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Original paper

***Lactobacillus* spp. and *Enterococcus faecium* strains isolation, identification, preservation and quantitative determinations from turkey gut content**

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Abstract

From gut content (ileum and cecum) of eight turkeys, 73 days old, was isolated, phenotypic identified and preserved, nine strains from *Lactobacillus* genus (*L. acidophilus* IBNA 11, *L. acidophilus* biotype 3 IBNA 12, *L. fermentum* biotype 1, IBNA 13-18 and *L. salivarius* IBNA 19) and one strain of *Enterococcus faecium* (IBNA 10).

The parallel identification of strains was made by apiwebTM API50CHL V.5.1, BioMerieux (France) software, API20STREP, and ABIS online software. Quantitative level of *Lactobacillus* spp. strains (10^6 – 10^9 CFU/g intestinal content) and *Enterococcus faecium* strain (10^6CFU/g intestinal content) in ecological niche was determined.

The viability of *Lactobacillus* spp. strains preserved at 4^0C (from 45 to 90 days) and at room temperature (under 90 days), and *Enterococcus faecium* at 4^0C (more than 11 months) was tested.

Keywords : Lactobacillus spp., Enterococcus faecium, gut, turkey

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Introduction

Lactobacillus spp. and *Enterococcus* spp. strains are part of the normal avian intestinal microbiota (R.M. DUAR & al. [1], D.W. WAITE & M.W. TAILOR [2], S. WEI & al. [3], J. Lu & al., [4]) with beneficial effects for host health. The lactobacilli have symbiotic role in host fitness, their enzymes and metabolites aid in the digestive process and organic acids produced from fermentation prevent pathogens (R.M. DUAR & al. [1], M. DUMITRU & al., [14], M. DUMITRU & Ș. JURCOANE [15]). *Lactobacillus* account 7% of bacterial 16S rRNA gene sequences recovered from turkey gut (S. WEI & al. [3]). Initial poultry caecal microbiota, derived from shell contaminants, consists predominantly of *Enterococcus*, coliforms and clostridia (M.E. COATES & R. FULLER [5]). Starting with the fourth day afterhatching from the egg, *Lactobacillus* becomes a significant component of the intestinal microbiota (X.Y. ZHU & al. [6]).

The aim of our work was to isolate, identify and preserve the *Lactobacillus* and *Enterococcus* strains from turkeys gut content, for subsequent ”in vitro” and ”in vivo” testing of their probiotic characters and selection of strains as intestinal flora stabilizers in turkey 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 (G. CIURESCU & O. PANĂ, [8]).

1. *Isolation of bacterial strains and determination of CFU/g intestinal content*

The MOUNTZOURIS method (K.C. MOUNTZOURIS & al. [7]) was followed, which was completed with Gram staining of smears from colonies appearing on selective media for their morphological confirmation by microscopic examination. Sample preparation: 1 g of intestinal content (ileum and cecum, respectively) per capita from eight turkeys (73 days old) was homogenized with 7 ml Oxoid BHI (Brain Heart Infusion) broth and 2 ml glycerol, and immediately frozen at – 20ºC until testing (no more three months). After defrost, decimal dilutions in Oxoid PBS (Phosphate Buffered Saline) were performed, and 0.1 ml from 10^{-4}, 10^{-5}, 10^{-6} dilutions, from every sample was inoculated on three Petri dishes with Oxoid MRS (Man, Rogosa, Sharpe) agar.

The MRS agar plates were cultural examined. The colonies of each cultural type were counted after 48 hours of anaerobic incubation in Oxoid jar with Anaerogen 2,5 L at 37ºC. Gram stained smears of each colony type were performed, for examining morphological characters and morphological confirmation of *Lactobacillus* or *Enterococcus* classification.

The catalase test was performed (negative for each genus) and CFU/g gut content was determined, as well. From every performed *Lactobacillus* colony type was inoculated one colony in one tube with Oxoid MRS broth for strains isolation and incubated 24-48 hours, in aerobic atmosphere, at 37ºC. From every presumptive Enterococcus colony type was inoculated one colony in one tube with Oxoid BHI broth for strains isolation and incubated 24 hours, aerobically, at 37ºC.

2. *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 (13), ABIS on line software (10), apiwebTM API50CHL software and API 20STREP BioMerieux (France). The results obtained by D.R. PELINESCU & al. [9] were also considered.

