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Original article

The effect of dietary supplementation of sage plant extract and Enterocin M on the mucus in the the small intestine and caecum in rabbits

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Abstract

The aim of this study was to determine the beneficial effect of natural substances – enterocin M (Ent M; the proteinaceous substance produced by *Enterococcus faecium* CCM8558) and sage plant (*Salvia officinalis* L.) extract on the production of mucus in the rabbits small intestine and caecum. Sixty four *post*-weaned rabbits (meat line M91) were divided into three experimental groups (EG – Ent M; SG – sage extract; ESG – combination Ent M with sage extract) and control group (CG). The experiment lasted for 35 days, the natural substances were administered during the first 21 days, Ent M in EG/ESG, sage extract in SG/ESG. The beneficial effect on mucus production quantity occured in the duodenum ($p<0.001$) and jejunum ($p<0.01$) in ESG compared to that found in CG on day 21, the prolonged effect in EG in the duodenum ($p<0.001$) compared to that observed in CG at the end of the experiment and to that in EG on day 21. The novelty of the study is in the application and monitoring the effect of non-rabbit-derived probiotic strain (*Enterococcus faecium* CCM8558) bacteriocin – Enterocin M and sage plant extract on mucus quantity (expressed in gram) in different segments of the rabbit small intestine as well as the caecum. The results obtained indicate that supplementation of selected natural substances in the feed has the potent stimulatory effects on mucus production in the rabbit small intestine.

Key words: rabbit, enterocin, sage, mucus production, intestine

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Introduction

The mucus layer is a viscoelastic gel that lubricates the intestinal mucosa and protects the epithelial layer from mechanical damage and pathogen invasion, and forms a protective biofilm on the mucosal surface. Mucus is a constantly changing mixture of many secretions and exfoliated epithelial cells. The main determinants of the functional and physical properties of mucus are highly glycosylated and high molecular weight proteins – mucins synthesized and secreted by specialized goblet cells located on the mucosal surface and in the invaginated epithelial lining of the intestinal crypts. Mucins are classified according to their ability to form a gel: gel-forming (secreted) or non-gel-forming (membrane-bound) mucins as well as according to the net charge of the molecule into neutral or acid mucins (differentiated into sulfate-containing sulfomucins and sialic acid-containing sialomucins). The mucin oligosaccharides in the mucous gel represent a direct source of peptides, carbohydrates, and nutrients that allow the colonization of bacteria in the mucus layer of the intestinal tract (Boonzaier et al. 2013).

The small intestinal epithelium is covered by a single unattached mucus layer consisting of a protective mucus gel composed predominantly of mucin glycoproteins synthesized and secreted by goblet cells. Goblet cell function can be exposed as well as disrupted by certain factors (*e.g.* microbes, microbial toxins, cytokines) affecting the integrity of the mucus barrier, continually producing mucins for the retention of the mucus barrier under physiological conditions, but different factors (*e. g.* microorganisms, microbial toxins, viruses, cytokines, and enzymes) can have profound effects on the integrity of intestinal epithelium covered by a protective mucus. Enterocytes forms the intestinal mucus covered by transmembrane mucins and goblet cells produce the secreted gel-forming mucins (MUC2), which is the major mucin produced and secreted by the goblet cells in the intestinal epithelial tissue. Under normal physiological conditions, MUC2 secretion is necessary to replenish and maintain a suitable thickness of the mucous layer in the intestine, because this layer is often sloughed off by intestinal movement, chemical compounds and microbial derived factors (Horn et al. 2009). Different factors such as microbial colonization in the intestine can effect the production, secretion and composition of mucin (Aliakbarpour et al. 2012). The mucus is organized in a single unattached mucus layer in the small intestine and in two mucus layers (inner, outer) in the colon. The intestine epithelium is covered by a mucus layer which acts as a medium for protection, lubrication, transport, a physical barrier and a positive environment for the beneficial endogenous microbiota adapted to symbiotic life. The mucus layer provides homeostasis in the intestine by affecting several aspects of the intestinal biology (physical/chemical protection, immunomodulation, mucus production and growth). An intestinal part modulating the communication between the luminal contents including bacteria and the mucosa is the mucus layer and its secretion, which plays on important role in controling pathogen behaviour in the intestinal ecosystem (Szabóová et al. 2018).

The probiotics as well as bacteriocins (which could be produced also by some probiotic strains) are involved in the mechanism of intestinal defense, support as antagonists against pathogens, adaptive immunity, improve intestinal epithelial layer and the mucus production (Rajput and Li 2011). Nowadays, there is increased interest in the application of natural substances (plant extracts/phytoadditives or microorganisms with probiotic properties and their products) to achieve increased financial returns and quality of breeding at rabbit farms. Innovative methods involve the use of natural substances to prevent/eliminate diarrhoea and other diseases occurring in rabbits, especially in the post-weaning period as critical period in the management of rabbits due to the increased risk of mortalities (El-Ashram et al. 2020).

