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Research Article

Effect of *Enterococcus faecium* on the Digestive Tract of Poultry as a Probiotic

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Abstract: This project investigates the microbiological and biochemical characteristics of chicken and turkey caeca. Microbiological characteristics were represented by CFU of *Escherichia coli*, faecal Enterococci and Lactobacilli determined in 1 g of chyme and biological ones by enzymatic activity of the cellulases expressed in CMC units.

In all experiments, a probiotic preparation based on *Enterococcus faecium* was added to the feeding mixture and drinking water at various concentrations or in the form of aerosol on the body or egg surface. Counts of *Escherichia coli* in CFU in 1 g of faecal chyme were determined on Endo agar, counts of CFU of faecal Enterococci on selective agar for faecal Enterococci and counts of Lactobacilli on Rogosa agar. Enzymatic activity of cellulases was determined according to the Miller method.

Counts of CFU of *Escherichia coli*, faecal Enterococci, Lactobacilli and enzymatic activity of cellulases were compared in experimental and control treatments. Theoretical and empirical evidence suggests that the counts of *Escherichia coli* CFU would be higher in control treatments and CFU counts of both faecal Enterococci and Lactobacilli as well as enzymatic cellulases activity (in CMC units) lower in control treatments compared to the experimental ones. Similar results were also obtained in our experiments with chickens and turkeys.

Polymerase chain reaction using specific primers was performed to identify *Enterococcus faecium*.

Key Words: Enterococci, enzymatic activity of cellulases, *Escherichia coli*, Lactobacilli, probiotic

Introduction

Probiotics nowadays offer broad application opportunities. They have a very favourable biological effect on the host organism, and pose neither side effects nor environmental risks. These facts form the prerequisites for the utilisation of probiotics in a larger extent than thus far. Probiotics are said to have come of age (1). Probiotics are mainly utilised in agriculture, the food industry, and medicine. They are applied in farm animal nutrition to improve feed conversion and increase weight gains (2), and to influence functional digestive system development in young animals (3). Furthermore, they are used as starting cultures in food products (4,5) and in the preventive therapy of human and animal diseases (6). As for probiotics use in human and veterinary medicine and farm animal nutrition, their biomedical impact is very significant, based on their inhibition effect against pathogens, their optimising influence on digestive processes, their stimulating effect on the immune system, and their anti-tumour and anti-cholesterol activity (7,8).

Our experiments with chickens and turkeys focused on testing probiotic preparations in which the active substance was represented by *Enterococcus faecium*, the lactic acid bacterium.

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We investigated the antagonism of *Enterococcus faecium* against *Escherichia coli*, its positive effect on propagation of Lactobacilli and stimulation of enzymatic activity, which would have a positive influence on the metabolism of the host macro-organism.

In general, the literature seems to have focused little on this topic. Instead, more attention had been devoted to the investigation of zootechnical characteristics, for example, the growth ability of animals, feed conversion, general well being of animals, and testing of probiotic preparations based on other micro-organisms.

Hence, a selection of appropriate micro-organisms is important. The present testing of a wide spectrum of randomly chosen natural and sometimes even collection strains of micro-organisms is time consuming and does not solve the problem sufficiently quickly. Apparently, an appropriate way of solving the problem is primary laboratory testing of the strains aimed at mutual antagonisms of the bacteria, growth rate in the intestine and adherence ability to intestine epithelium. Since this adherence is specific (especially of Lactobacilli) it will be necessary to examine whether it is not more purposeful to develop probiotics for individual categories of animals.

It is obvious that commercial preparations have to be standardised, produced in an appropriate applicable form and contain a declared number of exactly defined living micro-organisms.

The primary aim of this study was to investigate the effect of probiotic use on microbiological and biochemical characteristics in chicken and turkey caeca during fattening and detection of *Enterococcus faecium* by polymerase chain reaction. The investigated characteristics were verified by 2 trials.

