Effect of *Enterococcus faecium* M74 Strain on Egg Yolk Fat and Cholesterol

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Abstract
The aim was to evaluate the functional efficiency of a probiotic strain *Enterococcus faecium* M74 in the feed on egg yolk weight, egg yolk fat and cholesterol contents of Shaver Starcross 288 hens. Feed in the experimental group was enriched with a probiotic additive containing of $5*10^9$ viable *Enterococcus faecium* per g. Egg samples a total 30 pcs per group were collected during the first egg-laying period at week 28 and 38 of hens´ age. Non-significantly lower of egg yolk weight was observed in the experimental group at all sampling times compared with their respective controls. Non-significantly lower of egg yolk weight was observed in the experimental group at all sampling times compared with their respective controls. Significantly lower concentrations of egg yolk cholesterol were found in the experimental group at week 28, and week 38 in compare with controls. In conclusion, the addition of probiotic strains *Enterococcus faecium* M74 to the feed of Shaver Starcross 288 hens reduced cholesterol in egg yolk at all sampling times. Even though the hypocholesterolemic mechanism of probiotics has not yet been fully understood, it is an established fact that cholesterol and bile salt metabolism are closely linked. However, the hypocholesterolemic mechanism of probiotics based on the bile salt hydrolase activity hypothesis has not yet been sufficiently elucidated.

Keywords: cholesterol, egg yolk, fat, feed, *Enterococcus faecium* M74, probiotics

1. Introduction

Probiotics are living microbes given orally to proliferate in the gastrointestinal tract of the host and create beneficial conditions for nutrients' utilization [1-3]. Probiotic produce a positive balance of digestive microflora and limiting the damage caused by pathogenic bacteria, improves epithelial cell integrity and increased immune response [3-7]. Recently, emphasis has been given to the selection, preparation and application of probiotic strains, especially lactic acid bacteria [8]. Natural adaptation of lactic acid bacteria to intestinal environment and the lactic acid produced by them have provided advantages for these organisms over other microorganisms used bacteria. Species that have traditionally been regarded safe probiotics *are Enterococcus*, *Bifidobacteriaum* and *Bacillus* [10]. *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Escherichia coli*, *Lactococcus*, *Streptococcus*, *Pediococcus* species, and a range of yeast species and non-defined mixed cultures have been used [11-14]. However, even those belonging to the same species can have different strains and even these different strains from the same species can have different metabolic activities. These bacteria are used alone or in combination [15, 16]. Preventive application of probiotics achieved better utilization of nutrients and they have a positive effect on environment of gastrointestinal tract [17]. Several studies demonstrated that the supplementation of probiotics to poultry diets increased performance of the birds, stabilized the intestinal microbial flora [18, 19]. Other factors that might justify the variations in the effects of probiotics in poultry

