# COMBINED EFFECT OF BACTERIOCIN-PRODUCING *ENTEROCOCCUS FAECIUM* CCM4231 STRAIN AND SAGE IN RABBITS

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## ABSTRACT

The aim of this experiment was to study the combined effect of biological active matters – natural substances like a bacteriocin-producing strain with probiotic properties *Enterococcus faecium* CCM4231 and sage plant extract. Ninety six weaned rabbits (age of 35 days) were divided into three experimental (EG1, EG2, EG3) groups and one control (CG) group. Animals in EG1 received *E. faecium* CCM4231 strain ($10^9$ cfu/animal/day into water); for rabbits in EG2 the sage plant extract was applied at doses 10 μl/animal/day into water; rabbits in EG3 received both *E. faecium* CCM4231 strain and sage plant extract at the same doses as in the groups EG1 and EG2. The experiment lasted 42 days; natural substances were applied for 21 days. Faeces were sampled at the start of the experiment, at day 7 (1 week of administration), at day 21 (3 weeks of *E. faecium* administration) and at day 42 (3 weeks after cessation of application) to measure the counts of rabbit intestinal microflora as well as the occurence of *Eimeria* sp. oocysts. Samples of caecal contents (3 animals in each group) were collected at days 21 and 42 to determine bacterial counts. The immunological parameter - phagocytic activity was examined at days 1, 21, 42. The inhibitory effect of *E. faecium* CCM4231 strain (EG1) was observed at day 21 (3 weeks of CCM4231 application) by decrease of *E. coli* (difference of 1.84 $\log_{10}$ CFU/g) as well as *Clostridium*-like sp.(difference of 1.1 $\log_{10}$ CFU/g) in comparison to CG. Inhibitory effect against coagulase-negative staphylococci ($P<0.01$) was also noted at day 42 (3 weeks of in EG1 (*E. faecium* CCM4231) and in EG3 (the combination of *E. faecium* CCM4231 with sage) in comparison with CG. No changes in the other bacteria were noted. The bacterial counts in caecum were lower than those in faeces and no significant changes were found. At days 21 and 42, immunostimulative effect of the natural substances ($P<0.001$) was noted in the experimental groups (EG1, EG2, EG3) in comparison to CG. The reduction of *Eimeria* sp. oocysts was demonstrated after application of each natural substance in the EG1, EG2 and EG3 compared to CG at day 21. Thus, bioadditives represent promising alternatives to synthetic preparations.

**Key words**: Rabbit, Enterococci, Sage, Probiotic.

## INTRODUCTION

Rabbit production for meat is an important livestock activity in many countries. Advantages to grow rabbits are high fertility rate with rapid rate of growth; high feed efficiency and early marketing age; high muscle-bone ratio. Rabbit meat has high protein content, low level of cholesterol and low total lipids; so, it is very useful in human diets (Hernández *et al.*, 2000). Especially rabbits after weaning are susceptible to various infectious and diarrhoeal diseases caused by e.g. *Escherichia coli* or *Clostridium* sp, however. Moreover, eimeriosis represents permanent problem in rabbit breeding (Suvegová, 2004). Because the use of antibiotics as feed additives has been banned by EU and the use of coccidiostats will be forbiden in EU in near future, new alternative replacements are searched. Nowadays, natural substances such as probiotics; phytoaditives; enzymes; organic acids have been used in veterinary medicine with increasing frequency (Marounek *et al*., 2002; Lauková *et al.*, 2006a).