Materials and Methods

Quantitative microbiological and biochemical analysis

Applied methods:

Plate diluting method

Determination of CFU counts: Plate diluting was applied for quantitative CFU count determination of respective groups of micro-organisms in 1 g of substrate.

Gelatinous nutritive substrate in petri dishes was inoculated with 1 ml of chyme samples by the pour plate method (*Lactobacillus* sp.) and on the surface (*Escherichia coli*, faecal Enterococci) in 3 replications. Homogenised samples of faecal chyme (chyme was put into sterile petri dishes) were prepared in advance by sequential diluting based on decimal dilution.

Isolated species, genera and groups of micro-organisms and their fundamental identification signs are given in the literature (9).

Investigation of enzymatic activity of the cellulases

This was carried out according to the Miller method (10). Cellulase activity (from 1% carboxymethylcellulose) was measured by the method of releasing reducing sugars. Results were expressed in CMC units.

Verifying trials

In this experiment, quantitative counts of individual groups of micro-organisms in the caeca of 49-day-old chickens were investigated. The trial was carried out on an experimental basis of the Department of Poultry Keeping and Small Farm Animals at Slovak Agricultural University in Nitra.

Fattening itself went on from 1 to 49 days of chicken age. One-day-old chickens of the Inra Vedet 220 breed were randomly distributed into 4 groups as follows:

1. Treatment-control group without application of probiotic preparation,
2. Treatment-spraying of egg set with *Enterococcus faecium* at the concentration of 4.10^{11} CFU.g^{-1} at its displacing from prehatchery to posthatchery (after 18 days application of 1.5 g.l^{-1} H_2O per 100 eggs),
3. Treatment-spraying of chicken hatchlings with *Enterococcus faecium* at the concentration of 4.10^{11} CFU.g^{-1} on the body surface (application of 1.5 g.l^{-1} H_2O per 100 hatchlings),
4. Treatment-combination of 2nd and 3rd treatment (spraying of egg set at its displacing from prehatchery to posthatchery and spraying of hatchlings on their body surface).

The chickens were kept in one-storey cage technology. Microclimatic conditions were maintained on the level of large-scale production parameters. The

following feeding mixtures were used in the trials: HYD 01 (the first 2 weeks), HYD 02 (from the beginning of the 3rd to the end of the 5th week), HYD 03 (from the beginning of the 6th to the end of the 7th week)

In the trial with turkeys, the quantitative representation of micro-organism groups in the caeca of turkeys was researched after the application of a probiotic preparation based on *Enterococcus faecium*. Samples were taken at the turkey age of 6 and 11 weeks, respectively. Fattening itself went on from 1 to 77 days of the age of turkeys. One-day-old turkeys of the Large White hybrid were randomly distributed into 5 groups as follows:

1. Treatment-control without probiotic application,
2. Treatment-addition of probiotic preparation at the concentration of $5.10^{10}.g^{-1}$ into water,
3. Treatment-addition of probiotic preparation at the concentration of $5.10^{5}.g^{-1}$ into feed 3 times a week
4. Treatment-addition of probiotic preparation at the concentration of $5.10^{5}.g^{-1}$ into feed twice a week
5. Treatment-addition of probiotic preparation at the concentration of $5.10^{5}.g^{-1}$ into feed once a week.

Table 1. In the 1st treatment, the standard feeding mixture was applied. In the 2nd treatment, a probiotic preparation containing 5.10^{10} of *Enterococcus faecium* germs in 1 g of the preparation was added into the drinking water in the morning drinking at the following graded rates:

1st week	0.05 g per day	6th week	0.30 g per day
2nd week	0.10 g per day	7th week	0.35 g per day
3rd week	0.15 g per day	8th week	0.40 g per day
4th week	0.20 g per day	9th week	0.45 g per day
5th week	0.25 g per day	10th week	0.50 g per day

In the 3rd treatment, a preparation containing 5.10^{5} of *Enterococcus faecium* germs in 1 g of the preparation was added 3 times a week (Monday, Wednesday, Friday) at the rate of 3 g per 30 ml of drinking water in the morning in the 1st week and 0.5 g per turkey from the beginning of the 2nd week in the form of preparation mixture with a small amount of water applied to the feed.