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are: variations in the persistence of administered strains (relative intestinal concentration) [20, 21], stability during the manufacturing of feed [21], absence of statistical analysis of data in previous studies, experimental protocols not clearly defined, micro-organisms not identified [22], viability of organisms not verified [11, 22], as well as the fact that in many studies, the origin of microorganisms in probiotics was not reported [20]. La Ragione and Woodward [23] verified that the administration of viable spores of *Bacillus subtilis* to birds free from specific pathogens challenged with *C. perfringens* reduced the number of pathogens in the spleen, duodenum, colon and cecum, reporting similar results with a probiotic based on *Lactobacillus johnsonii* [24]. In experiment was tested effect of 0.10% of probiotics – *Enterococcus faecium*. *Enterococcus faecium* CECT 4515 was included in in the probiotical preparative protected by layers of polysaccharides, which are allowed to pass the stomach, protection is against its pH and gall salts, also partially are protected against high temperatures. The results of this experiment suggest that the use of probiotics as a feed supplement has a appropriate effect on the populating of the digestive tract. The obtained results suggest that the probiotic colonization positively affects the digestive tract and namely with the numbers of *Enterococcus* sp., and *Lactobacillus* sp. Which are higher compared with the numbers of control group. Compared with the control group were the numbers of *Enterobacteriaceae* sp. significantly lower. Numbers of *Lactobacillus* sp. ranged on average around 8.59 log CFU per g. That was decreased of the amount of *Enterococcus* sp. in average of 6.56 log CFU per g, which may be due to the high competition in the digestive tract [25]. Treatments of Aghai et al. [26] included three probiotic concentrations (0, 1000 and 2000 g per ton of feed). Commercial probiotic was used Bioplus 2B. The product contained 2 strains of bacilli, *Bacillus subtilis* (CH 201) and *Bacillus licheniformis* (CH 200) with a minimum of $3.2*10^9$ CFU per g of the product. Results showed that using probiotic had significant effect ($p<0.05$) on yolk cholesterol as addition probiotic decreased these trait (0 g per ton of feed 11.73 mg per g, 1000 g per ton of feed 10.10 mg per g, 2000 g per ton of feed 10.80 mg per g). Similarly, Haddadin et al. [27] states that the inclusion of *Lactobacillus acfdophflus* in which hen diets at levels up to four million live cells per gram of feed decreased cholesterol levels in egg yolk by 18.8%. Mahdavi et al. [28] states that the use of *Bacillus subtilis* and *Bacillus licheniformis* reduced cholesterol egg laying hens. Haddadin et al. [27] reported that probiotics reduce the plasma cholesterol and triglyceride. The use of probiotic may active the lactic acid producing bacteria, production of enzymes disintegrating bile salts and de-conjugating them. In low pH solvability of non-conjugated bile acids reduced, thus they absorbed decrease from the intestine [29]. The experiment was carried out on the broiler chicks. There was fed with feed enriched by probiotics *Bacillus subtilis*. Statistically significant ($P<0.05$) change of pH was confirmed in the intestine on the end experiment (42 days) in compare with control group [30]. Probiotic such as lactobacilli can assimilate cholesterol and de-conjugated bile acids and this leads to a reduction in serum cholesterol levels [31, 32]. Experimental was to evaluated the functional efficiency of a probiotic strain *Enterococcus faecium* M 74 in the feed mixture on selected biochemical, haematological and production parameters in blood of Isa Brown hens. Feed mixture in the experimental group was enriched with probiotic preparation containing of $5*10^9$ viable *Enterococcus faecium* M 74 per gram. Blood samples were collected during the egg-laying period at 5, 25 and 45 weeks of production. Significantly lower concentrations of total cholesterol and total lipids in blood plasma were observed in the experimental group at all sampling times compared with their respective controls. Concentrations of triglycerides did not differ. Average egg weight was not significantly affected by probiotic addition [33]. Elkin and Yan [34] reported that cholesterol content in the eggs is influenced by genetic factors, diet composition, lay intensity and layer age. Haddadin et al. [27] and Hargis [35] suggested a link between serum and egg lipid. So reduce serum cholesterol can lead to a reduction yolk cholesterol levels.

The aim was to evaluate the functional efficiency of a probiotic strain *Enterococcus faecium* M 74 in the feed on egg yolk weight, fat and cholesterol of Shaver Starcross 288 hens.

2. Materials and methods

Feed in the experimental group was enriched with a probiotic feed additive containing of $5*10^9$ viable *Enterococcus faecium* M74 per g. Egg samples a total 30 pcs were collected during the first egg-laying period at 28 and 38 weeks of production. Lactiferm is a feed additive containing viable cells of *Enterococcus faecium*. The probiotics is not pathogenic for the target species, the strain lacks the marker genes associated with human clinical isolates, the strain is free from acquired antibiotic resistance determinants, the end-products of the metabolism of the species are typical of lactic acid bacteria, and do not raise concerns, the additive does not contain excipients of concern. Powder form (basic 50): concentrate of *Enterococcus faecium* M74 NCIMB 11181 ca. 12-16%, maltodextrin as carrier ca. 84-88%, and guaranteeing minimum concentration of active agent of $5*10^{10}$ CFU per g. The strain is deposited at the National Collection of Industrial and Marine Bacteria collection (UK) with the accession number NCIMB 18111. The strain has been identified as *Enterococcus faecium* by Multilocus Sequence Typing of atpA, rpoA and pheS genes provided that the same dose is given [36].

3. Results and discussion

Experimental was to evaluated the functional efficiency of a probiotic strain *Enterococcus faecium* M 74 in the feed mixture determined for laying hens on egg yolk weight, fat and cholesterol in egg yolk of Shaver Starcross 288. Dry matter – drying the sample under prescribed cholesterol in egg yolk of Shaver Starcross 288. Dry matter – drying the sample under prescribed conditions in the oven, type J. R. Selecta s.a., at 105°C. The fat in the egg yolk – sample dried egg yolk with sea sand was quantitatively transferred to an extraction device type DET-GRAS N, which was extracted with petroleum ether solvent extraction. After extraction, the sample was evaporated to petroleum residues and dried in an oven at 100°C. Cholesterol in egg yolks – manually separated egg yolk was homogenized in a laboratory blender. The homogenized sample was weighed 5 g. To a sample was added to four times the pipetting amount of physiological solution 18.5 g of NaCl (1 L H_2O). The mixture was thoroughly homogenized. Cholesterol was determined by Ingr and Simeon [37].