The rabbit from small animal category belongs to the experimental animals used frequently as research models. The advantages of the rabbit as a model experimental animal are as follows: the animal is very docile and non-aggressive and hence easy to handle and observe, it is widely bred, very economical compared with the expense of larger animals and it has short vital cycles (gestation, lactation, and puberty) (Mapara et al. 2012).

At the same the rabbit has sufficient production/ /economic value and its breeding is intended mainly for meat production. Rabbit meat offers excellent dietary nutritional properties with protein contents as high as 22.4 % in the loin (*musculus longissimus thoracis* and *lumborum*). The leanest cut of meat in the rabbit carcass is the loin, which contains an average lipid content of 1.8 g/100 g of meat. Rabbit meat has a moderately high energy value (from 603 kJ/100 g in loin meat to 899 kJ/100 g in foreleg meat) that essentially depends on its elevated protein content, which accounts for 80% of its energy value. Together with its increased protein content, rabbit meat contains high levels of essential amino acids. Based on fatty acid composition, rabbit meat is highly suited to human consumption. Unsaturated fatty acids account for approximately 60 % and polyunsaturated fatty acids for 27 to 33 % of total fatty acids. Rabbit meat continues to be considered for rural usage or limited to ethnic groups in developed countries

despite its outstanding dietetic properties (Dalle Zotte 2015). Nevertheless, there is an increased demand for rabbit meat on the market (Bivolarski and Vachkova 2014).

The aim of this study was to observe the effect of the feed supplementation of natural substances including plant extracts and microbiota with probiotic properties and their antimicrobial products bacteriocins on mucus production in different parts of the small intestine and caecum in rabbits.

Materials and Methods

The experiment was performed in co-operation with the Institute of Animal Physiology in Košice (Centre of Biosciences of the Slovak Academy of Sciences, Košice, Slovak Republic) and National Agricultural and Food Centre in Nitra (NAFC, Slovak Republic). The experimental procedures and the guidelines for the care and use of animals were followed appropriately and approved by the Slovak Veterinary and Food Administration as well as Ethic Committees. The rabbits of meat line M91, maternal albinotic line (crossbreed New Zealand White, Bouscat rabbit, Argente Champagnet rabbit) and paternal acromalictic line (crossbreed Nitra's rabbit, Californian rabbit, Big light silver) were used in this experiment. The rearing conditions in a large-capacity hall at the National Agricultural and Food Centre (Institute of Small Farm Animals, Nitra-Lužianky, Slovak Republic) were intentionally similar to those present at farms practising intensive rabbit husbandry. Rabbits after the weaning period (aged 35 days) came from litters of the same breed and they were kept in the same conditions at the same breeding facility. The allocation of experimental rabbits into groups was fully randomized. A number of 64 post-weaned rabbits (aged 35 days, both sexes) were divided into 4 groups (1 control - CG and 3 experimental groups: EG – EntM application, SG – sage plant extract, ESG – EntM in combination with sage plant extract) with 16 animals in each group. Rabbits were kept in standard cages D-KV-72 (0.61x0.34x0.33 m; Kovobel company, Domažlice, Czech Republic), 2 animals *per* cage under cycle of 16 h light and 8 h dark. The temperature and the humidity in the building were recorded continuously with a thermograph located at the same height as the cages. The heating and forced ventilation systems allowed the building air temperature to be maintained at 16±4°C throughout the experiment. Relative huminidity was about 70±5 %.

The animals were fed a commercial granulated/ /pelleted diet (pellet length about 3.5 mm) for growing rabbits (Anprofeed, VKZ Bučany, Slovak Republic) during the experiment with access to feed and water *ad libitum*. The rabbits in groups EG and ESG were administered EntM (prepared according to Mareková et al. 2007; at a dose 50 µL/animal/day, with activity 12 800 AU/mL, from day 0-1 to day 21) in drinking water. Activity of EntM was tested by the agar spot test according to DeVuyst et al. (1996) against the principal indicator strain *E. avium* EA5 (isolate from faeces of piglet, our laboratory). Rabbits in groups SG and ESG received (from day 0-1 to day 21) sage plant extract at a dose of 10 µl/animal/day to water (*Salvia officinalis* L. extract contained thujone 24.0±1.0%, borneol 18.0±1.0% and cineole 15.0±1.0% - gas chromatography analysis; density: 0.915±0.001 g/cm3; refractive index: 1.469±0.001; Calendula a. s., Nová Ľubovňa, Slovak Republic). The natural substances were administered in all experimental groups from day 0-1 to day 21. The experiment lasted for 35 days.