In the 4th treatment, a probiotic preparation containing 5.10^{5} of *Enterococcus faecium* germs in 1 g of the preparation was added twice a week (Monday, Friday) at the rate of 3 g per 30 ml of drinking water in the morning during the 1st week and 0.5 g per turkey from the beginning of the 2nd week in the form of preparation mixture with a small amount of water applied to the feed.

In the 5th treatment, a probiotic preparation was added only once a week. The other conditions were the same as those in the 3rd treatment.

During the trials, the turkeys were kept in one-storey cage technology. Microclimatic conditions were maintained on the level of large-scale production parameters. During the trial, the following feeding mixtures were used: HYD 12 (the first 2 weeks), HYD 13 (from the beginning of the 3rd to the end of the 7th week), HYD 14 (from the beginning of the 8th to the end of the 11th week)

The results of determinations are arranged in the tables, and evaluated graphically and statistically by analysis of variance (Scheffe test) using the statistical software package SAS. For graphical and statistical evaluation of the results, logarithmised values of the real numbers were used.

PCR (polymerase chain reaction)

The PCR was used to confirm unambiguously the presence of *Enterococcus faecium* (applied into the digestive tract) in the group of faecal Enterococci.

DNA isolation from faecal chyme of chicken and turkey

A Sample of chyme - about 0.5 g - was homogenised in 1.5-3 ml of physiological solution (sodium chloride isotonic solution). Solid parts of the homogenisate were removed by filtration through 4 layers of gauze. Filtrate was centrifuged at 12,000 operating speed for 10 min. After pellet percolation in TE solution (1 M Tris HCl + 0.5 M EDTA) (twice), 450 µl of TE (wash) solution, 50 µl of SDS (5 M solution) and 50 µl of SDS (6.5% solution), 20 µl of proteinase K (20 mg in 1 ml) was added to the pellet and it was incubated at 60 °C overnight. After centrifugation at 10,000 operating speed for 5 min, 500 µl of phenol was added to the supernatant, which was mixed and left for 5 min and then again centrifuged at 10,000 speed for 5 min. Six hundred microlitres of chloroform + isoamylalcohol (1:24) was added to the water phase (in a clean Eppendorf test tube) and after mixing the samples were left for a short time and then

repeatedly centrifugated. This procedure (phenol + chloroform) was repeated twice. After the addition of 96% ethanol to the water phase in a ratio of 1:2 the samples were left at -20 °C for 10-12 h. After centrifugation at 10,000 speed for 10 min, the sediment was dried out and dissolved in 20 µl of TE solution.

PCR conditions

The experimental design of the study is explained at the beginning of the methodology by Dutka-Malen et al. (11). For amplification, the following sequences of primers were used: *Enterococcus faecium*, direct: 5´ TAGAGACATTGAATATGCC 3´; reverse: 5´ TCGAAATG TGCTACAATC 3´; length of the fragment: 550 bp.

Enterococcus faecalis, direct: 5´ ATCAAGTACAGT TAGTTCT 3´; reverse: 5´ ACGATTCAAAGCTAACTG 3´; length of the fragment: 941 bp.

PCR reaction was running in thermocycler MJ Research PT 150.

Temperature profile

The 1st cycle: 94 °C, 5 min. The next 32 cycles: 94 °C, 1 min; 54 °C, 1 min; 72 °C, 1.5 min. Last cycle: 72 °C, 10 min. Amplified products were evaluated electrophoretically on 3% agarose and visualised under UV transilluminator by means of ethidium bromide.

Results

The application of probiotic preparations based on *Enterococcus faecium* positively influenced the faecal microflora of chickens and turkeys (Figure 1).