The genus *Enterococcus* belongs to the group of microorganisms known as lactic acid bacteria (LAB). Enterococci are Gram-positive, facultative anaerobic bacteria and have the ability to produce bacteriocins, small peptides with antimicrobial activity towards more or less related bacteria; some of the bacteriocins have a narrow spectrum of activity while others inhibited a wide variety of bacteria. Bacteriocin-producing strain with probiotic properties *E. faecium* CCM4231, isolate from our Laboratory of Animal Microbiology was found as the first bacteriocin-producing strain of ruminal origin with probiotic character (Lauková *et al.*, 1993); it was successfully applied into many ecosystems to reduce spoilage flora (Lauková *et al.*, 1998; Lauková *et al.*, 1999). Moreover, this strain is able to transform linoleic acid to conjugated linoleic acid (CLA; Marciňáková, 2006). Based on our previous results with application of CCM4231 strain in various ecosystems, we decided to apply it in the rabbit breeding.

The genus *Salvia* (sage) encompasses about 900 species of plants belonging to the mint family Lamiaceae (Labiatae; Gali-Muhtasib *et al.*, 2000). Different types of extracts of *Salvia officinalis* has possessed anti-microbial, antioxidant, anti-inflammatory, hypoglycemic and anti-mutagenic bioactivities (Baricevic *et al.*, 2001; Alarcorn-Aquilar *et al.*, 2002).

The aim of this study was to test combinative use of bacteriocin-producing *Enterococcus faecium* CCM4231 strain with probiotic properties and sage plant extract. Their antimicrobial activity in the intestinal tract of rabbits; the impact on zootechnical, immunological and biochemical blood parameters and occurence of *Eimeria* spp. oocysts were investigated.

## MATERIALS AND METHODS

### Animals and experimental design

A group of 96 rabbits, 5-weeks old (male sex, Hy-Plus breed), were used in this experiment. All care and experimental procedures involving animals followed the guidelines stated in the Guide for the Care and Use of Laboratory Animals which was accepted by Slovak Governmental Veterinary Office. Rabbits were divided into 4 groups; the experimental groups (EG1, EG2, EG3) and one control group (CG) of 24 animals each. Rabbits were kept in standard cages, two animals per cage. All animals were fed a commercial diet for growing rabbits (ANPRO.FEED, VKZ Bučany, Slovakia, Table 1) with free access to water. Rabbits in EG1 received every day orally (in known amount of drinking water, dose of $10^9$ cfu/animal/day) bacteriocin-producing strain *E. faecium* CCM4231 with probiotic effect (rifampicin resistant variant- $rif^R$); rabbits in EG2 received sage plant extract (*Salvia officinalis* extract contained 24% of thujone, 18% of borneol, 15% of cineole; Calendula company, Nová Ľubovňa, Slovakia) in drinking water (dose 10 μl for animal per day); animals in EG3 received *E. faecium* CCM4231 (500 μl/animal/day) strain as well as sage plant extract (10 μl/animal/day). Experiment lasted for 42 days.

Samples of faeces were taken at the beginning of the experiment, at day 7 (1 week of natural substances administration-NSA), at days 21 (3 weeks of NSA) and 42 (3 weeks after cessation of NSA) to monitor the stability and effect of *E. faecium* CCM4231 as well as sage extract alone and in their combination in rabbits. The samples were treated by a standard microbiological method using appropriate dilutions in Ringer solution (pH 7.0; Oxoid Ltd., Basingstoke, Hampshire, England). The appropriate dilutions were plated onto Baird-Parker agar supplemented with egg yolk tellurite solution (Becton & Dickinson, Cockeysville, USA), Mannitol Salt Agar (Difco Laboratories, Detroit, USA) and *Clostridium difficile* agar with selective supplement (SR0096E) and 7% (v/v) defibrinated horse blood (SR0050, Oxoid Ltd., Basingstoke, Hampshire, England) to enumerate coagulase-positive staphylococci (CPS) including *Staphylococcus aureus*, coagulase-negative staphylococci (CNS) and *Clostridium*-like sp. Mac Conkey agar and Cetrimide agar (Becton & Dickinson) were used to count *E. coli* and *Pseudomonas* sp. The plates were incubated at 30°C and/or 37°C for 24-48 h depending on the bacterial species and bacterial counts were expressed in colony forming units ($log_{10}$ CFU) per gram. Three animals of each group were slaughtered at days 21, 42 and the caecal contents were

sampled to count bacteria. They were serially diluted in Ringer solution and plated on the media mentioned above.

