PHYLOGENY AND QUANTITATIVE ASSESSMENTS OF CULTIVABLE *LACTOBACILLUS* SPP. ISOLATED FROM BROILERS: A PROBIOTIC PROSPECTIVE

By

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ABSTRACT

Lactic Acid Bacteria (LAB) is a digestive tract microflora that have a beneficial role in fowl energy. The number and difference of LAB in the digestive tract are damaged by various determinants. The objective of this study to reach out isolate, identify, preserve and decide the quantitative level of the *Lactobacillus* strains from the gut content of 18-day-old chickens' broilers. *Lactobacillus* strains were phenotypically recognized and preserved from the gut content of 50 chickens' broilers. Identification was accomplished by morphological, cultural and biochemical characters. The all-inclusive level of *Lactobacillus* strains in stomach content (10^5 - 10^9 CFU/g). According to sequencing of the 16S ribosomal RNA gene fragment, strains of *L. crispatus* from the gut content of chickens were unique, phenotypically recognized, and which augments their request as a probiotic supplement. Conclusively, *L. crispatus* strains were technologically and ecologically appropriate probiotic.

Keywords:

Lactobacillus spp., Broilers, 16S rRNA, *L. crispatus.*

INTRODUCTION

Recent research on the construction of the usual intestinal microbiota of chickens disclosed the closeness of *Lactobacillus* spp. **(Lu *et al.,* 2003; Wei *et al.,* 2013; Waite and Tailor, 2014; Duar *et al.,* 2017)**, popular for enchantment advantageous on the host's well-being. *Lactobacilli* have a cooperative function in host appropriateness, their metabolites providing to the digestion process and preventing pathogens **(Duar *et al.,* 2017).**

Zou *et al.,*(2018) granted that *Lactobacillus* encourage a dividing effect on the chicken cecal microbiome, suggesting a big effective function concerning this type in local microbiome, including negative (*Ruminococcaceae*, *Lachnospiraceae*) or beneficial ones (*Lactobacilli*,

Bacteroides, *Clostridiales* and *Christensenellaceae*) equivalences. Fowl microbiota from cecum exists, mainly, as enterococcus, coliforms and clostridia **(Coates and Fuller, 1977).** From the 4th day of age, Lactobacillus is considered an important component of the intestinal microbiota **(Zhu *et al.,* 2002).** At the 7th day of age, the ileal mucosal microbiota is ruled by *Lactobacillus* spp., trailed by *Lachnospiraceae* and *Enterococcus* spp. **(Cressman *et al.,* 2010).** After the 14th day of age, cecum and part of digestive tract of broilers evolve miscellaneous societies **(Pedroso and Lee, 2015).** From days 21 to 42 of age, *Lactobacillus* is enhanced as ultimate plentiful organism in part of the digestive tract of which, *L. salivarius, L. johnsoni, L. reuteri, L. oris and L. crispatus* are common species **(Nakphaichit *et al.,* 2011).** This difference raises the issue of selecting high-quality strain for evolving bacterial-located feed supplements in fowl feed.

Lactobacilli are appendages of the lactic acid microorganisms group, a widely family of microorganism range of microorganisms that ferment differing hexoses to lactic acid. They are of low G+C, Gram-positive and catalase negative. These microorganisms settle in part of the digestive tract and caeca of chickens, a period afterwards breed **(Mead, 1997).** They help assert the everyday balance of structures (Microflora) in the entrails and help support an active digestive system **(Dunne *et al.,* 1999).** The use of lactobacilli as probiotics is referred to 1-Lactobacilli exhibit "cutthroat expulsion", a characteristic that inhibits the progress of bacterial microorganisms. 2- Lactobacilli, opportunely be part of the epithelial containers of the entrails and 3- Lactobacilli are famous as dependable collaborators for the host and improve the invulnerable structure function.

It became established that the use of probiotics have the potential of averting the progress of stomach bacteriome and counteracts poisoning caused by the eating food in chickens **(Pascual *et al.,* 1999).**

Kizerwetter-Swida and Binek (2009) showed that the *Lactobacillus salivarus* 3d strain weakened the number of *Salmonella enteritidis* and *Clostridium perfringens* in experimental chickens. **Lan *et al.,* (2004)** reported that *Lactobacilli* microorganisms, when second hand as a supporting-basic, keep the balance and assert everyday strength of microflora in the entrails of chickens following heat stress.

