https://doi.org/10.17221/143/2019-CJAS

Effect of probiotic *Clostridium butyricum* CBM 588 on microbiota and growth performance of broiler chickens

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Citation: Svejstil R., Plachy V., Joch M., Salmonova H., Duskova D., Hautekiet V., Vlkova E. (2019): Effect of probiotic *Clostridium butyricum* CBM 588 on microbiota and growth performance of broiler chickens. Czech J. Anim. Sci., 64, 387–394.

Abstract: *Clostridium butyricum* CBM 588 is used as a probiotic in eastern Asian countries and has been recently approved as an animal feed additive in the European Union. The purpose of this study was to evaluate the effect of *C. butyricum* MIYAIRI 588 (CBM 588) on abundance of selected genera of caecal and crop bacteria, volatile fatty acids and growth performance of broiler chickens. We studied counts of anaerobic bacteria in caeca and crops of broiler chickens by plate-count method and evaluated their growth performance. CBM 588 significantly reduced *E. coli* counts in caeca of broiler chickens at days 10 and 42 and also enhanced their growth performance. Additionally, it significantly increased the amount of butyrate in the caeca that provides energy to enterocytes, resulting in increased weight gains. Out of the obtained results we conclude that *C. butyricum* CBM 588 influences caecal microbiota of broiler chickens and positively affects their growth performance.

Keywords: probiotic feed additive; *E. coli*; bifidobacteria; butyrate; feed efficiency

Intestinal microbiota is known to be an important factor influencing health of living beings by protecting the body from various diseases (Guarner and Malagelada 2003), transforming indigestible parts of food and feed, and synthesising several vitamins and metabolising some xenobiotics (Cummings and Macfarlane 1997). Avian gut is intensively inhabited by bacteria that possess the above-mentioned functions (Kohl 2012). The main role of bacteria in the avian gut is to utilise substrates that cannot be digested by the metabolic processes of the bird (Vispo and Karasov 1997). Providing good nutritional factors is, therefore, one of the ways to influence the composition of the gut microbiota.

Broiler chickens are produced on a large scale in developed countries – reared in an environment with strict hygienic standards, where they never get in contact with broody-hens (Fuller 2001). Therefore, the chickens are colonised by microbiota present in the environment (Lutful Kabir 2009; Varmuzova et al. 2016) in which they are reared. Thus, hatching conditions, hygiene, stress, and medication have major influence on the microbiota of the chickens as well as on their resistance to the colonisation of pathogenic bacteria (Barrow 1992).

The concept of modulation of intestinal microbiota is well-known in poultry production, ever since Nurmi and Rantala (1973) managed to pro-

Supported by Huvepharma®, Antwerp, Belgium.

https://doi.org/10.17221/143/2019-CJAS

tect hatched chickens from *Salmonella enteritidis* infection by oral supplementation of faeces from healthy adult hens. Since then, many microorganisms belonging to the genera of *Lactobacillus*, *Streptococcus*, *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Aspergillus*, *Candida*, or *Saccharomyces* have been established in poultry production as probiotics. The effects of probiotics in poultry may possibly include modulation of intestinal microbiota, inhibition of pathogens, immunomodulation effects, and improvement of histological parameters of the gut. In addition, they may positively influence the growth performance as well as the meat quality, including its sensory aspects (Fuller 2001; Lutful Kabir 2009; Wang et al. 2017).

