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Original Article

Lactobacillus spp. Strains Isolation, Identification, Preservation and Quantitative Determinations from Gut Content of 45-Day-Old Chickens Broilers

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■Keywords

Chickens broilers, gut, Lactobacillus spp., phenotypic identification, preservation.

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ABSTRACT

The objective of this study was to isolate, identify, preserve and determine the quantitative level of the *Lactobacillus* strains from the gut content of 45-day-old chickens broilers; to test the viability of these strains preserved at 4 °C and room temperature (20 ± 2 °C). *Lactobacillus* strains were isolated, phenotypically identified and preserved from the gut content of 17 chickens broilers. Identification was performed by morphological, cultural and biochemical characters examination, using apiwebTM and ABIS online software. The quantitative level of *Lactobacillus* strains in intestinal content (10^5 – 10^9 CFU/g) and the viability of strains preserved at 4 °C and at room temperature (from 8 days to 9 months) was also determined. Twenty-three strains of *L. acidophilus*, *L. brevis*, *L. plantarum*, *L. fermentum* and *L. salivarius* from the gut content of chickens broilers were isolated, phenotypically identified, and preserved. Of these, *L. plantarum*, *L. fermentum* and *L. acidophilus* biotype 1 strains were technologically and ecologically suitable to continue the testing of probiotic traits.

INTRODUCTION

Recent research on the structure of the normal intestinal microbiota of chickens revealed the presence of *Lactobacillus* spp. (Lu *et al.*, 2003; Wei *et al.*, 2013; Waite & Tailor, 2014; Duar *et al.*, 2017), known for its beneficial effects on the host's health. Lactobacilli have a symbiotic role in host fitness, their metabolites contributing to the digestive process and counteracting pathogens (Duar *et al.*, 2017). Zou *et al.* (2018) showed that *Lactobacillus* induce a polarizing effect on the chicken cecal microbiome, suggesting a major influential role of this genus in local microbiome, with negative (with *Ruminococcaceae*, *Lachnospiraceae*) or positive (with other lactobacilli, *Bacteroides*, *Clostridiales* and *Christensenellaceae*) correlations. At hatching, the poultry microbiota from cecum consists, predominantly, as *Enterococcus*, coliforms and clostridia (Coates & Fuller, 1977) but, from the 4th day of age, *Lactobacillus* becomes a significant component of the intestinal microbiota (Zhu *et al.*, 2002). At the 7th day of age, the ileal mucosal microbiota is dominated by *Lactobacillus*, followed by *Lachnospiraceae* and *Enterococcus* (Cressman *et al.*, 2010). After the 14th day of age, cecum and small intestine of broilers chicks develop various communities (Pedroso & Lee, 2015) but, from day 21 to 42 of age, *Lactobacillus* became the most abundant organism in the small intestine. Of this genus, *L. salivarius*, *L. johnsoni*, *L. reuteri*, *L. oris* and *L. crispatus* were detected (Nakphaichit *et al.*, 2011). This diversity raises the issue of selecting the best strain for developing bacterial-based feed additives in poultry nutrition.

The objective of our work was to isolate, identify, preserve and assess the quantitative level of the *Lactobacillus* strains from the gut content

of 45-day-old chicken broilers, in order to further test their probiotic traits and to select the best strains as intestinal flora stabilizers in chicken nutrition.

MATERIALS AND METHODS

Birds were treated in accordance with Romanian legislation (law no. 305/2006) for handling and protection of animals used for experimental purposes. The birds' care and use protocol were approved by the Animal Care and Use Committee at the National Research-Development Institute for Biology and Animal Nutrition (INCDBNA-IBNA) Balotești, Romania, following the principles of EU Directive 2010/63/EU and Romanian Law on Animal Protection.