In addition, lactobacillus probiotic is thought-out as a main environmental determinant that decide main microorganisms in few environments like the entrails **(Busarcevic *et al.,* 2008).**

The selected probiotic bacterial strains must be innately constant and exhibit stable growth rate in vivo and artificial environments as well.

The objective of our work was to find out the overall level of the *Lactobacillus* spp. in the gut content of 18-day-old broiler chickens. The probiotic characteristics and potentials of the isolates were considered to select most capable ones to be used as stomach vegetation stabilizers in poultry feed.

MATERIAL AND METHODS

Isolation, initial identification of *Lactobacilli* and Determination of total bacterial count

The method of Mountzouris *et al.* (2007) completed by **Sorescu *et al.*, (2019)** was applied. One gram of both ileum and cecum content of 18 days old broilers was homogenized with 7 ml Oxoid BHI (Brain Heart Infusion) broth and 2 ml glycerol, and immediately frozen at - 20 ºC until testing (No more than three months).

After defrost, samples from each site were serially diluted in normal saline (From 0.1 to 0.001), plated onto Man Rogosa and Sharp (MRS) medium and incubated anaerobically at 37°C for 36 h. decimal dilutions from every sample were inoculated on Oxoid Man, Rogosa, Sharp (MRS) agar (Oxoid, UK).

The procedures of **Sorescu *et al.* (2019)** for the isolation and counting of *Lactobacillus* CFU/g was adopted.

Phenotypic identification of the lactobacilli isolates was performed by morphological, cultural and biochemical characters. The bacterial isolates were stored at 4°C for a short time and lyophilized in 15% sucrose for long-term storage.

Molecular identification of *Lactobacillus* isolates:

DNA was extracted from the bacteria as described by **Heilig *et al.* (2002)**. Lactobacillus identification was confirmed by using genus-specific primers targeting the 16S rRNA gene sequence via: F: 5'-CACCGCTACACATGGAG-3', R: 5'-AGCAGTAGGGAATCTTCCA-3' to amplify. a 341- bp fragment. The polymerase chain reaction (PCR) was started with heating at 94°C for 3 minutes followed by 35 cycles of 30 s at 92°C, 30 s at 55°C, 60 s at 72°C and a final extension step for 5 min. at 73°C.

The PCR products were analyzed on 1% agarose gel. The positive PCR products were eluted and extracted from the gel using a gel extraction kit (Bioneer, Korea).

Sequencing and phylogeny of 16S rRNA amplicons of genus *Lactobacillus:*

The DNA fragments were sequenced by the Seqlab Co. (Seqlab, Göttingen, Germany) and sequences were compared with available sequences in GenBank using the BLASTN tool through the National Center for Biotechnology Information (NCBI) server (http://blast.ncbi.nlm.nih.gov/Blast/, 2010). Sequence homologies of more than 97% regarded as belonging to the same species **(Tannock, 1999).**

RESULTS

Fifty isolates from broilers' gut were identified as *Lactobacillus*. According to macroscopic view these isolates had small to medium sized circular colonies. Their colors range from white to off white and they were transparent and greasy to dry textured on MRS agar plates. Microscopically, the isolates were Gram-positive, medium to large (1.1 to 5.7 µm) rods arranged singly, in pairs or in short or long chains. All identified isolates were catalase negative. Table (1) depicts the colony forming unit (CFU/g) counts of lactobacilli in ileum and cecum samples of 18 days old broiler chickens.

Table (1): The origin and the quantity of cultivable *Lactobacillus* spp. strains (18-day-old chickens' broiler intestinal content).