Raw data were statistically evaluated by analysis of variance (ANOVA) in SAS program system [38]. Differences were tested using t-test.

Table 1. Average egg yolk weight (g per pc)

	Week 28			Week 38		
Group	$\bar{x}$	SD	cv	$\bar{x}$	SD	cv
Control	15.2	14.85	5.55	16.37	1.00	6.10
Enterococcus faecium	0.84	1.20	8.08	14.72	1.13	7.71
t-test	p>0.05			p>0.05		

$\bar{x}$ – average, SD – standard deviation, cv – coefficient of variation, p>0.05 – statistically non-significant

We reached results of egg yolk weight, which confirmed the literature knowledge of the influence of probiotics on egg yolk weight [39-42]. In literary knowledge were not statistically significant differences p>0.05 between the group with probiotic supplement and a control group.

Table 2. Average total fat content in egg yolk (g per pc)

	Week 28			Week 38		
Group	$\bar{x}$	SD	cv	$\bar{x}$	SD	cv
Control	5.60	5.10	8.21	5.03	0.86	16.99
Enterococcus faecium	0.46	0.66	12.96	4.91	0.54	11.05
t-test	p<0.05			p>0.05		

$\bar{x}$ – average, SD – standard deviation, cv – coefficient of variation, p<0.05 – statistically significant, p>0.05 – statistically non-significant

Non-significantly lower of egg yolk weight was observed in the experimental group at all sampling times compared with their respective controls

Table 3. Average total cholesterol content in egg yolk (mg per pc)

	Week 28			Week 38		
Group	$\bar{x}$	SD	cv	$\bar{x}$	SD	cv
Control	221.35	191.46	6.76	258.48	18.74	7.25
Enterococcus faecium	14.97	33.86	17.68	224.29	21.60	9.63
t-test	p<0.05			p<0.05		

x̄ – average, SD – standard deviation, cv – coefficient of variation, p<0.05 – statistically significant

Recently, considerable attention has been paid to the potential of probiotics in altering lipid metabolism. This interest stems from the growing evidence that probiotics reduce cholesterol concentrations in egg yolk [27, 43, 44]. The mean yolk cholesterol levels in the experiment was reduced from 257.11 mg in the antibiotics group to 221.05 mg per egg yolk in the 500 mg of dried *Bacillus subtilis* culture per kg supplemented group [45]. These data were confirmed our results, which were statistically significant (p<0.05). It is possible that some of the organisms present in the probiotics preparation could assimilate the cholesterol present in the gastrointestinal tract for their own cellular metabolism, thus reducing the amount absorbed, as suggested by [46, 47]. Kalavathy et al. [48] indicated that lactic acid bacterial strains are able to alter the enterohepatic cycle and reduce cholesterol through the assimilation of dietary cholesterol into the bacterial cells and the bile salt hydrolase activity in the intestine. Another reason for the decrease of cholesterol in probiotics-fed hosts is that probiotics are able to inhibit hydroxymethyl-glutaryl-coenzyme A, an enzyme involved in the gastrointestinal tract [49]. Oral administration of probiotics has been shown to significantly reduce cholesterol levels by as much as 22 to 33% [50, 51] or prevent elevated cholesterol levels in mice fed a fat-enriched diet [52].

