New probiotic strain *Lactobacillus fermentum* AD1 and its effect in Japanese quail

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ABSTRACT: Probiotics have been used with increasing frequency in nutrition and for prophylactic purposes during the last years. In the present study we investigated the effect of *Lactobacillus fermentum* AD1 – canine isolate on selected intestinal microbial groups, weight gain, organic acids, haematology, glutathione peroxidase and phagocytosis of leucocytes in 2-days-old Japanese quail (*Coturnix coturnix japonica*). The results demonstrated that the 4-day application of this strain significantly increased the population of lactic acid bacteria – lactobacilli and enterococci in faeces ($P < 0.01$ and/or $P < 0.001$) and caecum of quail ($P < 0.001$) and significantly decreased the counts of *E. coli* in faeces ($P < 0.05$). The daily weight gain was increased by 14%. Although intestinal pH of both groups of birds was similar, the concentration of lactic acid was significantly increased in the experimental group ($P < 0.05$). The concentration of other organic acids (acetic, acetoacetic, formic, succinic, valeric, propionic, butyric) as well as blood glutathione peroxidase was not influenced. The index of phagocytic activity of leucocytes was significantly improved ($P < 0.01$).

Keywords: probiotic; *Lactobacillus* sp.; Japanese quail; effect

To maintain the intestinal microflora balance in animals it is important to prevent diseases by controlling the overgrowth of potentially pathogenic bacteria. The control of infections through a nonantibiotic approach is urgently requested. The natural bacterial flora (e.g. probiotic bacteria) represents a promising alternative therapy. Probiotics were defined as "living microorganisms that upon ingestion in certain numbers exert health effects beyond inherent basic nutrition" (Guarner and Schaafsma, 1998). They have been the object of studies at an international scale since the middle of the twentieth century. The use of probiotics in poultry was pioneered by Tortuero (1973), who reported an increase in growth rate in chicks given a *Lactobacillus acidophilus* culture in drinking water for 11 days from hatching. Similar results on the beneficial effects of *Lactobacillus* cultures on the growth of chickens were also reported by several researchers (Kalbane et al., 1992; Jin et al., 1998). Nahashon et al. (1996) described a positive effect of the applied *Lactobacillus* on egg production. The exclusion of pathogenic bacteria is especially important in newly hatched broiler chickens. Breeder vaccinations are routinely used in broiler breeders to provide the newly hatched chicks with yolk-derived maternal antibodies. Because in modern production methods the newly hatched chick has no contact with maternal faeces and thus no maternal spectrum of antigens is present, allowing the development of an active immune system. Probiotic supplementation of the intestinal mi-

Supported by the Grant Agency for Science of the Slovak Republic VEGA (Grant No. 2/5139/25).

croflora in poultry, especially with *Lactobacillus* species, showed beneficial effects on resistance to infectious agents such as *Escherichia coli* (Jin et al., 1996), *Salmonella* sp. (Pascual et al., 1999), *Campylobacter* sp. (Stern et al., 2001) and, more recently, *Eimeria acervulina* (Dalloul et al., 2003). Proposed mechanisms of pathogen inhibition by the probiotic microorganisms include competition for nutrients, production of antimicrobial conditions and compounds (volatile fatty acids, low pH, and bacteriocins), competition for binding sites on the intestinal epithelium, and stimulation of the immune system (Rolfe, 2000). These are not mutually exclusive mechanisms, and some microorganisms may effect the change due to a single mechanism whereas others may use several mechanisms.

Lactobacillus fermentum AD1 (formerly *L. casei)* is a new probiotic strain whose beneficial effects in the digestive tract of animals were presented in our previous studies (Strompfova, 2004; Strompfova et al., 2004). However, it was not applied to poultry yet. Japanese quail represents a useful bird model to test *in vitro* obtained results under *in vivo* conditions. Therefore the present study was conducted to determine the influence of orally administered AD1 strain on intestinal microflora, organic acids in intestinal contents, antioxidative enzyme glutathione peroxidase, haematology, phagocytic activity of leucocytes and daily weight gain of conventional Japanese quail.