Morphological characters of isolated strains were analysed by Gram staining method. Cultures were grown in MRS or BHI broth, 24-48 h, at 37ºC, in aerobic conditions, and 48 h in anaerobic atmosphere, on MRS agar. Mainly, the shape of bacteria, tinctorial affinity, sporulation, grouping and the culture purity were followed by microscopic examination.

In broth medium we analysed the turbidity, deposit and the presence of surface formations, respectively the size, type, shape and colonies pigmentation on MRS agar plates.

The biochemical characters of *Lactobacillus* strains were analysed by the catalase and carbohydrate fermentation test using API50CHL, Biomerieux (France) strips, according to manufacturer's instructions. The incubation of strips was performed at 37°C, under aerobic conditions, for 48-72/120h. *Lactobacillus* sp. identification was done based on morphological characters, cultural and catalase assay. In the case of Enterococcus strain identification, following by catalase assay, biochemical characterization was performed with API 20 STREP. Test interpretation was done at 4 and 24h, at 37□C. The sensitivity to Vancomycin (30 μg) Oxoid was tested on Oxoid BHI agar. The *Enterococcus* sp. identification was based on morphological, cultural and catalase assays.

3. Preservation of bacterial strains

The medium-term preservation (months) was done by culture in broth medium (MRS for *Lactobacillus* spp. and BHI for *Enterococcus faecium*). The viability of bacterial strains was evaluated after 45 days, 3 and 4 months. Long-time preservation (years) was done at -80^{0}C, with addition of glycerol 20%. Bacteria viability will be assessed every 2 years.

Results and Discussions

The taxonomic classification of bacterial strains in *Lactobacillus* sp. was performed by morphologically (Gram positive, non-spore forming rods), culturally (anaerobic growth) and biochemically characters (negative catalase test). Identification of the *Lactobacillus* spp. was performed based of biochemical characters. Thus, 9 strains of the genus *Lactobacillus (L. acidophilus* IBNA 11, *L. acidophilus* biotype 3 IBNA 12, *L. fermentum* IBNA 13-18 and *L. salivarius* IBNA 19) and one strain of *Enterococcus faecium* (IBNA 10) were isolated, identified and preserved from the intestinal content (ileum and cecum), from 8 turkey chickens with the age of 73 days.

Figures 1-3 show smears from *L. fermentum* IBNA 15, *L. fermentum* IBNA 17 and *L. salivarius* IBNA 19 cultures on agar medium.

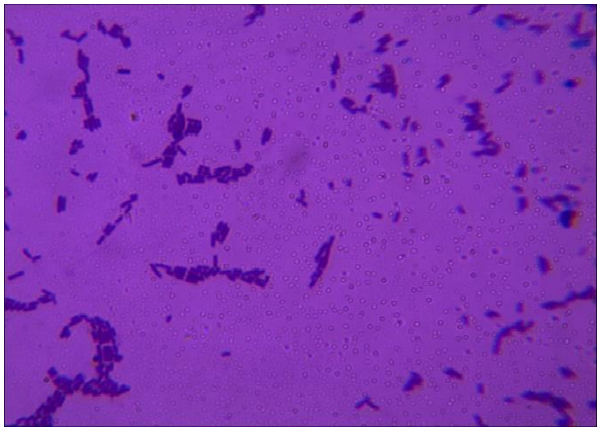

Figure 1. *L. fermentum* IBNA 15 anaerobe culture on MRS agar medium (Gram staining, x 1000)

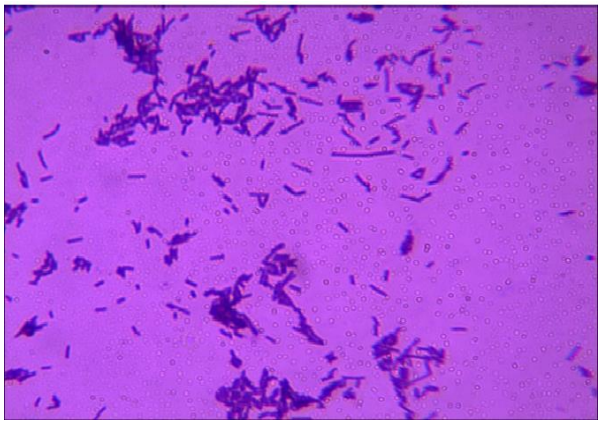

Figure 2. *L. fermentum* IBNA 17 anaerobe culture on MRS agar medium (Gram staining, x 1000)