Biochemical parameters were examined at days 1, 21, 42: serum levels of proteins and lipids (g/l), cholesterol (mmol/l), glucose (mmol/l), calcium (mmol/l), glutathion peroxidase (U/ml) using commercial kit Randox (England). Moreover, the phagocytic activity (PA) was monitored and expressed as percentage of bacteria ingested per phagocyte (100 neutrophils) during a limited period of incubation of particules suspension and phagocytes in serum (Hrubiško *et al.,* 1981).

The zootechnical parameters (feed consumption, weight gain, feed conversion, mortality) were evaluated daily.

*Eimeria* sp. oocysts were enumerated in the faecal samples microscopically at days 1, 7, 21 and 42 of the experiment and expressed as counts of oocysts per 1 g of faeces (OPG). The samples were stored at 4°C and then evaluated by the quantitative flotation technique - McMaster method (Ministry of Agriculture, Fisheries and Food, UK, 1986).

**Table 1**: Ingredients and chemical composition and nutritive value of diets

| Ingredients (%): | | Composition (g/kg) | |
|---|---|---|---|
| Clover (grass) meal | 27.00 | Crude protein | 197.00 |
| Extracted sugar beet pulp | 10.00 | Crude fibre | 166.50 |
| Barley | 15.00 | Crude fat | 39.00 |
| Oats | 13.00 | Ash | 80.00 |
| Wheat bran | 6.00 | Organic matter | 921.00 |
| Soybean meal | 7.50 | Starch | 178.00 |
| Sunflower meal | 14.00 | Lysine | 7.50 |
| Monocalcium phosphate | 0.60 | Methionine+cysteine | 6.50 |
| Dicalcium carbonate | 0.90 | Cholinchloride | 0.80 |
| Salt | 0.30 | ME (MJ/kg) | 10.0 |
| Carob-breadfruit of *Ceratonia siliqua* | 2.50 | | |
| DL-Methionine+wheat meal | 0.10+0.10 | | |
| Mineral and vitamin premix[1] | 3.00 | | |

[1]Premix (per kg diet): vitamin A, 10,000 IU; vitamin $D_3$, 2,000 IU; vitamin E acetate, 30 mg; vitamin $B_2$, 5 mg; vitamin $B_6$, 2 mg; vitamin $B_{12}$, 8 mg; Ca, 9.25 g; P, 6.2 g; Na, 1.6 g; Mg, 1.0 g; k, 10.8 g; Fe, 327.5 mg; Mn, 80 mg; Zn, 0.7 mg

### Statistical Analysis

The results were quoted as mean ± standard deviation (SD), statistical evaluation of the results was performed by the one-way ANOVA and the Tukey test.

## RESULTS AND DISCUSSION

The inhibitory effect of *E. faecium* CCM4231 strain (EG1) was observed at day 21 (3 weeks of CCM4231 application) by decrease of *E. coli* (difference 1.84 $\log_{10}$ CFU/g) as well as *Clostridium*-like sp. (difference 1.1 $\log_{10}$ CFU/g) in comparison to CG. Reductive effect in EG1 (*E. faecium* CCM4231) and in EG3 (the combination of *E. faecium* CCM4231 with sage) against coagulase negative staphylococci ($P<0.01$) was also noted at day 42 (3 weeks of CCM4231 strain cessation) in comparison to CG. Lauková *et al.* (2006b) observed in rabbits even 2 weeks after probiotic and enterocin A-producing strain *E. faecium* EK13 cessation significant diference in *E. coli* counts between control group and experimental group ($P<0.001$). Moreover, Strompfová *et al.* (2006) reported an antimicrobial activity of *E. faecium* EK13 in piglets by the reduction of *E. coli* at day 7 after EK13 strain application ($P<0.001$). In addition, Simonová *et al.* (2006) showed the antimicrobial effect of selected bacteriocin-producing and probiotic strain *E. faecium* 2019 (CCM 7420; isolate from rabbit ecosystem (faeces). The other bacteria were not influenced by additives. In general, the bacterial counts in caecum were lower than in faeces and no significant changes in bacterial counts were detected there. The results of the antimicrobial activity of the essential oils, described by Delamare *et*