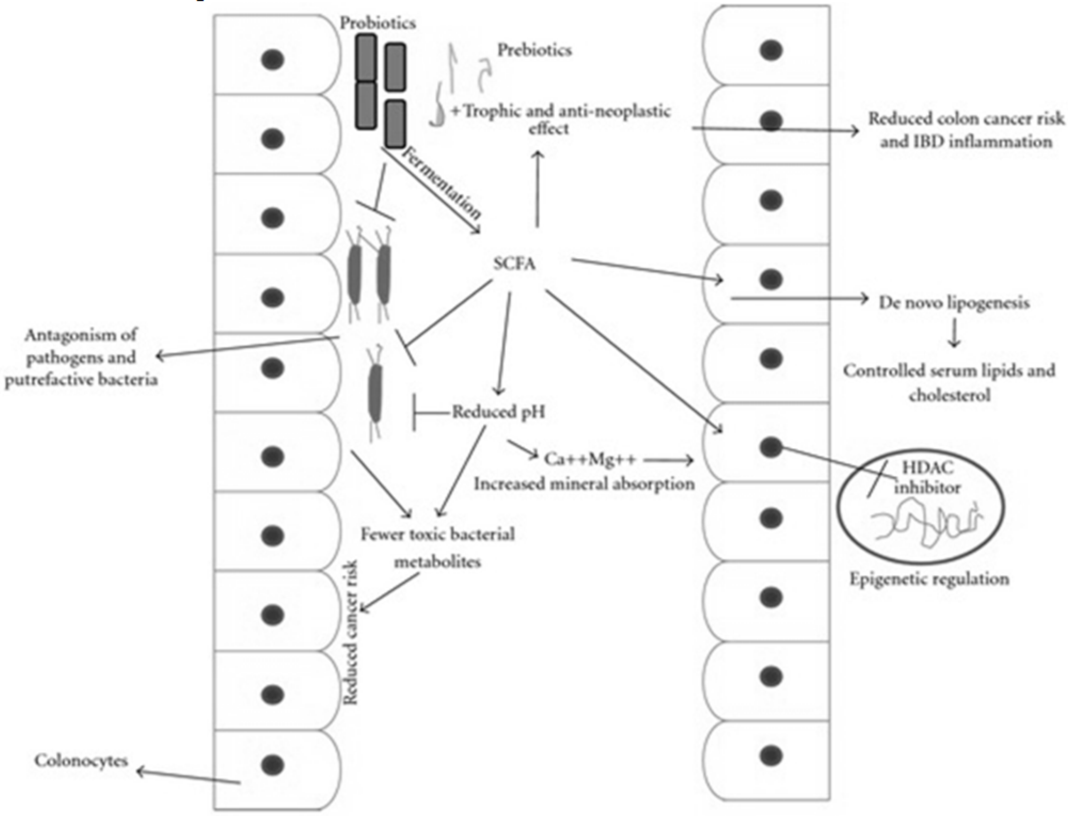

Figure 1. Role of probiotics' metabolites as epigenetic approach to control high cholesterol and colon cancer [53]

These cholesterol-lowering effects can be partially ascribed to bile salt hydrolase activity (other possible mechanisms include assimilation of cholesterol by the bacteria, binding of cholesterol

to the bacterial cell walls, or physiological actions of the end products of short-chain fatty acid fermentation (Figure 1) [53]. Deconjugated bile salts are less efficiently reabsorbed than their conjugated counterparts, which results in the feces. Also, free bile salts are less efficient in the solubilization and absorption of lipids in the gut. Therefore, deconjugation of bile salts could lead to a reduction in serum cholesterol either by increasing the demand for cholesterol for de novo synthesis of bile acids to replace those lost in feces or by reducing cholesterol solubility and thereby absorption of cholesterol through the intestinal lumen. Since unconjugated bile acids are less efficient than conjugated molecules in the emulsification of dietary lipids and the formation of micelles, bile salt hydrolase activity may compromise normal lipid digestion and the absorption of fatty acids and monoglycerides could be impaired [54].

4. Conclusions

Probiotic bacteria, especially feed addition *Enterococcus faecium* M74 NCIMB 11181 ca. 12-16%, maltodextrin as carrier ca. 84-88%, guaranteeing a with a minimum concentration of active agent of $5*10^{10}$ CFU per g, have demonstrated fat- and cholesterol-lowering efficacy in egg yolk in compare with control group. Non-significantly lower of egg yolk weight was observed in the experimental group at all sampling times compared with their respective controls. Non-significantly lower of egg yolk weight was observed in the experimental group at all sampling times compared with their respective controls. Significantly ($p<0.05$) lower concentrations of egg yolk cholesterol were found in the experimental group at week 28 from 221.35 to 191.46 mg per pc egg yolk, and at week 38 from 258.48 to 224.29 mg per pc egg yolk, respectively, in compare with controls. Probiotics have received much attention on their proclaimed health benefits. Despite these claimed benefits carried out for the last two decades, a decisive outcome has failed to be reached due to excretion of larger amounts of free bile acids in controversies raised. Even though the hypo-cholesterolemic mechanism of probiotics has not yet been fully understood, it is an established fact that cholesterol and bile salt metabolism are closely linked. However, the hypocholesterolemic mechanism of probiotics based on the bile salt hydrolase activity hypothesis has not yet been sufficiently elucidated.

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