In the first trial with chickens, decreasing counts of *Escherichia coli* CFU were found in all 3 experimental treatments, compared to the control treatment. This means that bacteria of *Enterococcus faecium* reduced moderately the CFU counts of *Escherichia coli*. Similarly, we did not detect an increase in CFU counts of faecal Enterococci in 1 g of faecal chyme in experimental treatments compared to the control treatment (Figure 1).

We established a marked increase in CFU counts of Lactobacilli in the 2nd treatment, compared to the control sample. Other experimental treatments (Tables 2 and 3) also yielded an increase in Lactobacilli counts, but not as marked as those in the 2nd treatment.

In the 3rd treatment, after the application of the probiotic preparation based on *Enterococcus faecium*, enzymatic activity of cellulases was approximately the same as that in the 3rd treatment and the control sample. Substantially higher enzymatic activity of cellulases in comparison to the control (Figure 1) as well as to the other 2 experimental treatments was determined in the 4th experimental treatment where a combination of spraying the set eggs and body surface of the chicken was applied.

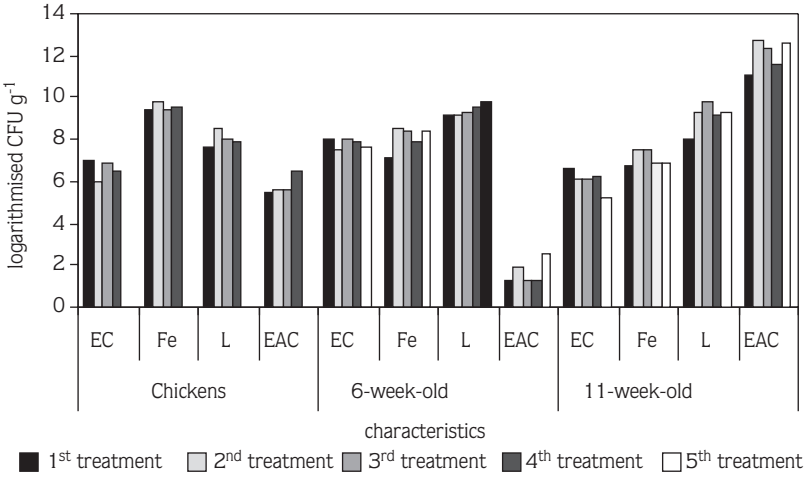

Figure 1. Effect of probiotics on respective characteristics in the caeca of chickens and turkeys.
EC – *Escherichia coli*, Fe – faecal Enterococci, L - Lactobacilli, EAC – enzymatic activity of cellulases in CMC units (carboxylmethylcellulase)

Table 2. Statistically significant achievement in the trials with 6-week-old turkeys.

Sources of variability	Sum of squares	Average of squares	F-value	Level of significance
E. coli	15.87050667	3.96762667	0.87	0.5173-
error	45.86666667	4.586666667		
total	61.73717333			
faecal Enterococci	3.91937333	0.97984333	53.54	0.0001+++
error	0.18300000	0.01830000		
total	4.10237333			
Lactobacilli	0.97133333	0.24283333	10.74	0.0012++
Error	0.22620000	0.02262000		
Total	1.19753333			
enzymatic activity	371642667	0.92910667	0.58	0.6865-
Error	16.12506667	1.61250667		
Total	19.84149333			

Table 3. Statistically significant achievement in the trials with 11-week-old turkeys.

Sources of variability	Sum of squares	Average of squares	F-value	Level of significance
E. coli	18.53062667	4.63265667	2.41	0.1184-
Error	19.23666667	1.92366667		
Total	37.76729333			
faecal Enterococci	17.71857333	4.42964333	1.42	0.2955-
Error	31.11106667	3.11110667		
Total	48.82964000			
Lactobacilli	4.81116000	1.20279000	8.67	0.0027++
Error	1.38760000	0.13876000		
Total	6.19876000			
enzymatic activity	5.56680000	1.39170000	1.54	0.2644-
Error	9.05260000	0.90526000		
Total	14.6194000			

Among the 6-week-old turkeys, the highest *Escherichia coli* CFU counts were detected in the control sample. All experimental treatments yielded lower counts compared to the control sample. However, the lowest count was found in the treatment where the probiotic preparation was applied to the drinking water, followed by the treatments with application of *Enterococcus faecium* at the concentration of 5.10^5.