Sample	Ileum content	Cecum content
	CFU/g intestinal content (log10)	CFU/g intestinal content (log10)
1	8.698	9.543
2	7.754	8.686
3	6.863	8.665
4	8.578	8.326
5	8.150	7.964
6	6.635	8.697
7	4.794	7.476
8	6.583	8.257
9	8.478	6.466
10	7.386	8.866
11	7.920	7.098
12	5.908	7.675
13	8.743	8.321
14	6.739	8.223
15	8.902	8.455
16	7.376	7.643
17	7.189	8.854
18	6.603	8.532
19	5.694	6.689
20	8.856	8.688
21	8.278	8.642
22	6.3845	6.347
23	8.029	8.555
24	7.836	8.900
25	8.183	7.481
26	8.520	8.846
27	6.947	9.892
28	4.837	6.845
29	5.036	7.182
30	8.392	8.291
31	8.430	8.038
32	8.183	8.633
33	7.111	8.128
34	8.409	8.343
35	7.294	8.392
36	8.854	8.049
37	6.620	7.698
38	7.303	8.348
39	8.398	8.603
40	7.644	7.168
41	7.541	8.234
42	7.023	8.765
43	8.473	8.284
44	6.322	7.154
45	5.216	8.854
46	6.009	8.223
47	8.626	9.675
48	8.478	7.432
49	6.493	6.398
50	7.433	7.456

Identification of *Lactobacillus* spp. by PCR detection of 16S rRNA gene:

PCR products of 341 bp were obtained from 14 tested lactobacilli isolates Fig. (1).

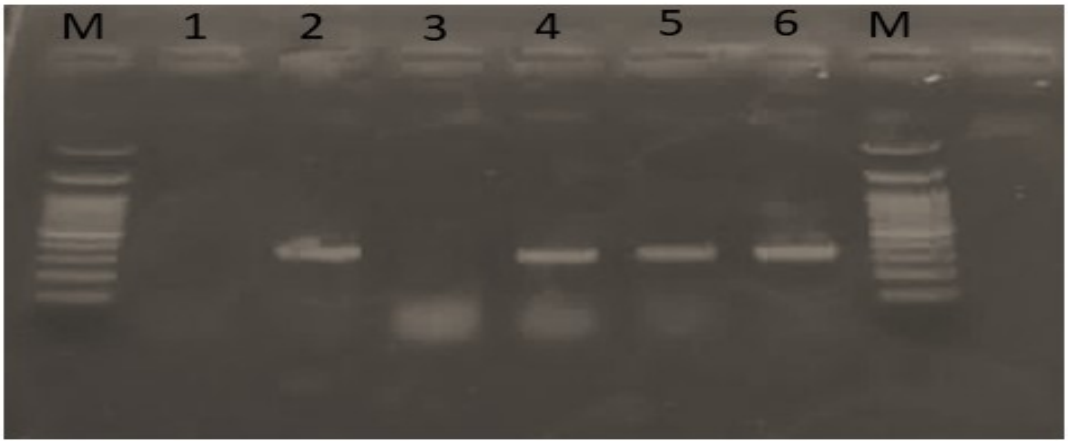

Fig. (1): PCR products by lactobacillus genus-specific primers. Lanes M: DNA ladder (100 bp), lane 1: Negaive control, lane 3: Negative, lanes 2- 6: *Lactobacillus* spp positive (341- bp amplicon)**.**

Sequencing of lactobacilli PCR products of lactobacilli 16S rRNA gene:

The results of the 16S rRNA gene sequencing showed that, the isolates had 97-99% similarity with 99% query coverage of 16S rRNA gene sequences 14 lactobacilli isolates. The sequences were deposited in the GenBank database under the accession numbers: *Lactobacillus crispatus* (OP744495.1, OP744494.1).

Neighbor joining Phylogenetic tree:

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree is shown Fig. (2). the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site.

The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each descendent clade is shown next to each internal node in the tree.

Fig. (2): Phylogenetic tree based on 16S rRNA gene sequence analysis, using the Neighbor-Joining method **(Saitou and Nei, 1987)**. The out group was *Lactobacillus crispatus* (OP744495.1, OP744494.1). Sequences belong to *Lactobacillus* species obtained from the Gene Bank (NCBI). Bootstrap test (1000 replicates) are indicated at the nodes of the tree.

This analysis involved 12 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 278 positions in the final dataset. Evolutionary analysis were conducted in MEGA X. Isolates of same species clustered together on phylogenetic tree. *L. crispatus* isolates from broiler (OP744495.1, OP744494.1)

were genetically close to other isolates. Distance based phylogenetic study of 16S rRNA gene sequence showed that *Lactobacilli* from same species were genetically close to each other.