It has been shown that the bacteria of the genus *Clostridium* can also be employed as probiotics. While some strains of clostridia are well-known pathogens, some are a part of normal intestinal microbiota, and others are considered as probiotics in Asian countries (Cassir et al. 2016). *Clostridium butyricum* CBM 588 (also labelled as MIYAIRI 588 or FERM BP-2789) that is used in the present study has been used as a probiotic in Japan. It has been tested in several clinical trials, which indicate that the strain can decrease the incidence of antibiotics-associated diarrhoea in children together with stabilisation of the population of bifidobacteria (Seki et al. 2003). Shimbo et al. (2005) and Imase et al. (2008) observed stabilising effects on the intestinal microbiota when CBM 588 was administered as a supplement during the antibiotic eradication of *Helicobacter pylori* infection. Furthermore, it has been found that CBM 588 is able to suppress the production of *Clostridium difficile* toxins after antimicrobial therapy in humans and also to inhibit some strains of *Vibrio cholerae*, *Aeromonas hydrophila*, and *Shigella flexneri* in mixed cultures (Kuroiwa et al. 1990). CBM 588 was also tested in rats, wherein it was able to mitigate the symptoms of dextran sulphate sodium-induced colitis and also increased the counts of lactobacilli in their gut (Okamoto et al. 2000). Takahashi et al. (2004) found that CBM 588 decreased the amount of Stx1 and Stx2 toxins produced by enterohemorrhaegic *Escherichia coli* O157:H7 in mice. Study by Yang et al. (2012) revealed that *Clostridium butyricum* HJCB998 decreased the counts of *E. coli*, *C. perfringens*, and *Salmonella* spp., and increased the counts of lactobacilli and bifidobacteria in caeca of broiler chickens and also stimulated their immune functions. Multiple studies exhibited positive effects of HJCB998 on the growth performance of broiler chickens (Yang et al. 2012; Zhang et al. 2014, 2016).

Clostridium butyricum CBM 588 is a key substance in the composition of Miya-Gold®, a zootechnical feed additive, which is claimed to be a gut flora stabiliser, authorised in accordance with the Regulation EC No. 1831/2003 (EFSA, 2013).

Thus, the aim of this study is to evaluate the effect of feeding *Clostridium butyricum* CBM 588 in the form of Miya-Gold® on the growth performance and microbiota of broiler chickens.

MATERIAL AND METHODS

The feeding trial was carried out at the Demonstration and Experimental Centre of the Czech University of Life Sciences Prague, Czech Republic (DEC). A total of 160 ROSS 308 broiler chickens were divided into two groups per 80 animals in control and experimental groups. The protocol for this study was approved by the Ethics Committee of the Czech University of Life Sciences Prague (permission No. CZ 02225). The broilers were housed on German Horse Span Classic bedding under a 16 h light : 8 h darkness cycle. The control group was fed BR-2-based feed *ad libitum* throughout the whole experiment – from the 1st to the 49th day of life. The experimental group received the same feed mixture but enriched with 1 g Miya-Gold® S (Huvepharma®, Antwerp, Belgium) per 1 kg of the feed. According to the safety data sheet of Miya-Gold® S, it contains a minimum of 5×10^8 CFU/g, i.e. log 8.70 CFU/g of spores of *Clostridium butyricum* CBM 588. The mixture was pelleted under conditions not exceeding a temperature of 60°C at the DEC. The processing did not affect the counts of viable bacteria as verified by cultivation analysis (data not shown). The feed mixture based on BR-2 consisted of the following ingredients: 60.17% wheat, 29.50% extracted soybean meal, 6.30% rapeseed oil, 0.16% DL-methionine, 0.25% sodium chloride, 1.35% monocalcium phosphate, 1.15% limestone, and 0.12% sodium carbonate. One kilogram of the feed (as fed) provided an energy of 12.73 MJ, 211.88 g of crude proteins, and 11.84 g of lysine.

Throughout the experiment, the average weight of all the individuals, their daily weight gain, and feed conversion ratio were recorded at multiple timepoints (at days 1, 7, 10, 20, 35, and 49). The caecal

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and crop microbiota were analysed at the beginning of the experiment (day 1), as well as at day 10, and day 42 of the experiment in 5 individuals from each group; volatile fatty acids in the caeca and crops at day 42 were also analysed.