The highest count of faecal Enterococci CFU in 1 g of faecal chyme of 6-week-old turkeys was detected in the treatment where the probiotic preparation based on *Enterococcus faecium* was added to the drinking water at the concentration of 5.10^{10}. About the same counts were also found in treatments where the preparation at concentration of 5.10^5 was applied once and 3 times a week. The lowest count of faecal Enterococci was recorded in the control treatment and the highest in the treatment with application of probioticum at 5.10^5 concentration to the feeding mixture of turkeys once a week (Figure 1).

Enzymatic activity of cellulases in the caeca of 6-week-old turkeys was the most intensive in the treatment where the probiotic preparation was applied to the feed once a week. It was twice as high as that in the control sample. When the probiotic preparation was applied to the drinking water, a higher value of enzymatic activity was also found, compared to the control (Figure 1).

Counts of *Escherichia coli* CFU in 11-week-old turkeys were, similar to the middle of the fattening period, the highest in the control treatment (Figure 1). The lowest count was detected in the treatment where the probiotic preparation was applied once a week. In other experimental treatments, the counts were approximately the same, but lower than those in the control sample. There was an obvious declining tendency of *Escherichia coli* CFU counts with increasing age of turkeys.

As for the faecal Enterococci, their counts declined in comparison with the 6-week-old turkeys. The highest counts were achieved when the probiotic preparation was added to the feed 3 times a week. If the probiotic preparation was applied once or twice a week, the counts of Enterococci were almost the same as those in the control treatment.

CFU counts of Lactobacilli in 11-week-old turkeys did not change markedly compared to 6-week-old ones. However, significant changes in counts occurred in the experimental treatments compared to the control sample. The highest count was found in the group where the probiotic preparation was added to the feeding ration 3 times a week.

Enzymatic activity of cellulases at this age increased several times when compared to 6-week-old turkeys. The highest activity was observed in the treatment with the probiotic preparation applied to the drinking water and the lowest in the control sample.

At the turkey age of 11 weeks, the counts of faecal Enterococci CFU changed not only within the framework of the individual treatments, but the counts were also reduced generally at this age in comparison to the 6-week-old ones, despite the fact that the digestive tract of turkeys was being supplemented with *Enterococcus faecium*, applied in the form of a probiotic preparation (Figure 1).

The presence of *Enterococcus faecium* (applied to the digestive system of poultry by the help of a probiotic preparation) in the caeca of chickens and turkeys was detected by means of the PCR. The amplification gene of *Enterococcus faecium* in the PCR produced 550 bp (Figures 2 and 3) and Enterococcus faecalis 941 bp (Figure 3).

In the trial with chickens, no statistically significant differences were found. In that with turkeys, such differences were established with the counts of faecal Enterococci and Lactobacilli at the age of 6 weeks and Lactobacilli themselves at the age of 11 weeks in turkeys (Tables 2 and 3).