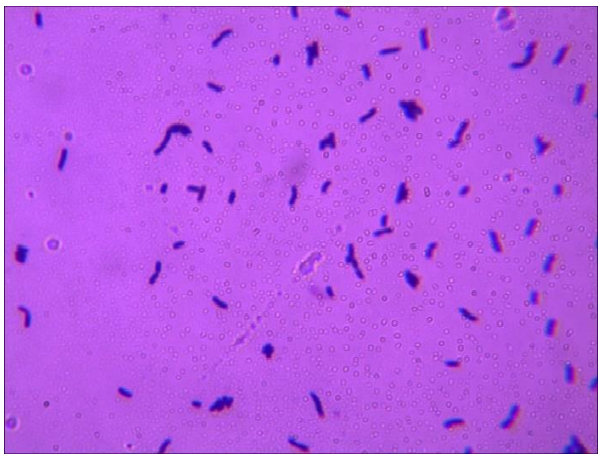

Figure 3. *L. salivarius* IBNA 19 anaerobe culture on MRS agar medium (Gram staining, x 1000)

The morphological, cultural and biochemical characteristics of these strains are presented in Table 1 and Table 2.

Table 1. Morphological, cultural and biochemical characteristics of the *Lactobacillus* strains isolated from intestinal contents of chicken turkey.

Tests	Strains								
	1	2	3	4	5	6	7	8	9
Morphological characters	a	a	b	a	a	a	a	a	b
Cultural characters	x	x	x	x	x	x	x	x	y
Catalase test	-	-	-	-	-	-	-	-	-
Fermentation (API50CHL, 48h)									
glycerol	-	-	-	-	-	-	-	-	-
erythritol	-	-	-	-	-	-	-	-	-
D-arabinose	-	-	-	-	-	-	-	-	-
L-arabinose	-	-	-	-	-	-	-	-	-
D-ribose	-	-	-	-	-	-	-	-	-
D-xylose	-	-	-	-	-	-	-	-	-
L-xylose	-	-	-	-	-	-	-	-	-
D-adonitol	-	-	-	-	-	-	-	-	-
Methyl-βD-xylopyran.	-	-	-	-	-	-	-	-	-
D-galactose	+	+/5 days	+	+/3 days	+	+/5 days	+/5 days	+	+
D-glucose	+	+/5 days	+	+	+	+	+	+	+
D-fructose	+	+	+	+	+	+	+	+	+
D-mannose	+	+/5 days	?/6 days	?	+/6 days	+/5 days	+/5 days	+/5 days	+
L-sorbose	-	-	-	-	-	-	-	-	-
L-rhamnose	-	-	-	-	-	-	-	-	-
dulcitol	-	-	-	-	-	-	-	-	-
inositol	-	-	-	-	-	-	-	-	-
D-mannitol	+/3 d...	-	-	-	-	-	-	-	+
D-sorbitol	-	-	-	-	-	-	-	-	+
Methyl-αD-mannopyr.	-	-	-	-	-	-	-	-	-
Methyl-αD-glucopyr.	-	-	-	-	-	-	-	-	-
N-acetylglucosamine	+	-	-	-	-	-	-	-	+
amygdalin	-	-	-	-	-	-	-	-	-
arbutin	+	-	-	-	-	-	-	-	-
esculin	+	+/5 days	+	+	+	-	+	+	?
salicin	+	+	-	-	-	-	-	-	-
D-cellobiose	+	-	-	-	-	-	-	-	-
D-maltose	+	+	+	+	+	+	+	+	+
D-lactose	+	-	+/6 days	+/3 days	+	-	+/5 days	+/5 days	+
D-melibiose	-	+	+	+	+	+	+	+	+
D-saccharose	+	+	+	+	+	+	+	+	+
D-trehalose	-	-	-	-	-	-	-	-	+
inulin	-	-	-	-	-	-	-	-	-
D-melezitose	-	-	-	-	-	-	-	-	-
D-raffinose	+	+	+	+	+	+	+	+	+
amidon (starch)	-	-	-	-	-	-	-	-	-
glycogen	-	-	-	-	-	-	-	-	-
xylitol	-	-	-	-	-	-	-	-	-
gentibiose	-	-	-	-	-	-	-	-	-
D-turanose	-	-	-	-	-	-	-	-	-

D-lyxose	-	-	-	-	-	-	-	-	-
D-tagatose	-	-	-	-	-	-	-	-	-
D-fucose	-	-	-	-	-	-	-	-	-
L-fucose	-	-	-	-	-	-	-	-	-
D-arabitol	-	-	-	-	-	-	-	-	-
L-arabitol	-	-	-	-	-	-	-	-	-
potassium gluconate	-	-	-	-	-	-	-	-	-
potassium 2-ketogluc.	-	-	-	-	-	-	-	-	-
potassium 5-ketogluc.	-	-	-	-	-	-	-	-	-