*al.* (2007) reveal that the oils of *Salvia officinalis* inhibited the growth of *Bacillus cereus, B. megatherium* and *B. subtilis*; partial inhibitory effect was observed against *E. coli* and *S. aureus.*

At days 21 and 42, immunostimulative effect of NSA (P<0.001) was noted in the experimental groups (EG1; EG2; EG3) in comparison to CG. In EG1, PA 38.0% ± 0.32 was measured; in EG2 27.7% ± 0.42; in EG3 33.0% ± 0.32 compared to CG (PA 22.5% ± 0.85) at day 21; while at day 42 (3 weeks after cessation of NSA) PA in rabbits of EG1 was 39.5% ± 0.65; EG2 (30.5% ± 0.96); EG3 (38.6% ± 0.24) in comparison to CG (Table 2). In spite of the higher % of PA in EG1, it seems that the combination of both, strain and sage extract have shown higher immunomodulatory effect (Table 2). Nofrarías *et al*. (2006) *e.g.* reported immunomodulatory effect of dietary plant extracts (carvacrol, cinnamaldehyd) which can affect intestinal morphology and immune cell subsets of gut tissues and blood in weaned pigs. Neutrophil polymorphonuclear leucocytes (granulocytes) are responsible for the non-specific immune response and in the first line share of phagocytosis intro-defence of the host to infectious and inflammatory actions (Escribano *et al*., 2005).

**Table 2**: Phagocytic activity during experiment in rabbits

| | Phagocytic activity (%) | | |
|---|---|---|---|
| | start of experiment | 21 days of experiment | 42 days of experiment |
| EG1 – EF CCM4231 | | 38.0 ± 0.32* | 39.5 ± 0.65* |
| EG2 – sage | 21.6 ± 4.22 | 27.7 ± 0.42* | 30.5 ± 0.96* |
| EG3 – sage with EF CCM4231 | | 33.0 ± 0.32* | 38.6 ± 0.24* |
| CG – control group | | 22.5 ± 0.85* | 20.4 ± 0.51* |

Phagocytic activity expressed in % as mean ± standard deviation (n=5); *P<0.001; day 21- 3 weeks after application, day 42- the end of experiment-3 weeks after cessation of application

The reduction of *Eimeria* sp. oocysts was demonstrated after application of each of natural substances (*E. faecium* CCM4231 strain; sage; combination of both substances) in EG1 (120.0 ± 11.5 OPG); in EG2 (30.0 ± 2.74 OPG); in EG3 (20.0 ± 2.74 OPG) comparing to CG (1184.0 ± 45.83 OPG) at day 21. Anticoccidial effects of green tea-based diets were evaluated in chickens by Seung *et al.* (2007). The green tea-fed chickens produced significantly reduced faecal oocysts (P<0.05) when compared to the *E. maxima*-infected group fed standard diet. The chamomile essential oil administration influenced *Eimeria* sp. oocysts in faeces of rabbits; the reduction of oocysts was recorded through the whole experiment (Simonová *et al.,* 2006).

The feeding of natural substances by rabbits did not influence biochemical and zootechnical parameters, as well as it has not negative effect on health status and growth performance of rabbits.

## CONCLUSIONS

Bacteriocin-producing *E. faecium* CCM4231 strain with probiotic properties in combination with sage showed the antimicrobial activity as well as immunomodulatory and anticoccidial effect in rabbit intestinal ecosystem. The other additional experiments are in process.

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