DISCUSSION

Different GI tract areas of chickens play various functions in feed digestion, mineral assimilation, and stomach well-being, all of which depend the environmental balance of the various microorganisms in different GI tract domains **(Pedroso and Lee, 2015)**. *Lactobacilli* are usually considered to rule the beginning part of digestive tract, crop, stomach and jejunal epithelial containers, and digesta of chickens. As a fundamental part of the stomach microbiota. Some experiments proved that microbial supplements permit an action to be used as a hint of choice for cures. In this way, it has endured seen that *Lactobacilli* microorganisms are fruitful on the progress of chicks without changing the cures **(Pandey *et al.,* 2000, Murry *et al.,* 2004, Kalavathy *et al.,* 2008).** It has speculated that probiotics developed the productive acting in chicken **(Russell and Grimes, 2009)** and reduced the carcass and antibody fat content **(Kalavathy *et al.,* 2006, 2008)**.

The counts and spread of various Lactobacillus variety indifferent GI tract domains of chickens play a main part in asserting the environmental balance of the various microorganisms **(Russell and Grimes, 2009).**

In the current study, we established total count of lactobacilli in the cecum of 18-days broiler chickens (Table 1). It was found that *Lactobacilli* had hostile variety in the cecum.

In a similar study, the bacterial counts in the constituents of various stomach portions in unoriginal and basic grill chickens was done by normal sophistication methods.The results granted that the lactobacilli counts of the crop were nearly the same as that of cecum and rectum in common grill for cookout chickens, and the lactobacilli numbers of the crop appeared above that of other parts of digestive tract, cecum and rectum **(Tamura *et al.,* 2004).**

Concerning 26-day-old chickens **(Sorescu *et al.,* 2020; Ciurescu *et al.,* 2020)** cases, in the stomach cecum content of 45-epoch-traditional chickens, plan of CFU *Lactobacilli* /g were greater (10^8 - 10^9) than in the part of digestive tract extent (10^5 - 10^8).

Concerning the molecular characterization, studies of 16S rRNA genes have been conducted on the chicken GIT lactobacilli **(Pedroso and Lee, 2015).**

Based on the results of this study, PCR using primers targeting *Lactobacillus* genus-specific primers confirmed the traditional identification of 14 chicken GIT isolates. The expected 341 bp specific product was obtained Fig. (1).

To figure out the phylogeny of isolates obtained in our investigation, the PCR products of 12 lactobacillus isolates were sequenced. Some reports have likewise examined the arrangement of the ileal and cecal microflora of broilers by reasoning of 16S rRNA deoxyribonucleic acid sequences as lactobacilli are abundant in different parts of the digestive tract **(Sorescu *et al* ., 2020).**

(Amit-Romach *et al.,* 2004) checked the stomach microflora of Cobb chickens by 16S rRNA gene targeting probes. In young chicks the major species present in the small intestines and ceca was *Lactobacilli*, accompanying a *Bifid bacteria* community flattering more prevalent in the ceca at earlier age.

It was indicated that the different breeds of chickens or the arrangement and construction of the feed or the dwelling environments in addition to the resolving procedures influence the arrangement of the stomach microbial society of chickens **(Bjerrum *et al.,* 2006)**.

In the study, we likewise driven the counts and spread of various Lactobacillus variety in different GI tract regions, and *L. crispatus* was ultimate plentiful *Lactobacillus* class. Moreover, the spread of few *Lactobacilli* in the residue of the gut was nearly comparable to the spread of *Lactobacilli* in the crop, that were present during the whole of the poultry digestive area, compatible accompanying prior studies **(Edelman *et al.,* 2002)** Nevertheless, the crop microflora acts as a bacterial inoculum for the residue of the gut **(Brambilla and Filippis, 2005). , (Van Coillie *et al.* 2007).** Reported that all the isolates in the drain and vulva of 22–44 weeks traditional laying hens, belongs to the *L. acidophilus, L. reuteri or L. salivarius* ethnic groups, accompanying the *L. reuteri* group being ultimate ruling group. **Bjerrum *et al.,* 2006**, stated that out of 41-day-old chickens, 61% of the ileal *Lactobacilli* clones were approximately had connection with *L. crispatus* and 20% to *L. salivarius*, In contrast, in nooriginal 40-day-old Ross chickens, the controlling phylotype (44% of the total) had connection with *L. salivarius,* attended by *L. johnsonii* (30%).