1=*L. acidophilus* IBNA 11, 2= *L. acidophilus* biotype 3 IBNA 12, 3= *L. fermentum* biotype 1 IBNA 13, 4= *L. fermentum* biotype 1 IBNA 14, 5= *L. fermentum* biotype 1 IBNA 15, 6= *L. fermentum* biotype 1 IBNA 16, 7= *L. fermentum* biotype 1 IBNA 17, 8= *L. fermentum* biotype 1 IBNA 18 and 9= *L. salivarius* IBNA 19.
a= Gram positive, non - spore forming rods, grouped in short chains; b= Gram positive short rods, with rounded end, non-spore forming, arranged in irregular clumps / palisade
x= small colonies, 1.0-1.5 mm in diameter, S type, round, semi-transparent / transparent, whitish / unpigmented;
y = large colonies, 2.0-4.0 mm in diameter, S type, round, opaque, white
- = negative; += positive; ?= dubious, weekly positive.

Table 2. Morphological, cultural and biochemical characters of *Enterococcus faecium* IBNA 10 strain, isolated from the intestinal content of a turkey chicken

Morphological characters		a
Cultural characters		x
Catalase test		-
Production of acetoin		+
Hippurine hydrolysis		?
Esculin hydrolysis		+
Pyrrolidonyl arylamidase		+
α-galactosidase		+
β-glucuronidase		-
β -galactosidase		+
Alkaline phosphatese		-
Leucinaminopeptidase		?
Arginindihidrolase		+
Acidification		
	ribose	+
	arabinose	+
	mannitol	+
	sorbitol	-
	lactose	+
	trehalose	+
	inulin	-
	raffinose	-
	starch	+
	glycogen	-
Vancomycin (30 μg)		R

a = Gram positive coc, diplo, in short chains and in small staph groups (on solid media).
x = small colonies, 1,0-1,5 mm diameter, S type, opaque, whitish, round, with regular margins.
- = negative; + = positive; ? = doubtful, weakly positive; R = resistant

From the analysis of Table 1, is observed that, the differentiation of *Lactobacillus* isolated strains was performed, mainly on the basis of morphological characters (aspect of bacilli and grouping of them), some cultural characters (colony size and degree of transparency / opacity) and especially, biochemical characters (fermentation of D-mannitol, N-acetylglucosamine, arbutin, esculin, salicin, D-cellobiose, D-lactose, D-melibiose). Thus, the use of biochemical tests is essential for the differentiation of *Lactobacillus* species and for the identification of the *Enterococcus* faecium strain.

Table 3. The origin and the level of *Lactobacillus* spp. and *Enterococcus faecium* strains presence in the ecological niche (73 days old turkey intestinal content).

Strains	**Origin, sample number**	**CFU/g intestinal content (log10)**
Enterococcus faecium IBNA10	ileum content, 7	7,698 + E
L. acidophilus IBNA 11	ileum content, 7	8,525 + E
L. acidophilus biotype 3 IBNA12	cecum content, 9	8,342 + E
L. fermentum biotype 1, IBNA 13	ileum content, 10	6,176 + E
L. fermentum biotype 1, IBNA 14	ileum content, 11	6,903 + E
L. fermentum biotype 1, IBNA 15	cecum content, 11	9,681 + E
L. fermentum biotype 1, IBNA 16	ileum content, 12	6,352 + E
L. fermentum biotype 1, IBNA 17	cecum content, 13	9,556 + E
L. fermentum biotype 1, IBNA 18	ileum content, 14	6
L. salivarius IBNA19	ileum content, 14	6,176 + E

Table 3 presents the origin (ileon or cecum content) and the quantitative level of the isolates presence in ecological niche.

It should be noted that, at the respective dilutions, *Lactobacillus* and *Enterococcus* strains appeared as pure culture (one type of colony developed), with the exception of sample 14, where co-exist *L. fermentum* IBNA 18 and *L. salivarius* IBNA 19.

In Table 4 are presented the results of strains identification by apiwebTM soft, API20STREP, API50CHL V.5.1, BioMerieux (France) and ABIS online software.

Table 4. The results of parallel identification of strains by apiweb™ soft, API20STREP, API50CHL V.5.1, BioMerieux (France) and ABIS online software.