MATERIAL AND METHODS

Experimental animals

Eighteen 2-days-old Japanese quail (*Coturnix coturnix japonica*) were selected for this study. The birds were divided into two groups, experimental (n = 9) and control (n = 9). The experiment lasted 7 days. All birds were fed the commercial diet BR1/FAT (Tatrafat s.r.o., Huncovce, Slovak Republic) and had access to feed and water *ad libitum*. On day 1, the experimental group was orally administered *L. fermentum* AD1 strain (0.1 ml per bird; 10^8 cfu/ml of saline solution) with a syringe. On the next three days AD1 strain was added into drinking water. The control group was given placebo – saline solution (0.85%, pH 7.0). Samples of faeces were collected from each quail on days 0, 1, 3, 5, 7. At the end of experiment, all animals were killed and their caecum was separated. The birds were weighed at the beginning and at the end of experiment.

Isolation and enumeration of intestinal microflora

The samples of faeces and caecal contents were transferred under aseptic conditions into a sterile plastic bag, diluted with saline solution (1:10) and mixed using Stomacher (80I, England) for 1–2 min. After dilution, 100 µl of each sample was plated onto the following media: Mac Conkey agar (Becton and Dickinson, USA) for *E. coli*, Mannitol salt agar (Becton and Dickinson) for staphylococci, M-*Enterococcus* agar (Becton and Dickinson) for enterococci, De Man-Rogosa-Sharpe agar (MRS, Merck, Germany) for lactobacilli and MRS agar with rifampicin (100 µg/ml) for *L. fermentum* AD1. Enterococci, staphylococci and *E. coli* were cultivated at 37°C for 24–48 h. Lactobacilli were incubated in 3% CO_2 atmosphere at 37°C for 48–72 h. The results are expressed as arithmetical means ± SD (in $\log_{10}$ cfu/g).

Preparation of *L. fermentum* AD1 culture

The rifampicin-resistant strain *L. fermentum* AD1 (isolated from canine faeces, own isolate) was inoculated into MRS broth (Merck) and incubated at 37°C for 24 h, then the bacterial cells were harvested by centrifugation at 2 000 g for 10 min at 4°C and the bacterial pellet was resuspended in a saline solution (0.85%, pH 7.0) to obtain the concentration 10^8 cfu/ml. The culture was stored at 4°C before application.

Haematology and determination of pH and organic acids

The leucocyte phagocytosis was detected using the diagnostic test Fago MSHP 53103-5 (Artim, Czech Republic). The activity of blood glutathione peroxidase was determined by a standard kit Ransel (Randox, England). Haemoglobin was analysed by a kit of Randox (England). At least 300 leucocytes were counted under low power microscopy to determine the differential percentages of white blood cells (lymphocytes, heterophils, basophils, monocytes and eosinophils).

The pH value in the contents of small intestine was determined immediately after their collection with an electronic pH meter (MS20, Tesla, Czech Republic). Approximately 1 g of intestinal digest was diluted in 50 ml of deionized water and 30 µl was applied for the analysis of organic acids. Capillary isotachophoresis (isotachophoretic analyser ZKI 01, Slovakia) was used for detection of formic, acetoacetic, lactic, succinic, acetic, propionic, butyric and valeric acids. As conducting and finishing electrolytes, 0.001 mmol/l hydrochloric acid (pH 4.25) and 5 mmol/l capronic acid (pH 4.5) were used.

Statistical analysis

Statistical evaluation of the results was performed by Student's *t*-test with the level of significance set at $P < 0.05$.

RESULTS

The results for the population of faecal bacterial groups are presented in Table 1. The application of *L. fermentum* AD1 to 2-days-old Japanese quail significantly increased the counts of lactobacilli (by 1.9 $\log_{10}$ cfu/g, $P < 0.01$) and enterococci (by 2.2 $\log_{10}$ cfu/g, $P < 0.001$) in faeces of birds in the experimental group compared to those in the control group on day 7 from the first administration of AD1 strain. The count of *E. coli* was reduced by 0.9 $\log_{10}$ cfu/g ($P < 0.05$). There were no significant differences in the population of staphylococci after AD1 culture application. *L. fermentum* AD1 achieved the amount 4.3 $\log_{10}$ cfu/g after 24 h, 8.1 $\log_{10}$ cfu/g after 3 days, 7.9 $\log_{10}$ cfu/g after 5 days and 5.2 $\log_{10}$ cfu/g on day 7 – it means 3 days after cessation of its administration.