Samples of the duodenum, jejunum, ileum and caecum were obtained from randomly selected animals (n=8) of each group at days 21 and 35 (two weeks after cessation of feed supplementation with sage or/and EntM). The animals were slaughtered and stunned with electronarcosis (90 V for 5 s) in an experimental slaughterhouse, immediately hung by the hind legs on the processing line and quickly bled by cutting the *jugular veins* and the *carotid arteries*. After exsanqiunation from samples from different segments of the small intestine (duodenum, jejunum, ileum) and caecum were processed duplicate for mucus determination according to Smirnov et al. (2004), modified by Faixová et al. (2012). The amount of produced mucus was determined/detected by the ELISA assay technique (Apollo LB 913, Berthold Technologies, Germany) at the wavelenght 630 nm using software PhotoRead version 2.2.2.1 (Berthold Technologies, Germany). The results, mucus production quantity, were expressed in grams (g) ± standard deviation (SD). The statistical analysis of the results as well as statistical significance of differences between all groups was determined and compared by one-way ANOVA test and confirmed by Tukey post test (MiniTab, Czech Republic) and the levels of statistical significance 0.01 and 0.001 were expressed by p value ($p<0.01$; $p<0.001$).

Results

The beneficial effect on mucus production quantity was found in the duodenum ($p<0.001$) and jejunum ($p<0.01$) in ESG compared to CG on day 21. The prolonged beneficial effect was found in EG in the duodenum ($p<0.001$) compared to CG at the end of the experiment. Mucus production was also significantly

Table 1. Mucus quantity production after Enterocin M and sage plant extract administration in different segments of the rabbit small intestine and caecum (rabbits *per* group, n=16, rabbits *per* sampling at day 21, n=8, rabbits *per* sampling at day 35, n=8).

Mucus quantity production (g±SD)	Day 21	Day 35
Duodenum		
EG	10.20 ± 3.58[d]	21.81 ± 6.66[c, d]
SG	14.53 ± 5.11	8.95 ± 2.62
ESG	38.59 ± 15.44[a]	5.51 ± 1.51
CG	8.65 ± 3.76[a]	13.59 ± 5.22[c]
Jejunum		
EG	8.00 ± 4.43	17.25 ± 10.10
SG	18.63 ± 7.42	11.67 ± 9.39
ESG	27.31 ± 14.96[b]	13.48 ± 7.66
CG	13.58 ± 4.79[b]	20.07 ± 8.02
Ileum		
EG	14.33 ± 5.52	10.70 ± 3.51
SG	19.42 ± 12.15	11.77 ± 3.36
ESG	20.53 ± 5.03	13.43 ± 2.22
CG	22.81 ± 4.97	13.08 ± 3.35
Caecum		
EG	14.21 ± 7.23	12.24 ± 3.18
SG	18.75 ± 13.87	11.12 ± 4.79
ESG	15.56 ± 4.70	9.97 ± 3.57
CG	15.68 ± 4.18	16.86 ± 1.92

Mucus production quantity was expressed in g ± SD (gram ± standard deviation); EG – Enterocin M group; SG – sage extract group; ESG – Enterocin M and sage extract group; CG – control group; the statistical analysis and statistical significance of differences between all groups were determined and compared by one-way ANOVA test, and confirmed by Tukey post test (MiniTab, Czech Republic); the same letters mean the significant value p at the level expressed in order: [a] $p<0.001$; [b] $p<0.01$; [c] $p<0.001$; [d] $p<0.001$

increased in the duodenum ($p<0.001$) 14 days after Enterocin M administration (at day 35) compared with day 21 in EG (Table 1). Increased prolonged but non-significant mucus production was noted in the Ent M experimental group (EG) in the jejunum at the end of the experiment (at day 35; 2 weeks after Ent M cessation) compared to that observed on day 21 (3 weeks after Ent M application). There was recorded no positive effect on the mucin production 2 weeks after administration, similarly as the prolonged effect of Ent M administration at the end of the experiment in the ileum and caecum except for the sage plant extract group (SG), where non-significant increase of mucus production at day 21 was observed (Table 1).

Disscusion

The overall composition of the mammalian intestinal microbiota varies between individuals: within each individual there are differences along the length of the intestinal tract related to host nutrition, intestinal motility and secretions. Mucus is a highly regenerative protective lubricant glycoprotein sheet secreted by host intestinal goblet cells. The mucin-containing mucus layer coating the gastrointestinal epithelium is the front line of innate host defense (Kim and Khan 2013).