Lactic acid bacteria isolation and determination of total bacterial count

The Mountzouris method (2007) completed by Sorescu *et al.*, (2019) was applied. Sample preparation: 1 g intestinal content (ileum and cecum, respectively) *per capita* from seventeen chicks (Cobb 500, 45-day-old) was homogenized with 7 ml Oxoid BHI (Brain Heart Infusion) broth and 2 ml glycerol, and immediately frozen at – 20 °C until testing (no more than three months). After defrost, decimal dilutions from every sample were inoculated on Oxoid Man, Rogosa, Sharpe (MRS) agar. Further instead, the procedure presented in other paper (Sorescu *et al.*, 2019) for the isolation and counting of *Lactobacillus* CFU has been applied.

Identification of bacterial strains

Phenotypic identification of isolated bacterial strains was performed by morphological, cultural and biochemical characters examination, according to Bergey's Manual of Systematic Bacteriology (Garrity *et al.*, 2009), ABIS on line software (Stoica & Sorescu, 2018) and apiweb™ API50CHL software BioMerieux (France), following the protocol described in (Sorescu *et al.,* 2019). The results obtained by Pelinescu *et al.* (2009) were also considered.

Preservation of bacterial strains

The medium-term preservation (months) was done by culture in MRS broth, the viability of bacterial strains being evaluated after 45 days, 3, 7 and 9 months.

Long-time preservation (years) was done at – 80 °C, with addition of glycerol 20%, and bacteria viability is to be assessed every 2 years.

RESULTS AND DISCUSSION

The taxonomic classification of bacterial strains in *Lactobacillus* spp. was performed through morphologically (Gram positive, non-spore forming rods), culturally (anaerobic growth) and biochemically characters (negative catalase test). Identification of the *Lactobacillus* spp. was performed based on their biochemical characters. Thus, twenty-three strains of the genus *Lactobacillus* (*L. acidophilus* biotype 1 IBNA 64, *L. acidophilus* biotype 3 IBNA 49, 51, 53, 55, 63, 65, *L. plantarum* biotype 1 IBNA 45, 46, 48, 61, *L. salivarius IBNA* 47, 52, 54, 59, 60, 62, 67, 68, *L. brevis* biotype 2 IBNA 50, and *L. fermentum* biotype 1 IBNA 56, 57, 69) were isolated, identified and preserved from the intestinal content (ileum and cecum), from seventeen 45 d-old chickens.

The morphological, cultural and biochemical characteristics of the identified strains are presented in Table 1.

Figures 1-4 show smears from *L. salivarius* IBNA 67, *L. plantarum* IBNA 46, *L. acidophilus* biotype 3 IBNA 65 and *L. fermentum* biotype 1 IBNA 57 cultures in/on MRS broth/agar (Gram staining, × 1000).

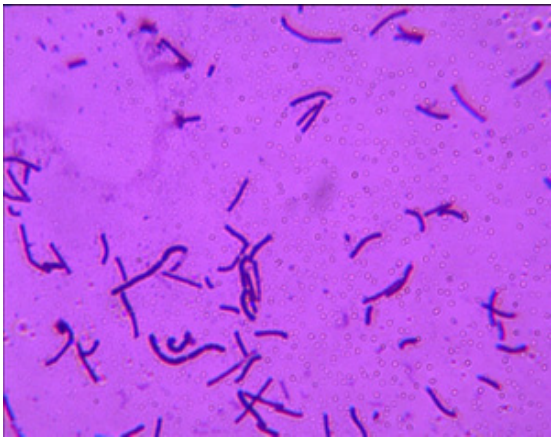

Figure 1 – *L. salivarius* IBNA 67 culture in MRS broth (Gram staining × 1000).

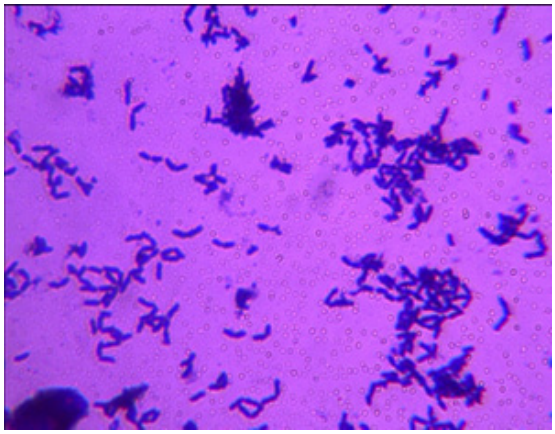

Figure 2 – *L. plantarum* IBNA 47 culture on MRS agar (Gram staining × 1000).