In the contents of caecum, a significant increase of lactobacilli (by 1.4 $\log_{10}$ cfu/g, $P < 0.001$) and enterococci (by 2.3 $\log_{10}$ cfu/g, $P < 0.001$) was noted but no significant differences in the counts of *E. coli* and staphylococci were detected.

Weight gains were improved in birds of experimental group by 14 % (control group 2.44 g/day; experimental group 2.83 g/day).

The intestinal pH values in quail were similar in both groups (control group 6.04; experimental group 6.21). The effect of *L. fermentum* AD1 on organic acid concentrations in the intestinal contents is shown in Table 2. The concentration of lactic acid increased significantly ($P < 0.05$) in the experimental group of animals. The composition of the other acids was not significantly affected despite of the higher values of concentrations in a majority of the studied acids (acetic, acetoacetic, formic, succinic, valeric).

No significant differences were detected in red blood cell count, leucocyte count, differential leucocyte counts, haematocrit, haemoglobin concentration and glutathione peroxidase (Table 3). Additionally, there was a significant increase in the phagocytic activity of leucocytes in experimental birds after the application of AD1 strain

Table 1. Total counts of microorganisms detected in faeces of Japanese quail before and after application of *L. fermentum* AD1

Genera	Faeces				Caecum day 7	
	day 0		day 7			
	CG	EG	CG	EG	CG	EG
Lactobacillus sp.	7.80 (0.33)	7.56 (0.20)	7.05 (0.42)	8.91 (0.21)**	7.30 (0.28)	8.73 (0.16)***
L. fermentum AD1	–	–	–	5.21 (0.16)	–	5.83 (0.25)
Escherichia coli	4.46 (0.42)	4.98 (0.38)	7.43 (0.21)	6.62 (0.27)*	7.47 (0.39)	8.17 (0.33)
Enterococcus sp.	7.26 (0.29)	7.04 (0.24)	5.17 (0.26)	7.33 (0.14)***	5.70 (0.20)	8.02 (0.24)***
Staphylococcus sp.	2.46 (0.31)	2.25 (0.47)	3.37 (0.36)	3.43 (0.41)	3.83 (0.49)	3.76 (0.21)

CG = control group, EG = experimental group; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Table 2. Concentration of organic acids in small intestinal contents of quail at the end of experiment

Acid (mmol/l)	Control group	Experimental group
Lactic	32.3 (6.2)	51.3 (16.9)*
Acetic	29.4 (10.5)	34.1 (5.6)
Acetoacetic	24.7 (8.6)	31.8 (7.8)
Formic	17.7 (3.5)	21.9 (12.1)
Succinic	10.9 (3.4)	14.4 (2.6)
Propionic	2.4 (1.2)	1.7 (0.2)
Butyric	1.5 (0.1)	1.3 (0.5)
Valeric	1.5 (0.1)	1.6 (0.4)

*$P < 0.05$

Table 3. Haematology, glutathione peroxidase and phagocytic activity of quail at the end of experiment

Parameter	Control group	Experimental group
Haemoglobin (g/l)	9.47 (0.65)	9.50 (0.65)
Haematocrit (l/l)	0.30 (0.03)	0.33 (0.12)
Red blood cell count (T/l)	1.50 (0.51)	1.48 (0.33)
Leucocyte count (G/l)	16.68 (5.43)	14.40 (2.54)
Heterophils (%)	16.20 (12.50)	26.40 (7.40)
Lymphocytes (%)	83.80 (12.48)	73.40 (7.81)
Monocytes (%)	0.0 (0.0)	0.2 (0.4)
Basophils (%)	0.0 (0.0)	0.0 (0.0)
Eosinophils (%)	0.0 (0.0)	0.0 (0.0)
Glutathione peroxidase (U/ml)	43.2 (1.4)	45.9 (4.6)
Phagocytic activity (%)	8.0 (1.6)	16.8 (6.3)*
Index of phagocytic activity	0.56 (0.19)	2.25 (0.95)**

*$P < 0.05$, **$P < 0.01$

($P < 0.05$, and/or in the index of phagocytic activity $P < 0.01$).