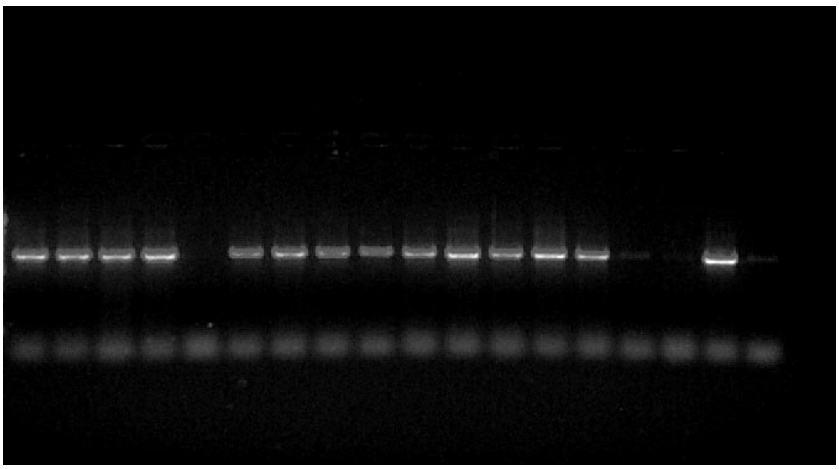

Figure 2. Agarose gel electrophoresis of PCR amplification product from DNA templates from *Enterococcus faecium*.

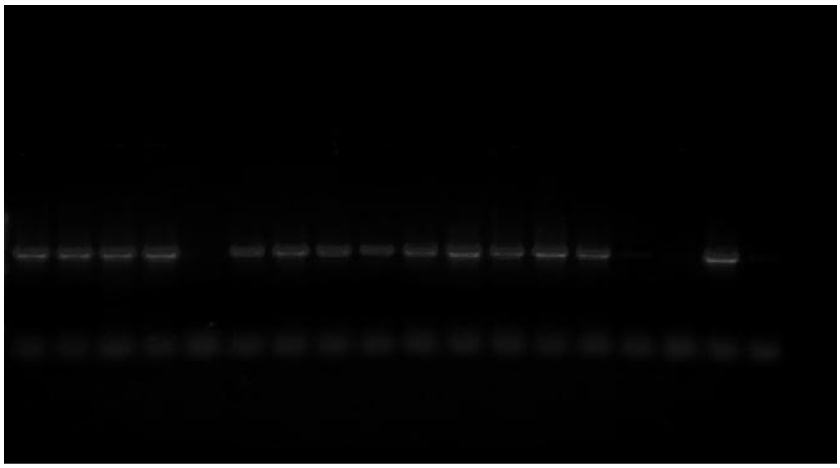

Figure 3. Specific PCR amplification product of the *Enterococcus faecium* and *Enterococcus faecalis*.

Discussion

The microbial populations in the gastrointestinal tracts of poultry play a key role in normal digestive processes and in maintaining animal health. Disease- and stress-induced changes in the physicochemical environment in the gastrointestinal tract, or simple changes in feed management practices can significantly influence the microbial populations and their effects on animal performance and health. In the last 5 decades, increased knowledge of the factors that influence the activities of micro-organisms in the alimentary tract has helped to define the critical role of these symbiotic organisms. Probiotics, competitive exclusion and direct-fed microbial feed supplements can be used as a strategic tool for managing these microbial populations. The aim of this trial was to study of effects of different levels of bacterial probiotic on broiler performance and some blood factors (12).

Probiotics are live, nonpathogenic bacteria that contribute to the health and balance of the intestinal tract. They are given orally to poultry to help the birds fight illness and disease (13).

The first recorded probiotics were fermented milks produced for human consumption. However, the subsequent development of the concept has been based on results obtained in animal experiments and the most current market in probiotics is for animal preparations (14). There is good evidence that the indigenous gut microflora provides protection against a wide range of infections. If that is so, do we need to resort to supplementation with probiotics? The answer lies in the way in which we maintain animals during the neonatal period (15).

This finding is in accordance with statements that probiotics on base *Enterococcus faecium* M-74 did not have a significant effect on the counts of Lactobacilli. Kačániová (16) found an increase in carboxymethylcellulase activity in faecal chyme after *Enterococcus faecium* application. According to Nava et al. (17), positive results were frequently achieved in trials with the application of probiotic preparations.

In contrast, if a probiotic preparation is applied to feed twice or 3 times a week, the values are lower compared to the control treatment. A positive influence of probiotics applied in the early age is stated by Hopkins et al. (18).