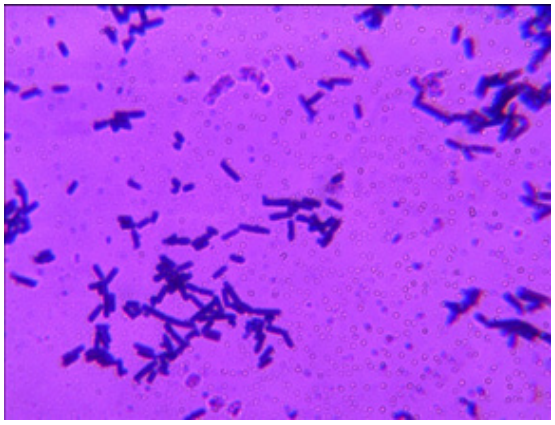

Figure 3 – *L. acidophilus* biotype 3 IBNA 65 culture in MRS broth (Gram staining × 1000).

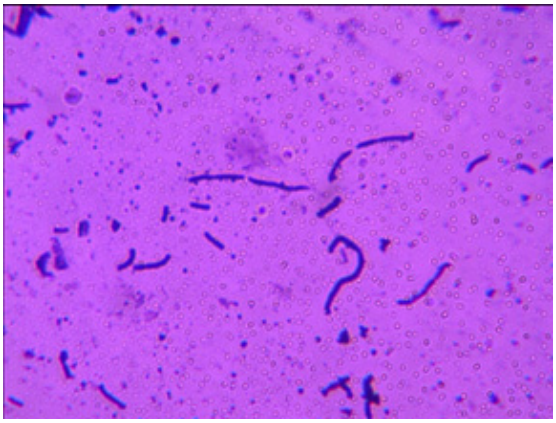

Figure 4 – *L. fermentum* biotype 1 IBNA 57 culture in MRS broth (Gram staining × 1000).

Table 1 – Morphological, cultural and biochemical characteristics of the *Lactobacillus* strains isolated from intestinal contents of broiler chickens at 45 days.

Tests	1	2	3	4	5	6
Morphological characters	a	a	b	a	a	b, c
Cultural characters	x	x	x, y, z	y	x	y
Catalase test	0(1)*	0(6)	0(4)	0(1)	0(3)	0(8)
Fermentation (API50CHL)						
glycerol	0(1)	0(6)	1(4)	0(1)	0(3)	0(8)
L-arabinose	0(1)	0(6)	3(4)	1(1)	0(3)	0(8)
D-ribose	0(1)	0(6)	4(4)	1(1)	0(3)	0(8)
D-xylose	0(1)	0(6)	0(4)	1(1)	0(3)	0(8)
D-galactose	1(1)	5(6)	4(4)	1(1)	3(3)	8(8)
D-mannose	1(1)	3(6)	4(4)	0(1)	2(3)	8(8)
L-rhamnose	0(1)	0(6)	0(4)	0(1)	0(3)	0(8)
inositol	0(1)	0(6)	0(4)	0(1)	0(3)	7?(8)
D-mannitol	1(1)	0(6)	4(4)	0(1)	?(3)	8(8)
D-sorbitol	0(1)	0(6)	2(4)	0(1)	0(3)	0(8)
Methyl-αD-mannopyr.	0(1)	0(6)	1(4)	0(1)	0(3)	0(8)
N-acetylglucosamine	1(1)	6(6)	4(4)	0(1)	0(3)	8(8)
amygdalin	1(1)	0(6)	3(4)	0(1)	0(3)	0(8)
arbutin	1(1)	3(6)	4(4)	0(1)	0(3)	0(8)
esculin	1(1)	6(6)	4(4)	0(1)	0(3)	0(8)
salicin	1(1)	3(6)	4(4)	0(1)	0(3)	0(8)
D-cellobiose	1(1)	1(6)	4(4)	0(1)	0(3)	0(8)
D-maltose	1(1)	3(6)	4(4)	1(1)	3(3)	8(8)
D-lactose	1(1)	0(6)	4(4)	1(1)	3(3)	8(8)
D-melibiose	1(1)	?(6)	4(4)	1(1)	3(3)	8(8)
D-trehalose	1(1)	0(6)	4(4)	0(1)	0(3)	8(8)
D-melezitose	0(1)	0(6)	1(4)	0(1)	0(3)	0(8)
amidon (starch)	1(1)	2?(6)	1(4)	0(1)	0(3)	0(8)
gentibiose	1(1)	2?(6)	4(4)	0(1)	0(3)	0(8)
D-tagatose	0(1)	0(6)	1(4)	0(1)	0(3)	0(8)
D-arabitol	0(1)	0(6)	0(4)	1(1)	0(3)	0(8)
potassium gluconate	0(1)	0(6)	3(4)	1(1)	0(3)	0(8)