In the microbiological analysis, total counts of anaerobic bacteria as well as counts of bifidobacteria, lactobacilli, enterococci, and *E. coli* were determined by plate-count method using a ten-fold dilution of each sample up to 10^{-9} dilution. Prior to the analysis, the birds were slaughtered by stunning and cervical dislocation. Approximately 1 g of the caecum (faeces) and crop (chyme) content of each slaughtered chicken was immediately and aseptically transferred to the sterile tubes. The tubes were pre-weighed in order to determine accurate weight of collected caecal/crop content and to adjust the dilution to standardized volume of the sample. The CO_2-flushed sterile tubes contained Nutrient Broth No. 2 (5 g/l; procured from Oxoid, UK), tryptone (5 g/l; Oxoid), yeast extract (2.5 g/l; Oxoid), Tween 80 (0.5 ml/l; Sigma-Aldrich, USA), and L-cysteine (0.25 g/l; Sigma-Aldrich). The identical medium was used for dilution of the samples. The collected samples were homogenised by vortexing immediately after sampling, and underwent the analysis straight away. For microbiological analysis, 5 chickens from each group were sampled. To determine total anaerobes, Wilkins-Chalgren anaerobe agar (43 g/l; Oxoid) was used (Wilkins and Chalgren 1976; Rada and Petr 2000), with the addition of Veggietone Soya Peptone (5 g/l; Oxoid), L- cysteine (0.5 g/l; Sigma-Aldrich), and Tween 80 (1 ml/l; Sigma-Aldrich). An identical medium was enriched with the antibiotic, mupirocin (100 mg/l; Oxoid), and glacial acetic acid (1 ml/l) according to Rada and Petr (2000) and used for the determination of bifidobacteria. Culture plates for the growth of anaerobes and the bifidobacteria were incubated in anaerobic jars (Anaerobic Plus System; Oxoid) at 37°C for 48 h. Lactobacilli were cultured using Rogosa agar (82 g/l; Oxoid) adjusted to pH 5.4 by glacial acetic acid for 48 h under micro-aerophilic conditions (Corry et al. 2003). Counts of *E. coli* were determined using TBX medium (36.6 g/l; Oxoid) by incubating the plates aerobically at 37°C for 24 h (Verhaegen et al. 2015). Enterococci counts were determined using the Slanetz and Bartley medium (42 g/l; Oxoid) by incubating the plates aerobically at 37°C for 48 h (Niemi and Ahtiainen 1995). Total counts of anaerobes and bifidobacteria were cultured using a pour-plate method, lactobacilli were cultured using a double layered pour-plate method (Geigerova et al. 2016) and enterococci and *E. coli* were cultured using a spread-plate method.

Analysis of the volatile fatty acids of caeca and crops was performed by gas chromatography using a Stabilwax®-DA column (Restek, USA) with Flame-Ionisation detector (GC-FID) and H_2 as a mobile phase; the flow was 120 ml/min and the injection and detection temperature was 200°C. Briefly, the samples were vortexed and 0.1 ml of 3 M formic acid and 0.03 ml of internal standard (2-ethylbutyric acid) were added to 0.8 ml of each sample. After centrifugation, 1 µl of the sample was injected into the column (Joch et al. 2017).

Statistical evaluation was carried out by Statgraphics Centurion XV 15.2.05/2007 (StatPoint Technologies, Inc., USA) using two-sample *t*-test for comparison between the groups. The data were checked for normality by Shapiro-Wilk test prior to the statistical analysis.

RESULTS AND DISCUSSION

Weight gains of broiler chickens were significantly higher when they were fed a mixture containing Miya-Gold® than of those fed with regular feed throughout the whole trial, as shown in Table 1. There was a significant difference between the body weights of the individual chickens at day 7 ($P < 0.05$), day 10 ($P < 0.001$), day 20 ($P < 0.01$), and day 49 ($P < 0.001$); however, no significant difference ($P > 0.05$) between the body weights was observed at day 35. These findings are supported by values of daily weight gains (Table 2) and feed conversion ratio (Table 3). The results indicate that Miya-Gold® supported the growth of broiler chickens and their feed conversion, although there was an equalisation between the groups at day 35. This could be due to certain forms of social hierarchy that could result in aggressive pecking, thereby reducing access to the feed (Nicol et al. 1999). We found significantly more butyrate and isocapronate ($P < 0.05$) in the caeca of chickens from Miya-Gold® group than in those from control group (Table 4); and therefore, we assumed that it was the result of the metabolic activity of CBM 588. The increased weight gains in the experimental group could have been the result of butyrate production by CBM 588 (Hu and Guo 2007; Matis et al. 2013). Butyrate provides 60–70% of energy

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Table 1. Average weights of broiler chickens in the course of the experiment. Values are means ± standard error

Group	Average weight (g)					
	day 1	day 7*	day 10***	day 20**	day 35	day 49***
Control	44.10 ± 3.71	130.96 ± 13.19	195.04 ± 20.38	760.08 ± 79.59	1767.92 ± 242.09	2780.91 ± 445.41
Miya-Gold®	44.71 ± 4.01	138.09 ± 15.85	217.25 ± 21.83	810.26 ± 73.56	1787.23 ± 215.09	3231.67 ± 509.15

significant differences between the groups:*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Table 2. Average daily weight gains of broiler chickens. Values are means ± standard error