Gong *et al.* (2007) established that *L. aviarius* and *L. salivarius* were the ruling varieties with lactobacilli in the GIT of 5-week-old (Ross X Ross) grill for cookout chickens. The results of **Hilmi *et al.* (2007)** revealed that *L. reuteri, L. crispatus, and L. salivarius* were ultimate plentiful varieties in the crops of chickens risinging from four various farms utilizing two various marketing feeds. Therefore, we projected that *L. reuteri, L. acidophilus, L. crispatus,*

and *L. salivarius* were four conventional and ruling *Lactobacillus* class and present during the whole of the hen digestive area.

Phylogenetic forest established 16S rRNA deoxyribonucleic acid order study, utilizing the Neighbor-Joining form **(Saitou and Nei, 1987)**. *L. crispatus* isolates from grill for cookout (OP744495.1, OP744494.1) were innately nearly isolates. Sequences obtained from our study aligned with twenty sequences of *Lactobacillus* genus acquired from the Gene Bank (NCBI).

Distance located ethnic study of 16S rRNA deoxyribonucleic acid order revealed that *Lactobacilli* from unchanging variety were innately nearly. Boot leash principles until 100 % are pointed out at the knots of the wood.

Decision for draft of ideal strain for probiotic purposes maybe troublesome regard to diversified tests. Characterization of tests are beneficial to establish their significance for the option of best choice microorganisms as a probiotic. The summary of multiples form ending score for each strain. The tests that include for cutthroat forbiddance or inhibitory have extreme cooperative (cooperative=2 and 3 individually) and hostility seasoning fighting (cooperative=2). Since the pH awareness may be removed by encapsulation of microorganisms, depressed cooperative was likely to it (cooperative=1.5). These talents, in addition to the larger total scores, form *L. crispatus* for probiotic purposes. Further experiments are inevitable to judge the in vivo characteristics of these strains.

CONCLUSIONS

There is diversification in dispersion of *Lactobacillus* in broiler's gut. That is because various cultivable lactobacilli are established in different domains of broiler gastro intestinal tract. In this study, 50 strains of the *Lactobacillus* type have been isolated from the gut content (part of digestive tract and cecum). The total bacterial was 10^9 -10^8 in cecum and in another part of digestive tract. Some isolates are particularly present in a region. Neighbor joining phylogenetic tree of lactobacillus isolates demonstrated that isolates from fowl GIT were innately nearer to *Lactobacillus crispatus* that is technically and ecologically acceptable as potential probiotics.

REFERENCES

Amit-Romach E, Sklan D and Uni Z. (2004): Microflora ecology of the chicken intestine using 16S ribosomal DNA primers. Poult Sci, 83: 1093-1098.

Bjerrum L, Engberg RM, Leser TD, Jensen BB, Finster K. and Pedersen K. (2006): Microbial community composition of the ileum and cecum of broiler chickens as revealed by molecular and culture-based techniques. Poult Sci, 85: 1151-1164.

Brambilla G. and Filippis SD. (2005): Trends in animal feed composition and the possible consequences on residue tests. Anal Chim Acta, 529:7-13.

Busarcevic M, Kojic M, Dalgalarrondo M, Chobert JM, and Haertle T, Topisirovic L. (2008): Purification of bacteriocin LS1 produced by human oral isolate *Lactobacillus salivarius* BGHO1. Oral. Microbiol. Immunol, 23: 254-258.

Ciurescu G, Dumitru M, Gheorghe A, and Untea AE, Draghici R. (2020): Effect of Bacillus subtilis on growth performance, bone mineralization, and bacterial population of broilers fed with different protein sources. Poultry Science; 99 (11):5960-5971.

Coates ME, Fuller R. (1977): The gnotobiotic animal in the study of gut microbiology. In: Clarke RTJ, Bauchop T, editors. Microbial ecology of the gut. London: Academic Press; p.311-346.