Strains	API, % ID	ABIS, % SIM
1. Enterococcus faecium IBNA 10	*E.faecium*, 99.2%	*E. mundtii*, 96% *E. gallinarum*, 92% *E. moraviensis*, 92%
2. *Lactobacillus acidophilus* IBNA 11	*L.crispatus*, 38.4% *L. acidophilus*, 37.2%	*L. acidophilus*,95%
3. *Lactobacillus acidophilus* 3 IBNA 12	*L. acidophillus 3*, 96.8%	*L. acidophilus*,92%
4. *Lactobacillus fermentum* 1, IBNA 13	*L. fermentum* 1, 90.6%	*L.acidophilus*,87.7% *L.fermentum*, 81.8%
5. *Lactobacillus fermentum* 1, IBNA 14	*L. fermentum* 1, 84.7%	*L.acidophilus*, 88% *L.fermentum*, 81.8%
6. Lactobacillus fermentum 1, IBNA 15	*L. fermentum*, 90.6%	*L.acidophilus*, 88% *L.fermentum*, 81.8%
7. Lactobacillus fermentum 1, IBNA 16	*L. fermentum 1*, 94.9%	*L. kefiranofaciens* subsp. *kefiranofaciens*, 94.8% *L. fermentum*, 81.9%
8. *Lactobacillus fermentum* 1, IBNA 17	*L. fermentum 1*, 88.3%	*L. fermentum*, 88%
9. *Lactobacillus fermentum* 1, IBNA 18	*L. fermentum 1*, 88.3%	*L. fermentum*, 88%
10. Lactobacillus salivarius IBNA 19	*L. salivarius*, 99.9%	*L. salivarius*, 97%

For apiweb identification is presented the % ID (percentage of identification), respectively % SIM for ABIS (percentage of similarity with respectively specie). % SIM for ABIS represents the percentage of similarity with taxa from the database, wich containing a matrix where probabilistic incidence values are allocated for every taxon and their corresponding morpho-biochemical characters. Apiweb™ % ID is a probabilistic calculation using bioMerieux own system procedure. API identification is based on the use of all 49 fermentation tests, while ABIS identification is based on 27 fermentation tests, generally those that differentiate between species. According to CATO & al. [11] *L. crispatus* are synonymous with *L. acidophilus* group A2 because the type strains of *L. crispatus* and *L. acidophilus* group A2 have 100% DNA homology.

In Table 5 are presented the results of viability test for *Lactobacillus* strains which are preserved at 4°C and at room temperature.

Table 5. Testing the viability of *Lactobacillus* and *Enterococcus faecium* strains preserved at 4°C and room temperature.

Strains	Viability at 4°C	Viability at room temperature
1. E. faecium IBNA 10	+/ 11 months	ND
2.*L. acidophillus* IBNA 11	-/4 months; +/45 days	-/4 months
3. *L. acidophillus* 3 IBNA 12	-/3 months; +/45 days	-/3 months
4. *L. fermentum* 1, IBNA 13	-/3 months; +/45 days	-/3 months
5. *L. fermentum* 1, IBNA 14	+/3 months	-/3 months
6. L. fermentum 1, IBNA 15	+/3 months	-/3 months
7. L. fermentum 1, IBNA 16	+/3 months	-/3 months
8. L. fermentum 1, IBNA 17	-/3 months; +/45 days	-/3 months
9. L. fermentum 1, IBNA 18	-/3 months; +/2 months	-/3 months
10. L. salivarius, IBNA 19	-/45 days	-/3 months

+ = positive, - = negative.

Note that, for E. faecium IBNA 10, 11 months is the minimum duration of resistance at 4ºC and their viability will be tested at 15 months. *L. acidophilus* IBNA 11, 12 resisted 45 days at 4ºC, a single passage. *L. fermentum* IBNA 14 and 15 strains survived 3 months at 4ºC, a single passage. *L. fermentum* IBNA 17 resisted 45 days at 4ºC, a single passage.

L. salivarius is included in the vertebrate adapted lifestyle lactobacilli group, with bile resistance and bacteriocins production as lifestyle-associated traits (R.M. DUAR & al. [1]). *L. salivarius* was isolated from human, pigs, chickens, hamsters and horses (R.M. DUAR & al. [1]). *L. acidophilus* belongs to the same group of lactobacilli and has recognized probiotic properties (R.M. DUAR & al. [1]). *L. fermentum* is included in the nomadic species group of lactobacilli (R.M. DUAR & al. [1]). *L. fermentum* was isolated from fermenting plant material, manure, sewage, milk products, mouth and faeces of humans, and intestines of pig, birds, cattle, mouse and rat (C. STOICA & I. SORESCU [10]).