Mucins are likely to be the first molecules that invading pathogens interact with at the cell surface and thus, can limit binding to other glycoproteins and neutralize the pathogen (Kim and Khan 2013). Bacterial species in the mucus present differential proliferation and resource utilization. Functional competition in this layer is likely to be a major determinant of microbiota composition and microbial molecular exchange with the host (Li et al. 2015). Lauková et al. (2012, 2016) reported reduction of coliforms, staphylococci, pseudomonads and clostridia in the intestinal tract of rabbits after suffiecient colonization and application of beneficial Ent M-producing strain *Enterococcus faecium* AL41 (CCM5885) and its enterocin M. The combined application of *Enterococcus faecium* CCM5885 with *Eleutherococcus senticosus* also significantly stimulated phagocytic activity in rabbits ecosystem ($p<0.001$; $p<0.05$). The combined effect of Ent M, synthesized by *Enterococcus faecium* CCM5885, with

another plant extract obtained from sage also singnificantly stimulated the production of mucus quantity in rabbits duodenum ($p<0.001$) and jejunum ($p<0.01$) after 3 weeks dietary supplementation. The higher amount of produced mucus in our experiment could also influence the status of microbiome and reduce the potentional risk of intestinal infection development.

Probiotic bacteria or their antimicrobial proteinaceous substances called bacteriocins (enterocins) hold promising prophylactic potential for animal breeding (Lauková et al. 2018). The effect on mucus production in the intestinal tract after application of Enterocin M has never been studied in rabbits before. The majority of application-oriented studies are focused on bacteriocins from Gram-positive microorganisms, mostly lactic acid bacteria or enterococci, in animal breeding. In-depth studies of a selected few bacteriocins opened exiting new research fields and broadened the novel application of these antimicrobial peptides. The possibility of developing bacteriocins into next generation antibiotics, accompanied with the rapid development in genetics and nanotechnology, paves the way to even more fascinating applications such as novel carrier molecules (delivery systems) and the prevention/treatment of e. g. intestinal disorders (Chikindas et al. 2018). Bacteriocins, such as enterocins, considered to be colonizing substances of the animal intestinal tract are probably involved in influencing of mucus production. This is proved by the results obtained in our experiment, which showed the significant prolonged effect of Ent M application in the rabbit duodenum ($p<0.001$).

The significant effect of adding different probiotic strains (*Lactobacillus casei, Lactobacillus acidophilus* and *Enterococcus faecium*) was assessed on the increased production of sulfated acid mucins, non-sulfated acid mucins and neutral mucins in weaned piglets (Ciro Galeano et al. 2015).

According to Pogány Simonová et al. (2020), Ent M and sage application, alone or in combination, improve the small intestine histomorphology morphometry ($p<0.001$) in rabbits. Feeding probiotics and their antimicrobial products help maintain a beneficial intestinal microflora, enhances the host's resistance to enteric pathogens, and results in a healthy gastrointestinal environment with an improved intestinal function (Aliakbarpour et al. 2012). Similarly, Hassain et al. (2015) studied the synergistic effects of dietary supplementation with sodium butyrate and synbiotic (Poultry-Star®) on the productive performance and intestinal morphometry in rabbits and reported positive results/effect on the intestinal morphometry, providing/enhancing intestinal homeostasis and health.

Aliakbarpour et al. (2012) also found that the inclusion of probiotic lactic acid bacteria in the diet increased the jejunal villus height and goblet cells density ($p<0.05$) and the expression of intestinal MUC2 mRNA numerically was higher in broilers supplemented by lactic acid bacteria diet. The mucus production in selected experimental groups in this study was probably increased due to proliferation of goblet cells as confirmed by the study of Smirnov et al. (2005). They found that changes in the intestinal bacterial populations by use of probiotic can affect processes of mucin dynamics and biosynthesis, which is affected by conditions or agents that affect differentiation of precursor cells into mature goblet cells. The amounts of duodenal and jejunal mucin mRNA and proteins increased in response to a 72 hour fast without any increase in goblet cell numbers during this time. Different factors including microbial colonization (e. g. with probiotics) can affect/regulate goblet cell activity and mucin production, secretion and composition by activating different signaling cascades and secretory chemical agents (Dharmani et al. 2009).

The present study has revealed that supplementation of either natural substances, Ent M and sage plant extract, in feed has the same stimulatory effects and improves mucus production quantity especially in the proximal segment of the small intestine in rabbits.

Conclusions

The results of this study obtained after application of enterocin M as well as sage extract in rabbits are original, giving us further opportunity to continue these investigations and to show the benefits of natural substances dietary supplementation on the amount of mucus production in the small intestine selected segments/ /caecum in rabbits ecosystem.

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