Cressman MD, Yu Z, Nelson MC, Moeller SJ, Lilburn MS, Zerby HN. (2010): Interrelations between the microbiotas in the litter and in the intestines of commercial broiler chickens. Applied Environmental Microbiology; 76(19):6572-6582.

Duar RM, Lin XB, Zheng J, Martino ME, Grenier T, Munor MEP, (2017): Lifestyles in transition: evolution and natural history of the genus Lactobacillus. Review. FEMS Microbiology; 30 (41):27- 48.

Dunne C, Murphy L, Flynn S, O'mahony L, O'halloran S, Feeney M, Morrissey D, Thornton G, Fitzgerald G, Daly C, Kiely B, Quigley EM, O'sullivan GC, Shanahan F, Collins JK. (1999): Probiotics: from myth to reality. Demonstration of functionality in animal models of disease and in human clinical trials. Antonie Van Leeuwenhoek, 76: 279-292.

Edelman S, Westerlund-Wikstro¨m B, Leskela¨ S, Kettunen H, Rautonen N, Apajalahti J and Korhonen TK. (2002): In vitro adhesion specificity of indigenous lactobacilli within the avian intestinal tract. Appl Environ Microbiol, 68: 5155–5159.

Felsenstein J. (1985): Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.

Gong J, Si W, Forster RJ, Huang R, Yu H, Yin Y and Yang C. (2007): 16S rRNA gene-based analysis of mucosa-associated bacterial community and phylogeny in the chicken gastrointestinal tracts: from crops to ceca. FEMS Microbiol Ecol, 59: 147–157.

Guan LL, Hagen KE, Tannock GW, Korver DR, Fasenko GM and Allison GE. (2003): Detection and identification of Lactobacillus species in crops of broilers of different ages by using PCR-denaturing gradient gel electrophoresis and amplified ribosomal DNA restriction analysis. Appl Environ Microbiol, 69: 6750–6757.

Heilig, H.G., Zoetendal, E.G., Vaughan, E.E., Marteau, P., Akkermans, A.D. and De Vos, W.M. (2002): Molecular diversity of Lactobacillus spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. Appl Environ Microbiol 68, 114 -123.

Hilmi HTA, Surakka A, Apajalahti J. and Saris PE. (2007): Identification of the most abundant Lactobacillus species in the crop of 1-and 5-week-old broiler chickens. Appl Environ Microbiol, 73: 7867–7873.

Kalavathy R, Abdullah N, Jalaludin S, and Wong MCVL, Ho YW. (2006): Effects of *Lactobacillus* feed supplementation on cholesterol, fat content and fatty acid composition of the liver, muscle and carcass of broiler chickens. Anim. Res., 55: 77 - 82.

Kalavathy R, Abdullah N, Jalaludin S, Wong CMVL, Ho YW. (2008): Effect of *Lactobacillus* Cultures and Oxytetracycline on the Growth Performance and Serum Lipids of Chickens. Int. J. Poult. Sci., 7: 385 - 389.

Kizerwetter-Swida M, Binek M. (2009): Protective effect of potentially probiotic *Lactobacillus* strain on infection with pathogenic bacteria in chickens. Pol. J. Vet. Sci., 12: 15-20.

Kumar S., Stecher G., Li M., Knyaz C., and Tamura K. (2018): MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution 35:1547-1549.

Lan PTN, Sakamoto M, Benno Y. (2004): Effects of Two Probiotic *Lactobacillus* Strains on Jejunal and Cecal Microbiota of Broiler Chicken under Acute Heat Stress Condition as Revealed by Molecular Analysis of 16S rRNA Genes. Microbiol. Immunol, 48: 917-929.

Lu J, Idris U, Harmon B, Hofacre C, Maurer JJ and Lee MD. (2003): Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. Appl Environ Microbiol, 69: 6816–6824.

Macpherson AJ and Harris NL. (2004): Interactions between commensal intestinal bacteria and the immune system. Nat Rev Immunol, 4: 478-485.

Mead GC. (1997): Bacteria in the gastrointestinal tract of birds. In: Mackie RJ, White BA and Isaacson RE (eds) Gastrointestinal Microbiology. Chapman and Hall, New York, pp. 216-240.