Group	Daily weight gains (g)				
	days 1–7	days 1–10	days 11–20	days 21–35	days 36–49
Control	14.48 ± 0.07	16.77 ± 0.11	56.56 ± 0.12	67.19 ± 0.17	72.36 ± 0.23
Miya-Gold®	15.56 ± 0.10	19.17 ± 0.10	59.30 ± 0.14	65.13 ± 0.23	103.17 ± 0.28

Table 3. Average feed conversion ratio of broiler chickens

Group	Feed conversion ratio (kg)						
	days 1–7	days 1–10	days 11–20	days 21–35	days 36–49	days 1–35	days 1–49
Control	2.09	1.90	1.81	1.82	2.76	1.90	2.08
Miya-Gold®	1.53	1.71	1.70	2.22	2.04	1.79	1.84

to the enterocytes present in the gut (Roediger 1995); thus, its production by gut bacteria could increase the overall energy intake, thereby enhancing the weights. Zhang et al. (2011) have reported that dietary inclusion of *C. butyricum* increased the heights of jejunal villi and relative lengths of the caeca. Similarly, Kotunia et al. (2004) found a proliferative effect of butyrate on the jejunum and ileum. Thus, we speculate that the production of butyrate by CBM 588 in our experimental chickens could have resulted in improved digestion and absorption of the nutrients, thereby leading to an increased energy intake. Improvement of the growth performance of broiler chickens has been found by multiple authors when these chickens were administered probiotic lactobacilli (Jin et al. 1998; Kalavathy et al. 2003; Apata 2008), such as *Enterococcus faecium* (Owings et al. 1990) and *Bacillus subtilis* (Khaksefidi and Ghoorchi 2006), or sodium butyrate alone (Zhang et al. 2011). Effects

Table 4. Analysis of volatile fatty acids (VFA) in the caeca and crops of broiler chickens by gas chromatography, day 42. Values (in mmol) are means ± standard error

VFA	Caecum			Crop	
	Control	*P*	Miya-Gold®	Control	Miya-Gold®
Acetate	441.52 ± 32.86		381.08 ± 63.11	59.42 ± 35.15	58.79 ± 29.03
Propionate	183.42 ± 41.20		196.96 ± 57.42	1.14 ± 2.20	0.27 ± 0.38
Isobutyrate	3.37 ± 0.68		2.14 ± 1.23	2.19 ± 2.85	1.85 ± 1.52
Buytrate	103.41 ± 14.78	*	132.12 ± 18.51	ND	ND
Isovalerate	7.51 ± 2.43		4.08 ± 3.46	0.48 ± 0.62	ND
Valerate	8.24 ± 2.33		4.80 ± 3.46	2.46 ± 3.71	1.25 ± 1.32
Isocapronate	0.91 ± 0.56	*	2.74 ± 1.63	4.64 ± 7.37	3.70 ± 4.45
Capronate	1.18 ± 2.63		0.28 ± 0.40	0.84 ± 0.91	2.78 ± 3.54
Heptanoate	ND		ND	ND	ND
∑VFA	749.57 ± 59.11		724.19 ± 96.92	71.16 ± 28.36	68.63 ± 25.95

ND = below detection limit

significant differences *$P < 0.05$

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of Miya-Gold® on growth performance of broiler chickens has already been demonstrated by several studies conducted for EFSA (2013), wherein two of three trials demonstrated a significantly higher final body weight. Additionally, the average daily weight gain in broilers receiving Miya-Gold® at feed doses of 2.5×10^8 CFU/kg in one trial and 5.0×10^8 CFU/kg in the other one was significantly higher; the dosage administered in the latter is similar to that used in our experiment. Although Zhang et al. (2011), using a different strain of *C. butyricum*, found increased levels of butyrate in caeca but no effect on the growth performance of broilers. On the other hand, Yang et al. (2012) and Zhang et al. (2014, 2016) observed significant improvement in the broilers' growth performance after supplementation with *C. butyricum* HJCB998 strain.

