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Effect of two plant extracts and *Lactobacillus fermentum* on colonization of gastrointestinal tract by *Salmonella enterica* var. Düsseldorf in chicks*

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Abstract: According to published data, it is well known that essential oils from plants possess antimicrobial activity against a wide range of pathogens, including *Salmonella* strains. The influence of the administration of essential oils from oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*) as well as *Lactobacillus fermentum* on crop, caecum, liver and spleen colonization by *Salmonella enterica* var. Düsseldorf in chicks was investigated in this study. For the experiment, one hundred 1–day old chicks were used, and they were divided into four groups: (i) untreated (C); (ii) treated with *L. fermentum* (L); (iii) treated with a mixture of oregano and thyme essential oils (OT); and (iv) treated with a combination of *L. fermentum* and a mixture of oregano and thyme essential oils (LOT). Essential oils from oregano and thyme were mixed with commercial poultry diet, which was offered ad libitum to chicks in appertaining groups. *L. fermentum* was added daily to drinking water. After 3 days all the chicks were challenged orally with *S. enterica* var. Düsseldorf. The crops, ceca, spleens and livers of the birds were examined for *S. enterica* var. Düsseldorf colonization 5 days after the challenge. Our results showed that a combined administration of *L. fermentum* and essential oils (oregano and thyme) in group with combined application of essential oils and lactobacillus strain reduced the percentage of colonized crops and ceca when compared to the control group without any treatment.

Key words: essential oils; *Lactobacillus*; *Salmonella*; chicks.

Introduction

Enteric diseases caused by *Salmonella* spp. are an important concern of the poultry industry because of lost productivity, increased mortality, and the associated contamination of poultry products (Patterson & Burkholder, 2003).

Newly hatched commercial chicks are raised under controlled conditions which can delay the establishment of a definitive cecal bacterial community and increase the susceptibility of these chicks to salmonellae cecal colonization (Tellez et al., 2001). The main site of salmonellae colonization is the caecum (Hinton et al., 1991).

The use of antibiotics as a feed supplement for broilers in many countries has been limited by the European Union, which is why there is a trend to find some alternatives to antibiotics for in-feed use (Lee et al., 2004). Many authors have compared various plant essential oils as alternatives to antibiotics in animal production (Langhout, 2000; Mellor, 2000a,b; Taylor, 2001).

Essential oils should be regarded as one of several available feed additives that have been shown to have antibacterial activity against undesirable pathogenic bacteria, such as *Salmonella* spp. and others (Elgayyar et al., 2001). Essential oils consist of a number of active compounds with some of them comprising more than sixty individual components that can inhibit the growth of certain microorganisms (Russo et al., 1998). The chemical composition of essential oils is variable. For example, the concentrations of the main components of thyme essential oil (thymol and carvacrol) can range from 3–60% of the total essential oil (Lawrence & Reynolds, 1984). Major components can constitute up to 85% of the essential oil, whereas other components are present only as a trace (Senatore, 1996; Bauer et al., 2001); nevertheless they are also very im-

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portant. The primary components are the major active ingredients, while the secondary components act synergistically to increase the total effectiveness (Tzakou et al., 2001).

Many essential oil isolates exhibit inhibitory properties in challenge tests against microorganisms (Deans & Ritchie, 1987; Beuchat, 1994; Dorman & Deans, 2000). Herbs have been found to possess antimicrobial activity and anti-viral properties (Smith-Palmer et al., 1998; Hammer et al., 1999) and have been reported to activate the immune system (Chang et al., 1995; Barak et. al., 2001). The antimicrobial properties of plant volatile oils and their constituents from a wide variety of plants have been assessed (Lis-Balchin & Deans, 1997) and reviewed (Pattnaik et al., 1995; Carson et al., 1996). The mechanisms of action may vary greatly, and depend mainly on the composition of the essential oil (Cowan, 1999). The effect of essential oils can be enhanced through the synergistic effects both between individual essential oil and by combination with other feed additives, e.g. *Lactobacillus* strains, which can regulate the gut microbial flora (Si et al., 2006).

The main goal of the present study was to investigate the influence of the administration of essential oils from oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*) as well as *Lactobacillus fermentum* on crop, caecum, liver and spleen colonization by *Salmonella enterica* var. Düsseldorf in chicks.

Material and methods

Bacterial strains

Lactobacillus fermentum CCM 7158 was isolated from the ileum of a healthy hen. This strain was selected for rifampicin resistance. Resistance to rifampicin enabled differentiation between the administered organism and indigenous strains. The strain was cultured in MRS (Biomark Laboratories) broth. The numbers of lactobacilli were determined on MRS agar (Biomark Laboratories) containing rifampicin (100 μg/mL) and incubated under anaerobe conditions for 48 hours at 37 °C. The MRS broth containing the *Lactobacillus* strain (1×10^8 CFU/mL) served as a storage medium for the whole experiment and was being kept at 4 °C before use in the trials.

Salmonella enterica var. Düsseldorf SA31 (supplied by Dr.Vasiľková from the Institute of Parasitology, Slovak Academy of Sciences, Košice, Slovakia), resistant to nalidixic acid was used as a challenge strain. Salmonella for infection was cultured in PYG broth (yeast extract 10 g, D(+) glucose 10 g, peptone bacteriological 5 g, trypticase peptone 5 g/L, pH 6.8–7.2). The viable cell concentration of the inoculum was determined by counting of cherry-red colonies ($1x10^5$ CFU/mL) grown on Brilliant green agar plates (Biomark Laboratories) containing nalidixic acid after 24 hour incubation at 37 °C.