DISCUSSION

In our case, although only a limited number of birds was involved, the addition of AD1 strain increased the weight gain of quail by 14% after 7 days from the beginning of its application. Numerous forms of microbial culture products have been used in poultry feed, but the response of growing broilers to the microbial products has been inconsistent. Jin et al. (1998) reported that the addition of *L. acidophilus* I26 strain or a mixture of 12 lactobacilli to the basal diet of broilers increased significantly their body weight for 0–6 weeks. Similarly, Kim et al. (1988) presented an increase in the body weight of chickens after supplementation of their diet with commercial probiotic. On the contrary, several authors (Watkins and Kratzer, 1984, Maiolino et al., 1992) reported that there were no significant differences in weight gains of chickens given diets with or without *Lactobacillus* cultures.

Probiotics are known to benefit the host animals by improving their intestinal microflora balance (Fuller, 1989). The administration of *L. fermentum*

AD1 to quail lead to significant increase of lactic acid bacteria and significant decrease of *E. coli*. Similarly, Jin et al. (1998) showed a decrease of the coliform population in the caecum of broilers after the addition of *L. acidophilus* or a mixture of lactobacilli to broilers while the counts of lactobacilli were not significantly influenced. A reduction of pathogenic *E. coli* was also observed in the gastrointestinal tract of gnotobiotic chickens dosed with *L. acidophilus* (Watkins et al., 1982). Several mechanisms have been proposed to explain this reduction effect: competition for receptor sites, production of antimicrobial products (e.g. bacteriocins), production of volatile fatty acids (acetate, butyrate and propionate) or stimulation of the host immune system (Nemcová, 1997). Volatile fatty acids are known to reduce the counts of enterobacteria and the hypothesis is that the undissociated form of volatile fatty acids and lactic acid reduces their counts. In our experiment, the reduction of *E. coli* could be caused by lactic acid production by AD1 strain because a significantly higher concentration of lactic acid was detected in the experimental group of quail. Other acids were not significantly influenced. Surprisingly, the pH values of small intestinal contents did not differ significantly. Probably, pH value depends on a quantitative proportion of L(+) lactate isomer and D(–) lactate isomer produced by the applied strain. Vahjen et al. (2002) also found a significant increase of lactic acid content in the jejunum and ileum of turkeys after the application of *Enterococcum faecium*. The study of Jin et al. (1998) showed that lactobacilli added to the diet of broilers increased the concentrations of volatile fatty acids in the ileum and caecum and decreased the pH values in the caecum. In their study, the non-volatile fatty acids, lactic and succinic acids, in the caecum and ileum of broilers were not influenced.

Concerning the haematology, no significant differences were observed after *L. fermentum* AD1 application. These results agree with the findings of Zhou et al. (2000), who tested potential probiotic lactic acid bacterial strains *L. rhamnosus*, *L. acidophilus* and *Bifidobacterium lactis* in mice. The enzyme glutathione peroxidase, a component of the antioxidative defence system, was not affected. It indicates that *L. fermentum* AD1 does not induce an oxidative stress in quail. Although the antioxidative properties of lactobacilli *in vitro* were studied (Kullisaar et al., 2002), the knowledge of the effect of potential probiotics on the values of antioxidative enzymes in animals is limited. An important result is a significant increase of the component of nonspecific immunity – phagocytic activity of leucocytes as well as index of phagocytic activity which were significantly higher in the experimental group of quail. A significant increase of peripheral blood leucocytes exhibiting phagocytic activity was also observed by Shu and Gill (2002), who studied the effects of feeding the immunoenhancing probiotic *L. rhamnosus* HN001 against *E. coli* O157:H7 infection in mice. Jahreis et al. (2002) demonstrated the influence of probiotic sausage (*L. paracasei* LTH 2579) on immunological parameters in healthy volunteers.

Overall, the present results indicated the ability of a canine isolate – *L. fermentum* AD1 to survive and to colonize the digestive tract of young Japanese quail during its application, to increase lactic acid bacteria population, body weight, lactic acid concentration, phagocytic activity of leucocytes and to decrease the population of *E. coli* in faeces. Moreover, the applied strain did not induce an oxidative stress in quail. Therefore, *L. fermentum* AD1 may have the potential to enhance intestinal health in birds after its preventive application. Whether a longer time of its application will lead to the same results remains to be determined.

Acknowledgements

Margita Bodnarova's excellent technical assistance is gratefully acknowledged. Our thanks belong to Dr. Bojana Bogovic-Matijasic from Zootechnical Department, Biotechnological Faculty, University of Ljubljana, Slovenia, for species identification of strain ADI.

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Received: 05–06–30
Accepted: 05–09–09

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