1 = L. acidophilus biotype 1 IBNA 64; 2 = L. acidophilus biotype 3 IBNA 49, 51, 53, 55, 63, 65; 3 = L. plantarum biotype 1 IBNA 45, 46, 48, 61; 4 = L. brevis biotype 2 IBNA 50; 5 = L. fermentum biotype 1 IBNA 56, 57, 69; 6 = L. salivarius IBNA 47, 52, 54, 59, 60, 62, 67, 68.

a = Gram positive, non-spore forming rods, grouped in pairs, chains, filaments and, rare, in palisade; b = Gram positive short rods, with rounded end, non-spore forming, arranged in pairs, short chains or irregular clumps; c = Gram positive short and thick rods or coccoid cells, non-spore forming, arranged in short chains and irregular clumps.

x = small colonies, 0.5-1.5 mm in diameter, rarely larger, up to 2.0 mm, smooth type, round, opaque, semi-transparent or transparent, and whitish, grey or colourless on MRS agar; y = large colonies, 2.0-4.0 mm in diameter, rarely smaller, smooth type, round, opaque, white or whitish; z = small colonies, 1.0-1.5 mm in diameter, rough type, transparent, flattened, round, colourless.

* = number of positive strains from number of tested strains; ? = dubious, weekly positive.

All strains were negative for the fermentation of erythritol, D-arabinose, L-xylose, D-adonitol, methyl-βD-xylopyranoside, L-sorbose, dulcitol, methyl-αD-glucopyranoside, inulin, glycogen, xylitol, D-turanose, D-lyxose, D-fucose, L-fucose, L-arabitol, potassium 2-ketoglucunonate and potassium 5-ketoglucunonate. All strains were positive for the fermentation of D-glucose, D-fructose, D-saccharose and D-raffinose.

Table 2 presents the origin (ileum or cecum content) and the quantitative level of the isolates presence in ecological niche.

Table 3 presents the results of strains identification by apiwebTM soft, API50CHL V.5.1, BioMerieux (France), and ABIS online software.

Details on the meaning and mode of calculation of %SIM for ABIS and API %ID were presented in a previous article (Sorescu *et al.,* 2019).

Table 4 presents the results of viability test for *Lactobacillus* strains which are preserved at 4 °C and at room temperature (20 ± 2 °C).

Table 2 – The origin and the level of *Lactobacillus* spp. strains presence in the ecological niche (45-day-old chickens' broiler intestinal content).