Mountzouris KC, Tsirtsikos P, Kalamara E, Nitsch S, and Schatzmayr G, Fegeros K. (2007): Evaluation of the efficacy of a probiotic containing Lactobacillus, Bifidobacterium, Enterococcus, and Pediococcus strains in promoting broiler performance and modulating cecal microflora composition and metabolic activities. Poultry Science; 86(2):309-317.

Murry AC, Hinton JA, Buhr RJ. (2004): Effect of a Probiotic Containing Two *Lactobacillus* Strains on Growth Performance and Population of Bacteria in the Ceca and Carcass Rinse of Broiler Chickens. Poult. Sci., 83: 322.

Nakphaichit M, Than omwon gwattana S, Phraephaisarn C, Sakamoto N, Keawsompong S, Nakayama J. (2011): The effect of including Lactobacillus reuteri KUB-AC5 during post-hatch feeding on the growth and ileum microbiota of broiler chickens. Poultry Science, 90 (12): 2753-2765.

Ouweh and A, Isolauri E and Salminen S. (2002): The role of the intestinal microflora for the development of the immune system in early childhood. Eur J Nutr., 41: i32–i37.

Pandey A, Franco SG, Soccol CR, Reque EDF, (2000): Isolation, Identification and physiological study of *Lactobacillus fermentum* LPB for use as probiotic in chickens. Braz. J. Microbiol., 31: 303-307.

Pascual M, Hugas M, Badiola JI, and Monfort JM, Garriga M. (1999): *Lactobacillus salivarius* CTC2197 prevents *Salmonella enteritidis* colonization in chickens. Appl. Environ. Microbiol, 65: 4981- 4986.

Pedroso AA, Lee MD. (2015): The composition and role of the microbiota in chickens. In: Niewold TA, editor. Intestinal health. Wageningen: Wageningen Academic Publishers; p.21-25.

Russell SM, Grimes JL. (2009): The effect of a direct-fed microbial (Primalac) on turkey live performance. J. Appl. Poult. Res., 18: 185- 192.

Saitou N. and Nei M. (1987): The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, 4:406-425.

Scholz-Ahrens KE, Ade P, Marten B, Weber P, TimmW, A6il Y and Glu¨er CC. (2007): Prebiotics, probiotics, and synbiotics affect mineral absorption, bone mineral content, and bone structure. J Nutr, 137: 838S-846S.

Sorescu I, Dumitru M, Ciurescu G. (2019): Lactobacillus spp. and Enterococcus faecium strains isolation, identification, preservation and quantitative determinations from turkey gut content. Romanian Biotechnological Letters, 24 (1):41-49.

Sorescu I, Dumitru M, Ciurescu G (2020): Lactobacillus spp. Strains Isolation, Identification, Preservation and Quantitative Determinations from Gut Content of 45-Day-Old Chickens Broilers. Brazilian Journal of Poultry Science, 23(1):1-8.

Tamura K., Nei M., and Kumar S. (2004): Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences (USA), 101: 11030-11035.

Tannock GW. (1999): Identification of lactobacilli and bifidobacteria. Mol. Biol., 1: 53-64.

Van Coillie E, Goris J, Cleenwerck I, Grijspeerdt K, Botteldoorn N, Van Immerseel F and De Buck J. (2007): Identification of lactobacilli isolated from the cloaca and vagina of laying hens and characterization for potential use as probiotics to control *Salmonella* Enteritidis. J Appl Microbiol., 102:1095-1106.

Waite DW, Tailor MW. (2014): Characterizing the avian gut microbiota: membership, driving influences, and potential function. Frontiers in Microbiology, 5 (223):1-12.

Wei S, Morrison M, Yu Z. (2013): Bacterial census of poultry intestinal microbiome. Poultry Science, 92(3):671-683.

Zhu XY, Zhong T, Panya Y, Jorger RD. (2002): 16S rRNA-based analysis of microbiota from the cecum of broiler chickens. Applied and Environmental Microbiology, 68(1):124-137.

Zou A, Sharif S, Parkinson J. (2018): Lactobacillus elicits a 'Marmite effect' on the chicken cecal microbiome. NPJ Biofilms and Microbiomes, 4(27):1-5.