These strains isolated from intestinal content of turkeys will be important for the development of a probiotic compound for same bird species, because the host-adapted strains of lactobacilli have a higher ecological fitness in their respective hosts and will be more competitive as probiotic when compared to strains that don't share an evolutionary history with the host (R.M. DUAR & al. [1]). The probiotic characters of these strains will be tested further. Higher fitness is relevant to outcompete pathogens, to have higher metabolic activity and to have an increased production of metabolic compounds. Moreover, tolerance interactions with the host immune system will be established. The count of lactobacilli/gram of intestinal content methodology of K.C. MOUNTZOURIS & al. [7] was completed/modified for the morphological confirmation of colonies which appear on selective medium by Gram staining of smears and microscopic examination. It was found that the microscopic confirmation is necessary because some colonies with common traits with *Lactobacillus* spp., by Gram staining, appear as Gram positive cocci or, more seldom, as Gram negative coccobacilli. Thus, the morphological confirmation of the presumptive positive colonies is necessary for fitting into the group of lactobacilli.

In the intestinal cecum content, the numbers of CFU lactobacilli/g are much higher (10^8-10^9) than in the ileum area (10^6-10^8). *L. fermentum* has a superior presence to other lactobacilli. This observation is very interesting, because the *L. fermentum* is considered to be nomadic specie, while the *L. acidophilus* and *L. salivarius* strain are adapted to vertebrate species. The higher numbers of CFU *Lactobacillus*/g intestinal content can suggest a better adaptation to the ecological niche, so a potential probiotic advantage.

As identification system, both software (apiwebTM and ABIS) are proved to be useful. In general was obtained the same taxonomic classification, sometimes even with identical percentage results, although the way of calculating them is totally different. The use of biochemical tests is essential for the differentiation of *Lactobacillus* species and for the identification of the *Enterococcus faecium* strain.

The resistance at 4ºC is a relevant technical character of the strains. Thus, *E. faecium* IBNA 10 resisted, at 4ºC, for at least 11 months, while three of the six isolated strains of *L. fermentum* resisted for 3 months. *L. salivarius* strain IBNA 19 did not survive 45 days at 4ºC, whereas the *L. acidophilus* strains survived only 45 days a single passage. Obviously, a longer resistance is a plus, a positive character to the strains during their selection.

These results are very useful in screening the phenotypic characters of the candidate strains to prepare a probiotic product, involving resistance from the isolated strains, of at least 45 days at 4ºC, without significant loss of the biological value (viability, CFU/ml). From this point of view, *L. fermentum* and *E. faecium* strains will be selected for further testing of the probiotic characteristics. We did not find this type of information in the literature, perhaps because of the very specific benefices for industrial producers of probiotic products. However, it is accepted that the ability of probiotics to remain viable during culture handling, storage and gastric passage is an important

criterion during strain selection (A. UPADRASTA & al. [12]).

Conclusions

The gut content (ileum and cecum) of eight turkeys, 73 days old, was used to isolate, identify phenotypically and preserve nine strains of the *Lactobacillus* genus (two strains of *L. acidophilus*, six strains of *L. fermentum* and one strain of *L. salivarius*) and one strain of *E. faecium*.

The count of lactobacilli per gram of intestinal content methodology of K.C. MOUNTZOURIS & al. [7] was followed for the morphological confirmation of colonies which appear on selective media, by Gram staining of smears and microscopic examination. The quantitative level of *Lactobacillus* spp. strains (10^6 – 10^9 CFU/g intestinal content) and *E. faecium* strain (10^6 CFU/g intestinal content) in the ecological niche was determined.

It was found that the number of lactobacilli in cecum content is higher (10^8-10^9 CFU/g), than in the ileum (10^6-10^8 CFU/g) and *L. fermentum* is superior to other lactobacilli. The parallel identification of strains was made by apiwebTM API50CHL V.5.1, BioMerieux (France) software, API20STREP, and ABIS on line software.

Both softwares proved to be useful, generating the same taxonomic classifications. The viability of *Lactobacillus* spp. strains preserved at 4ºC (from 45 to 90 days) and at room temperature (under 90 days) and *Enterococcus faecium* at 4ºC (more than 11 months) was tested. From isolated *Lactobacillus* strains, those from *L. fermentum* are technically suitable for continual testing of probiotic qualities, as well as the strain of *E. faecium*.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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