolates from chicken intestinal samples.

Working# of LAB isolate	Gentamycin, 10 μg (CN 10)	Kanamycin, 30 μg (K 30)	Neomycin, 30 μg (N 30)	Streptomycin, 10 μg (S 10)	Penicillin G, 1 IU (P 1)	Ampicillin, 10 μg (AMP 10)	Oxacillin, 1 μg (OX 1)	Tetracycline, 30 μg (TE 30)
LC2	10	10	12	0	20	32	15	30
LC3	0	0	0	0	0	22	0	40
LC4	22	22	18	14	14	26	30	40
LC10	9	9	10	0	10	23	0	31
LC18	ND	10	ND	12	ND	28	ND	ND
LC21	28	30	24	18	30	ND	30	32
LC26	10	11	12	0	0	20	0	32
LC33	0	0	18[b]	14[b]	14	22	11[a]	10
LC37	10	0	0	10	14	24	9	34
LC40	0	0	0	9	0	20	0	28
LC49	18	14	17	12	18	24	0	0
LC50	10	0	10	9	24	25	0	0
LC52	22	24	20	18	28	32	26	34

Note:ND – no data.
a 1–2 mm around of antibiotic disc slight stimulation of growth.
b Incomplete growth inhibition.

Objectives and methods

Four domestic chickens have been selected in villages of 4 different districts of Adjara, Georgia. Chickens were killed by cervical dislocation and the ileum, cecum and rectum were removed. Each segment of intestines with content were placed into a sterile flasks and added sterile 0.9% NaCl solution at a ratio 1:9; Homogenization have been carried out at a rotary shaker for 1 h at room temperature. Then the samples were subjected to serial dilution up to 10^8 using physiological solution [4]. For isolation of lactic acid bacteria (LAB), 0.1 ml of each dilution was plated on three different nutrient media: MRS agar (supplemented with 0.5 g/l L-cysteine and 1 ml/l Tween-80), M17 and bifidobacteria selective agar. Incubation was conducted anaerobically at 37 °C for 48 h. The colony-forming unit (CFU) per gram of sample was expressed as logarithm at the base of 10. The enumeration of LAB was conducted in triplicates.

For obtaining of pure cultures 3-fold plating on the same media and same conditions were used. Pure colony of isolates, which were Gram-positive rods and coccus, were then transferred to MRS broth and incubated at 37 °C for 24 h. The pure LAB culture was kept in MRS broth supplemented with 20% (v/v) glycerol and stored at −20 °C until further analysis.

For extraction of DNA from the certain isolates, overnight culture in MRS broth was centrifuged and collected. Extraction of DNA was performed using Bio-Rad Instagene Matrix, according to the manufacturer's instructions. PCR reactions of bacteria were performed using Promega Gotaq Green Master Mix and primers: Forward primer – 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and Reverse primer – 1492R (5′-GGTTACCTTGTTACGACTT-3′). Successful amplification was confirmed via electrophoresis in 1% agarose gel.

LAB susceptibility to different antibiotics was determined using the agar diffusion method. MRS agar was used as a basal medium for bacterial growth. LAB isolates were adjusted absorbance to 0.08–0.1 at 625 nm and spread on the surface of MRS agar by three way swabs [5]. Antimicrobial resistance were tested to 17 antibiotics: gentamicin, 10 μg (CN 10), tetracycline, 30 μg (TE 30), penicillin G, 1 IU (P 1), metronidazole, 5 μg (MTZ 5), erythromycin, 15 μg (E 15), oxytetracycline, 30 μg (OT 30), oxacillin, 1 μg (OX 1), neomycin, 30 μg (N 30), tylosin, 30 μg (TY 30), norfloxacin, 10 μg (NOR 10), bacitracin, 10 IU (BA 10), rifampicin, 5 μg (RD 5), ampicillin, 10 μg (AMP 10), kanamycin, 30 μg (K 30), ciprofloxacin, 5 μg (CIP 5), streptomycin, 10 μg (S 10), nystatin, 100 IU (NY 100). The diameter of inhibitory zones was measured after 18 h of incubation at 37 °C under anaerobic condition.

Inhibitory activity of LAB isolates against bacterial indicator strains (*Campylobacter jejuni* ATCC 33291, *Staphylococcus aureus* ATCC 25923, *Shigella flexneri* ATCC 29903, *E. Coli* ATCC 8739, *Enterococcus faecalis* ATCC 29212, *Bacillus cereus* ATCC 10876, *Salmonella enterica* DSH 50912, *Salmonella typhimurium*, *Listeria monocytogenes*) was studied by agar diffusion method (the so-called method of agar blocks) [6] after 18 h of incubation at 37 °C under anaerobic condition and by determination of inhibition zone diameter. The incubation medium for LAB was MRS agar, for test-organisms – Mueller-Hinton agar.

The antibiotic and antimicrobial assay was conducted in triplicates.