Strains	Origin, sample number	CFU/g intestinal content (log10)
L. acidophilus biotype 1 IBNA		
64	ileum content, 91	8.301
49	cecum content, 63	8.698
51	ileum content, 68	7.301
53	cecum content, 72	8.662
55, 63, 65	ileum content, 76, 90, 91	7.602; 8.176;7.698
L. plantarum biotype 1 IBNA		
45	cecum content, 59 (large colonies)	8.518
46	59 (small colonies)	9.431
48, 61	ileum content 63, 88	6.301; 7.301
L. brevis biotype 2 IBNA 50	ileum content, 66.	5.477
L. fermentum biotype 1 IBNA		
56	cecum content, 78	9.079
57	ileum content, 81	7.954
69	cecum content 97	9.672
L. salivarius IBNA		
47, 52	ileum content, 63, 72	7.531; 6
54	cecum content, 73	8
59	ileum content, 82	7.176
60	cecum content, 87	8
62, 67, 68	ileum content, 90, 93, 95	8.301; 7.113; 8.397

L. salivarius and *L. acidophilus* are included in the vertebrate adapted lifestyle lactobacilli group and were isolated from human, pigs, hamsters, horses, chickens (Duar *et al.,* 2017) and turkeys (Sorescu *et al.*, 2019). *L. fermentum* and *L. plantarum* belongs to the nomadic species group of lactobacilli (Duar *et al.*, 2017). *L. fermentum* was isolated from fermenting plant material, sewage, milk products, mouth and faeces of humans, and intestines of pig, rat, cattle, mouse and birds (Garrity *et al.,* 2009), including turkeys and 26-day-old chicks (Sorescu *et al.*, 2019). *L. plantarum* was isolated from fruit flies, vertebrate digestive tract, plants, dairy products, environments, silage (Duar *et al.*, 2017). *L. brevis* has a free-living lifestyle (Duar *et al.*, 2017) and was isolated from silage, beer, milk, cheese, sauerkraut, sourdough, cow manure, mouth, feces and intestinal tract of human, cattle, rats, pigs and birds (Garrity *et al.,* 2009), including 26-day-old chicks (Sorescu** *et al.,* 2020).

The strains described in this paper, isolated from the intestinal content, can be important for developing probiotic compounds for the same bird species because they are host-adapted and have a high ecological fitness. Moreover, this higher fitness is relevant in the process of outcompeting the pathogens.

Differentiation of Lactobacillus strains was performed as described before by Sorescu *et al.* (2019) (on turkeys) and Sorescu** *et al.* (2020) (on 26-day-old chickens), mainly on the basis of some morphological characters (aspect of bacilli and grouping of them), some cultural characters (colony size, smooth or rough type, colour and degree of transparency/opacity) and especially, biochemical characters (fermentation of glycerol, L-arabinose, D-ribose, D-xylose, D-galactose, D-mannose, L-rhamnose, inositol, D-mannitol, D-sorbitol, Methyl-αD-mannopyranoside, N-acetylglucosamine, amygdalin, arbutin, esculin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-trehalose, D-melezitose, starch, gentibiose, D-tagatose, D-arabitol and potassium gluconate). It can be noticed that Lactobacillus strains isolated from chickens aged 45d, generally fermented more carbohydrates (31) than those from 26-day-old chickens (21) and turkeys (15), which may interfere

Table 3 – The results of parallel identification of strains by apiweb™ soft, API50CHL V.5.1, BioMerieux (France) and ABIS online software.