An analysis of caecal and crop microbiota was carried out at days 1, 10, and 42 of the experiment; the results are shown in Table 5. We found that caecal counts of *Escherichia coli* were significantly lower in the experimental group at both days 10 ($P < 0.05$) and 42 ($P < 0.05$). No difference at the first time-point (day 1) was observed, since it was only the first day of feeding trial and the clostridia would not have been established at this point of time in the caecal milieu. Use of probiotics is usually recommended for several days (Shimbo et al. 2005; Islam 2016) to produce a significant effect. Our results are in accordance with the findings of Yang et al. (2012), who also observed a significant decrease of *E. coli* in caeca of broiler chickens, despite being supplemented by only 2×10^7 CFU/kg compared to 5×10^8 CFU/kg in our trial. The antagonistic effect of CBM 588 against pathogenic *E. coli* O157:H7 strain was shown in gnotobiotic mice by Takahashi et al. (2004), wherein the strain demonstrated a preventive as well as therapeutic effect. Contrastingly, Shimbo et al. (2005) did not observe any decrease of *E. coli* counts in humans receiving CBM 588 prior to antibiotic therapy, which could be due to the fact that some strains of *E. coli* are species-specific (McLellan et al. 2003; Zhi et al. 2015) and human patients in the above-mentioned study possessed the strains that were not susceptible to CBM 588.. These data suggest the possibility that broiler chickens may possess *E. coli* strains that are specifically susceptible to CBM 588. The hypothesis of the suppression of *E. coli* by CBM 588 is supported by the fact that we found significantly lower counts of these bacteria ($P < 0.01$) even in the crop (Table 5) at day 42. Unfortunately, we failed to detect *E. coli* at day 10 in the crops of both groups. The inhibitory effect of CBM 588 on *E. coli* can be attributed to the combined effect of its anti-adhesive properties and the production of butyrate. Takahashi et al. (2004) observed an inhibitory effect of CBM 588 on the adhesion of enteroheamorrhagic *E. coli* (EHEC) to Caco-2 cells and also observed an inhibitory effect of butyric acid on EHEC even at a neutral pH. Besides, Zhang et al. (2016) observed an increased immune response in broiler chickens challenged

Table 5. Analysis of caecal microbiota and crop microbiota of broiler chickens in the course of the experiment. Values are means log CFU/g ± standard error

Bacterial group	Day 1		Day 10			Day 42		
	Control	Miya-Gold®	Control	*P*	Miya-Gold®	Control	*P*	Miya-Gold®
Caecal microbiota								
Total anaerobes	10.08 ± 0.26	10.06 ± 0.28	10.09 ± 0.20		10.25 ± 0.32	10.09 ± 0.26		10.13 ± 0.23
Bifidobacteria	4.92 ± 1.27	5.09 ± 1.09	9.18 ± 0.23		8.83 ± 1.35	9.92 ± 0.36		9.55 ± 0.19
Lactobacilli	5.91 ± 0.47	6.87 ± 0.91	9.04 ± 0.19		8.55 ± 0.56	8.53 ± 0.31		9.01 ± 0.40
Enterococci	9.55 ± 0.43	8.90 ± 1.75	8.64 ± 0.35		7.82 ± 0.61	8.10 ± 0.17	*	7.55 ± 0.39
E. coli	9.60 ± 0.06	9.03 ± 1.70	8.47 ± 0.81	*	7.29 ± 0.61	8.22 ± 0.64	*	7.00 ± 0.92
Crop microbiota								
Total anaerobes	8.95 ± 0.31	9.01 ± 0.12	9.32 ± 0.39		9.58 ± 0.46	8.74 ± 0.56		9.65 ± 0.33
Bifidobacteria	ND	5.40 ± 0.46	5.04 ± 0.39	*	4.29 ± 0.55	5.20 ± 0.32		4.73 ± 0.96
Lactobacilli	5.54 ± 1.72	7.29 ± 0.78	8.82 ± 0.11		8.69 ± 0.63	8.29 ± 0.59		9.04 ± 0.44
Enterococci	7.73 ± 0.78	7.84 ± 0.28	8.08 ± 0.33		8.34 ± 0.63	7.18 ± 0.43		6.94 ± 0.40
E. coli	7.90 ± 0.78	7.96 ± 0.75	ND		ND	6.73 ± 0.52	**	5.39 ± 0.49

ND = below detection limit; significant differences *$P < 0.05$, **$P < 0.01$

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with *E. coli* K88 when they were fed *C. butyricum* HJCB998. *E. coli* is a normal inhabitant of both mammalian and avian intestines; however, it has been reported that chickens can carry pathogenic strains that can cause diseases in humans and the birds themselves (Dho-Moulin and Fairbrother 1999; Manges 2016). Thus we consider the reduction of *E. coli* as a positive outcome and conclude that CBM 588 has similar effects on *E. coli* and broiler chicken performance as HJCB998.