The effect of probiotics is derived from their capability to reduce the counts of undesired micro-organisms in the digestive tract mucous membranes. Thus, the host organism need not be focused on a permanent influence of unwanted micro-organisms present directly in the oral, nasal and intestinal mucous membranes. The role of protective microflora is irreplaceable in relation to the general well being of animals and achieving optimum weight gains (19).

References

1. Lee, Y.K., Salminen, S.: The coming of age of probiotics. Trends Food Sci. Technol., 1995; 6: 241-245.
2. Burgstaller, G., Ferstl, R., Alps, H.: Zum Zuchsatz von Milchsäurebakterien (*Streptococcus faecium SF - 68*) in Milchaustauschfuttermittel fur Mastkälber. Züchtungskunde, 1984; 56: 156-162.
3. Wallace, R.J., Newbold, C.J.: Probiotics for ruminants. In: Fuller, R.: Probiotics: the Scientific Basis. Chapman and Hall, London. 1992; 317-353.
4. Angelovičová, M., Michalik, I.: A test of enzymatic preparation in relation to performance and commercial utilization of feeds in broiler chickens. Zivocisna Vyroba, 1997; 42: 175-180.
5. Kivanc, M.: Antagonistic lactic cultures toward spoilage and pathogenic microorganisms in food. Nahrung, 1990; 34: 273-277.
6. Watkins, B.A., Miller, B.F., Neil, D.H.: In vivo inhibitory effects of *Lactobacillus acidophilus* against pathogenic *Escherichia coli* in gnotobiotic chicks. Poult. Sci., 1982; 61: 1298-1308.
7. Gibson, G.R., Beatty, E.R., Wang, X., Cummings, J.H.: Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin. Gastroenterology, 1995; 108: 975-982.
8. Christl, S.U., Gibson, G.R., Cummings, J.H.: Role of dietary sulphate in the regulation of methanogenesis in the human large intestine. Gut, 1992; 33: 1234-1238.
9. Holt, G.J., Krieg, N.R., Sneath, P.H.A., Staley, J.T., Williams, S.T.: Bergey's Manual of Determinative Bacteriology. Baltimore, Williams and Wilkins. 1994; 787.
10. Miller, G.L., Blum, R., Glennon, W.E., Buton, A.L.: Measurement of carboxymethylcellulase activity. Anal. Biochem., 1960; 23: 257-270.
11. Dutka-Malen, S., Evers, S., Courvalin, P.: Detection of glycopepetide resistance genotypes and identification to the species level of clinically relevant eneterococci by PCR. J. Clin. Microbiol., 1995; 33: 24-28.
12. Tannock, G.W.: Probiotics and prebiotics: Scientific aspects. Caister Academic Press, Wymondham, UK, 2005, 230.
13. Qureshi, M.A., Ali, R., Cheema, M.A., Ahmed, Z., Roth, H.: Immunmilk feeding increases growth and immune responses in broiler chicks. Int. J. Poult. Sci., 2004; 3: 305-312.
14. Fuller, R.: Probiotics 2: applications and practical aspects. Chapman and Hall, London UK, 1-209.
15. Tannock, G.W.: Probiotics and prebiotics: where are we going? Caister Academic Press, Wymondham, UK. 2002, 336.
16. Kačániová, M.: The effect of probiotics to microbiological and biochemical indexes in the ceacum of chickens and turkeys. PhD Dissertation. Slovak University of Agriculture in Nitra, SK. 2001.
17. Nava, G.M., Bielke, L.R., Callaway, T.R., Castañeda, M.P.: Probiotic alternatives to reduce gastrointestinal infections: the poultry experience. Anim. Health Res. Rev., 2005, 6: 105-118.
18. Hopkins, M.J., Sharp, R., MacFarlane, G.T.: Age and disease-related changes in intestinal bacterial populations assessed by cell culture, 16S rRNA abundance and community cellular fatty acid profiles. Gut, 2001, 48: 198-205.
19. Angelovičová, M.: The effect of *Streptococcus faecium* M-74 based probiotic on the performance of laying hens. Zivocisna Vyroba, 1996; 41: 391-395.