Strains		API, %ID	ABIS, %SIM
L. acidophilus biotype 1 IBNA 64	IBNA 64	*L. acidophilus* 1, 74.4	*L. kalixensis,* 94
		L. crispatus, 19.0	*L. manihotivorans,* 92
			L. acidophilus, 88
L. acidophilus biotype 3	IBNA 49	*L. acidophillus* 3, 99.5	*L. kefiranofaciens* ssp. *kefiranofaciens,* 88
			*L. acidophilus,*85
			L. curvatus ssp. *melibiosus,* 90
	IBNA 51	*L. acidophillus* 3, 97.9	*L. acidophilus,*82
			L. acidophilus, 88
			L. curvatus ssp. *melibiosus* 90
	IBNA 53	*L. acidophillus* 3, 99.8	*L. acidophilus,* 82
	IBNA 55	*L. acidophillus* 3, 97.7	*L. gigeriorum*, 85
			L. acidophilus, 84
			L. delbrueckii ssp. *delbrueckii*, 88
	IBNA 63	*L. acidophillus* 3, 99.8	*L. acidophilus,* 77
	IBNA 65	*L. acidophillus* 3, 95.6	
L. plantarum biotype 1:	IBNA 45	*L. plantarum* 1, 35.7	*L. plantarum*, 89
	IBNA 46	*L. plantarum* 1, 70.4	*L. plantarum*, 88
	IBNA 48	*L. plantarum* 1, 99.7	*L. plantarum*, 86
	IBNA 61	*L. plantarum* 1, 47.8	*L. plantarum*, 91
L. brevis biotype 2 IBNA 50		*L. brevis* 2, 98.5	*L.fermentum* (possibility of *L. reuteri*), 95
			L.brevis (possibility of *L. buchneri*), 94
L. fermentum biotype 1:	IBNA 56	*L. fermentum* 1, 73.2	*L. kefiranofaciens* ssp. *kefirgranum*, 97
			L.fermentum (possibility of *L.reuteri*), 91
			L. kefiranofaciens ssp. *kefiranofaciens,* 91
			L. fermentum (possibility of *L.reuteri*), 88
	IBNA 57	*L. fermentum* 1, 97.5	*L. kefiranofaciens* ssp. *kefirgranum*, 96
	IBNA 69	*L. fermentum* 1, 92.4	
L. salivarius:	IBNA 47	*L. salivarius,* 23.3	*L. salivarius,* 91
	IBNA 52	*L. salivarius,* 23.3	*L. salivarius,* 94
	IBNA 54	*L. salivarius,* 23.3	*L. salivarius,* 93
	IBNA 59	*L. salivarius,* 23.3	*L. salivarius,* 93
	IBNA 60	*L. salivarius,* 23.3	*L. salivarius,* 93
	IBNA 62	*L. salivarius,* 23.3	*L. salivarius,* 93
	IBNA 67	*L. salivarius,* 23.3	*L. salivarius,* 93
	IBNA 68	*L. salivarius,* 23.3	*L. salivarius,* 93

For apiweb identification is presented the %ID (percentage of identification), and %SIM for ABIS (percentage of similarity with respectively specie).

with the absorption and metabolism of these carbohydrates in the host gut, if these strains are used in poultry nutrition. L. delbrueckii subsp. delbrueckii was identified only from 26-day-old chickens and *L. plantarum* only from 45-day-old chickens.

As in turkeys (Sorescu *et al.*, 2019) and 26-day-old chickens (Sorescu** *et al.*, 2020; Ciurescu *et al.*, 2020) cases, in the intestinal cecum content of 45-day-old chickens, the numbers of CFU lactobacilli/g were higher (10^8 - 10^9) than in the ileum area (10^5 - 10^8), obviously especially in the case of isolation of the same species from both intestinal segments (*L. acidophilus* biotype 3, *L. plantarum*, *L. fermentum*, *L. salivarius*). Unlike the results on turkeys (probably due to the age difference – 73d versus 26d, and 45d and the species) and similar to the results on 26-day-old chickens, *L. fermentum* and *L. plantarum* strains had relative higher presence (up to 10^9 CFU/g) than other lactobacilli (up to 10^8 CFU/g) in the intestinal content of the 45-day-old chickens, which suggests a possible ecologic and, therefore, probiotic advantage for them. This fact is interesting, because the *L. fermentum* and *L. plantarum* are considered to be nomadic species, while the *L. acidophilus* and *L. salivarius* strains are adapted to vertebrate species.

As identification systems, both software (apiwebTM and ABIS) proved to be appropriate, especially for *L. plantarum*, *L. salivarius* and *L. brevis* biotype 2, where the same taxonomic classification was obtained, but with different percentage results, the way of calculating them being totally different. Instead, for *L. acidophilus* biotype 1, *L. acidophilus* biotype 3, which

Table 4 – Testing the viability of *Lactobacillus* spp. strains preserved at 4°C and room temperature.