In our experiment, counts of enterococci were significantly reduced ($P < 0.03$) in the caeca of experimental group at day 42 compared to those in the control group (Table 5). Enterococci are a part of normal microbiota of broiler chicken; however, they are not known to be infectious agents. Nevertheless, some strains can play the role of opportunistic pathogens (Stepien-Pysniak et al. 2016). Moreover, it has been reported that enterococci isolated from poultry often carry multiple resistance to antimicrobials administered in human medicine (Hayes et al. 2004). In this study, we observed that they were significantly less abundant only at the last time-point. The reduction at day 10 was not significant due to heterogeneity of the results; consequently, we cannot clearly deduce whether CBM 588 was able to suppress enterococci throughout the whole experiment.

Counts of bifidobacteria were significantly lower in the crops of experimental group at day 10 ($P < 0.05$), but were not significantly lower at day 42 than those in control group (Table 5). Additionally, bifidobacteria counts in the caeca of experimental chickens were also not significantly lower throughout the experiment. Yang et al. (2012) tested a different strain of *C. butyricum* and found that it increased the counts of bifidobacteria in caeca. Moreover, Zhang et al. (2014) found that *C. butyricum* HJCB998 increased the population of bifidobacteria and lactobacilli in broiler chickens. Our findings seem to be different in comparison with the data obtained by these investigators; however, most of the differences observed are non-significant. Although data on bifidobacteria in caeca is available, there is a lack of knowledge about bifidobacteria in the crop of broiler chickens.

It is an obvious fact that counts of *Clostridium* spp. should be determined when analyses of microbiota in feeding trials with clostridia added to a diet are performed. Unfortunately, it is very difficult to determine the clostridia in the faecal samples due to insufficient selectivity of media for the cultivation of clostridia for such types of samples. *Clostridium* spp. are able to grow in the mupirocin-containing medium that we used for the analysis of bifidobacteria (Vlkova et al. 2015). Nevertheless, due to high counts of bifidobacteria in the samples, we could not enumerate the counts of clostridia by the plate-count method and a ten-fold dilutions up to 10^{-9} used in this study. Thus, using alternative methods, such as microscopy and MALDI-TOF mass spectrometry (Bruker Daltonik, Germany) using MALDI Biotyper RTC with DB-5989 MSP library for identification of these bacteria (data not shown), we verified that the most abundant colonies on the agar plates were bifidobacteria.

CONCLUSION

In the present study we analysed the effect of *Clostridium butyricum* CBM 588 on the growth performance of broiler chickens *in vivo* and also its influence on caecal and crop microbiota. We found that CBM 588 was able to positively affect the growth performance of broiler chickens. Moreover, CBM 588 was able to increase the content of butyrate in the caeca by its metabolic activity and influence the composition of the intestinal microbiota by reducing the counts of *E. coli*. Elevated amount of butyrate can contribute to gut health and improve weight gain. Some strains of *E. coli* can act as opportunistic pathogens; thus, their decrease can be beneficial to the host. Since the administration of *C. butyricum* did not completely suppress *E. coli* or the other tested bacterial genera, it did not disrupt the microbial balance in the caecum. In conclusion, we consider *C. butyricum* CBM 588 as a potentially beneficial additive to the feeds of broiler chickens.

Acknowledgement. We would like to express our thanks to Huvepharma® for providing us with MiyaGold® and for their financial support. We would like to thank the Demonstration and Experimental Centr of the Czech University of Life Sciences Prague for providing space to conduct these experiments. We are also grateful to Jana Veselá and Luci Glanc, BSc. and MSc. students, who kindly helped us with the analysis of the microbiota. Last, but not least, we thank late professor Vojtěch Rada for his kind advice and help with the experiments.

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Received: 2019–06–18
Accepted: 2019–08–16