Results and discussion

It has been proved that the composition of chicken microbiota together with many other factors responds to feed [7–9] and antibiotic treatment [10,11] so selection criteria of chicken were as followed: 1. Batched and bred in family farm; 2. No antibiotic treatment; 3. Fed with ecologically pure food. The selected chickens were clinically healthy according to their bloods hematological, biochemical characters, and internal organs (heart, liver, kidneys, lungs, gastrointestinal tract) and tissues macro-morphologically observation (unpublished data). It is also proved by inoculation of intestinal samples on selective media of different microorganisms (Bard Parker agar,

Oxytetracycline, 30 μg (OT 30)	Metronidazole, 5 μg (MTZ 5)	Erythromycin, 15 μg (E 15)	Tylosin, 30 μg (TY 30)	Norfloxacin, 10 μg (NOR 10)	Ciprofloxacin, 5 μg (CIP 5)	Rifampicin, 5 μg (RD 5)	Bacitracin, 10 IU (BA 10)	Nystatin, 100 IU (NY 100)
32	0	14	26	0	12	13	24	0
31	0	22	30	11	20	24	20	0
28	0	10	30	0	20	30	40	0
30	0	28	26	20	22	16	22	0
ND	0	ND	36	ND	0	34	40	0
34	0	0	32	ND	ND	30	28	0
34	0	11	24	9	11	11	18	0
10	0	18	16	0	0	30	32	0
30	0	34	34	0	0	30	33	0
36	0	20	26	10	20	16	18	0
0	0	14	12	0	0	32	36	0
10	0	36	36	0	0	32	32	0
36	0	0	28	24	29	26	29	0

Campylobacter agar base blood free, Hektoen enteric agar, CLED agar, SS agar, WLN, Czapek-Dox modified agar, PCA).

Bacteria were grown on selective media for different pathogenic and opportunistic microorganisms but none of them was characterized by features characteristic for causative organisms (*S. aureus*, *Salmonella enterica*, *C. jejuni*, *Shigella*, *Proteus*). Only single cases of growth on Czapek-Dox agar was observed for micromycetes and on WLN – for yeasts.

Study of microbial composition in different segments of chicken intestines reveals qualitative similarities but quantitative differences. In cecum, CFU was richer by several factors compared to those in ileum and rectum. In addition, CFU of bacteria on MRS agar, M17 agar and bifidobacteria selective agar was similar in different chickens (Table 1). It may be explained by importance of avian ceca in digestion, especially for chicken. It is a multi-purpose organ vital to the bird's physiology; a complex system inhabited by a very dense microbial community that converts the cecal pouches into fermentation powerhouses. Members of the cecal microbiota have the ability to digest cellulose, starch and other stable polysaccharides [12,13].

The 66 certain colony of different morphology and consistence were isolated from MRS agar, M17 agar and bifidobacteria selective agar and selected based on their cell morphology and gram staining. Gram-positive rods and coccus were identified by genus specific PCR. Bacterial isolates have been renovated on MRS agar and DNA and their PCR-products obtained from their overnight liquid culture. Electrophoresis of PCR products of some LAB isolates DNA are shown at Fig. 1.

As seen from the obtained results, certain pure cultures give positive response on PCR lactobacteria primer couples that proves that the mentioned bacteria belong to lactic acid bacteria.

Selected strains were assayed for their susceptibility to 17 antibiotics (Table 2).

Table 2 shows the inhibitory zone of antibiotic susceptibility for 13 LAB isolates obtained from intestinal samples.

Table 3 – Inhibitory activity of selected LAB isolates against bacterial indicator strains.

Test-culture	LAB isolate					
	LC2	LC 10	LC 26	LC 33	LC 37	LC 40
Campylobacter jejuni ATCC 33291	14	14	14	16*	11	18*
Staphylococcus aureus ATCC 25923	14	13	15	0	0	14*
Shigella flexneri ATCC 29903	20	20	20	14	14	22
E. Coli ATCC 8739	15	14	15	13	0	17
Enterococcus faecalis ATCC 29212	14*	13*	18	0	0	0
Bacillus cereus ATCC 10876	14*	12*	12*	11	11	12*
Salmonella enterica DSH 50912	15	14	13	0	0	19*
Salmonella typhimurium	13	13*	12*	0	0	17*
Listeria monocytogenes	17	17	21	12*	0	16*

Note: * – incomplete growth inhibition.

Antibiogram provides qualitative results by categorizing bacteria as susceptible, intermediate or resistant [14]. All tested isolates were resistant to metronidazole and nystatin and sensitive to ampicillin, tylosin, rifampicin and bacitracin. Susceptibility to other tested antibiotics was variable and depending on the strain. Most of isolates have intermediate sensitivity to the majority antibiotics. Ampicillin, tylosin, rifampicin, bacitracin, tetracycline and oxytetracycline had significant higher inhibitory zone than other tested antibiotics. Exceptions are only some isolates that have resistance to tetracycline and oxytetracycline. The four LAB isolates (LC2, LC4, LC10, LC52) had similar antibiotic susceptibility profiles: they are resistant to 3–4 antibiotics among them 2 are the same. Other LAB isolates have resistance more then to 5 antibiotics.

According to European Commission, strains carrying the acquired resistance due to acquisition of exogenous resistance genes are unacceptable for use as animal feed additives [15]. However, this study was conducted with the purpose of verifying their ability to survive if they are taken simultaneously with an antibiotic therapy.

For further research were taken 6 LAB isolates, which demonstrate high growth intensity and was studied antimicrobial activity towards 9 test organisms. Results are given in Table 3.

As it seen from the table, three LAB isolates have antibacterial sensitivity to all tested nine test-organism. However, inhibition towards *E. faecalis*, *B. cereus* and *S. Typhimurium* were not complete. The highest inhibition zones were observed in LC2, LC10, LC26, to *Sh. flexneri*, in LC33 – to *C. jejuni*, *and* LC40 – also to *Shigella flexneri*.

Conclusion

Thus, selected LAB isolates have antibacterial action against several bacterial indicator strains, which makes them effective remedy to control antibiotic-independent pathogen through competitive exclusion and promotion of good protective microbiota and perspective probiotic additives for chicken food. Future investigations, proving the safety of the strains and their antimicrobial compounds, will enable to apply *in vivo* probiotic properties on poultry production.

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