Strains		Viability at 4 °C	Viability at room temperature
L. acidophilus biotype 1			
	IBNA 64	66 days	45 days
L. acidophilus biotype 3:			
	IBNA 49	8 days	8 days
	IBNA 51	45 days	45 days
	IBNA 53	45 days, a single passage	< 45 days
	IBNA 55	45 days	50 days, a single passage
	IBNA 63	68 days	< 45 days
	IBNA 65	45 days	< 45 days
L. plantarum biotype 1:			
	IBNA 45	7 months	< 3 months
	IBNA 46	7 months	3 months
	IBNA 48	9 months	3 months, a single passage
	IBNA 61	3.5 months	< 3 months
L. brevis biotype 2 IBNA 50		3 months	45 days
L. fermentum biotype 1:			
	IBNA 56	3 months	45 days
	IBNA 57	4 months	45 days
	IBNA 69	3 months	< 45 days
L. salivarius: IBNA 47			
	IBNA 47	45 days	< 45 days
	IBNA 52	45 days	< 45 days
	IBNA 54	45 days	45 days, a single passage
	IBNA 59	< 45 days	< 45 days
	IBNA 60	< 45 days	< 45 days
	IBNA 62	45 days, a single passage	< 45 days
	IBNA 67	< 45 days	< 45 days
	IBNA 68	< 45 days	< 45 days

are biochemically close to *L. fermentum*, and for *L. fermentum* biotype 1, ABIS software is not yet refined enough for an exact identification.

The resistance at 4 °C and room temperature are relevant technologically characters of the strains. A longer resistance is a positive trait of the strains during their selection. *L. plantarum*, *L. fermentum* biotype 1, *L. brevis* biotype 2 and *L. acidophilus* biotype 1 isolates resisted for the longest period of time, 66 days to 9 months at 4 °C and 45 days to 3 months at room temperature. These results are useful in screening the phenotypic characters of the candidate strains in order to prepare a probiotic product, involving resistance at least two months at 4 °C. Considering the presence of just 10^5 CFU/g for *L. brevis*, the *L. fermentum* biotype 1, *L. plantarum* and *L. acidophilus* biotype 1 strains only were selected for further testing of the probiotic characteristics. Same reasons led to the selection of strains in previous studies: *L. fermentum* - from turkey's gut for same reasons and other strains of *L. fermentum* and *L. brevis* - from 26-day-old chicks (Sorescu *et al.*, 2020).

Capability of probiotics to remain viable during storage and gastrointestinal passage is an important trait during strain selection (Upadrasta *et al.*, 2011; Dumitru *et al.*, 2020). Also, viability, the cell wall condition and the growth stage of the probiotic have an important influence on its performance (Papadimitriou *et al.,* 2015). Therefore, the commercially successful probiotics were based on their technological robustness, they retaining viability during product shelf-life (O'Toole *et al.*, 2017).

CONCLUSION

In this study 23 strains of the *Lactobacillus* genus (*L. acidophilus* biotype 1-one strain*, L. acidophilus* biotype 3 - six strains*, L. plantarum* - four strains*, L. brevis* biotype 2 - one strain*, L. fermentum* biotype 1 - three strains and *L. salivarius* - eight strains) have been isolated from the gut content (ileum and cecum) of 17 broiler chickens.

The total bacterial cell was counted as 10^8-10^9 in cecum and 10^5-10^8 in ileum. *L. plantarum*, *L. fermentum* biotype 1, *L. brevis* biotype 2 and *L. acidophilus* biotype 1 isolates resisted for the longest period of time. From all isolated strains, the *L. fermentum* biotype 1, *L. plantarum* and *L. acidophilus* biotype 1 are technically and ecologically suitable as potential

probiotics and worth continuing the testing of their probiotic properties.

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