Chickens

One hundred 1–day-old New Hampshire broiler chicks were obtained from a local hatchery at Nižná Kamenica, Slovakia.

Essential oils

Oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*) essential oils were purchased from Calendula, Ltd., Nová Ľubovňa, Slovakia.

Enumeration of bacteria

This was carried out by the conventional pour plate technique. The salmonella colonization was expressed as the percentage incidence per infected organ in each experimental group.

Experimental procedure

One hundred 1–day-old broiler chicks were randomly divided into four experimental groups (25 chicks per group). The birds were kept in 4 breeding pens and maintained under continuous lighting. The pens were situated in an insulated room with facilities to control light, temperature and humidity, which were 32 °C and 40–60% respectively. The basic diet fed was a commercial diet NORM TYP HYD-04 (Tajba, a.s., Čaňa, Slovakia), that contained no antibiotics and no coccidiostat (Table 1). The diet was fed *ad libitum* and the birds had free access to drinking water.

The four experimental groups were as follows: (i) C, untreated group; (ii) L, group treated with *Lactobacillus fermentum*; (iii) OT, group treated with a mixture of oregano and thyme essential oils; LOT, group treated with a combination of *Lactobacillus fermentum* and a mixture of oregano and thyme essential oils.

One-day-old chicks in treatment groups L and LOT were treated orally through a thin plastic tube with *Lactobacillus fermentum* (10^8 CFU/mL) at a dose of 0.2 mL of the inoculum per chick. This procedure was performed before the birds were allowed access to food or water. From day 2 the same strain was added to drinking water from the stored suspension at the same dose of 0.2 mL per chick for the all remaining days of the experiment. Birds from C and OT groups received orally 0.2 mL of saline solution per chick as a placebo.

Birds in groups OT and LOT were fed daily with commercial diet and a mixture of thyme and oregano essential oils, which were added to the feed in a concentration of 0.05% during the whole experiment.

At day 3 all the birds were challenged orally also through a thin plastic tube with approximately 10^5 CFU/mL of *Salmonella enterica* var. Düsseldorf at a dose of 0.2 mL of the inoculum per chick. No waste of inoculum was seen.

Five days after challenge with the salmonella strain, all the chicks were killed and the crop, caecum, liver and spleen of each bird were collected aseptically and evaluated for *S. enterica* var. Düsseldorf colonization.

Whole crops were aseptically removed from each bird and were placed in separate sterile plastic bags. Twenty millilitres of sterile distilled water was added to each of the bags, and the contents were blended in a Stomacher Lab-Blender for 1 min. Serial dilutions were made in 0.1% peptone water and plated on an appropriate agar medium. Salmonellas were enumerated on BGA agar with nalidixic acid and lactobacilli on MRS agar with rifampicin.

The liver and spleen from each chick was minced with scissors, and incubated in 30 mL of tetrathionate broth (Difco) for 24 h at 37 °C. After incubation, samples from the broth were streaked on BGA plates containing nalidixic

Table 1. Composition of the feedstuff (NORM TYP HYD-04) in 1 kg.

Substance	Amount
ME	Minimum 11.5 MJ
NS	Minimum 175 g
Fibre	Maximum 50 g
Ash	Maximum 80 g
Lys	Minimum 8 g
Met	Minimum 3.5 g
Met+Cys	Minimum 7 g
Linoleic acid	Minimum 10 g
Ca	Minimum 8 g
P	Minimum 5 g
Na	1.2–2.5 g
Fe	Minimum 60 mg
Zn	Minimum 50 mg
Cu	Minimum 6 mg
Mn	Minimum 50 mg
Vitamin A	Minimum 8,000 IU
Vitamin D_3	Minimum 1,500 IU
Vitamin E	Minimum 12 mg
Vitamin B_2	Minimum 4 mg
Vitamin B_{12}	Minimum 10 μg
Choline	Minimum 300 mg

acid, incubated for 24 h at 37°C, and examined for *S. enterica* var. Düsseldorf colonies.

The similar procedure was performed with a caecum of the birds. The caecum was removed aseptically, minced with scissors, and incubated in 30 mL of selenite-cysteine broth for 24 h at 37°C. After incubation the broth was streaked on BGA plates with containing nalidixic acid and incubated for another 24 h at 37°C, and examined for typical *S. enterica* var. Düsseldorf colonies.

Results and discussion

Figure 1 shows that combined administration of *L. fermentum* and essential oils (LOT group) reduced the percentage of colonized crops and ceca by 86% and 57%, respectively, when compared to the control group. Administration of *L. fermentum* alone (L group) or essential oils (OT group) reduced the percentage of colonized crops by 43% and 57% in comparison to the control. The percentage of invasion of the liver and spleen was 57% and 43%, respectively, in the control group. No invasion was observed in the livers and spleens of the LOT group.

Pathogens have to overcome numerous obstacles in order to colonize the intestinal tract of animals and cause an infection (Patterson & Burkholder, 2003). Probiotics (mainly lactobacilli strains) are able to alter the intestinal microbiota to protect the birds against salmonellae and coliform colonization (Corrier et al., 1994).

The gastrointestinal tract of newly hatched chicks is almost sterile (Ewing & Cole, 1994). Either from exogenous preparations or through direct contact with the environment, the microflora with *Lactobacillus* appeared in the crop and caeca as early as on the 3rd day of age.

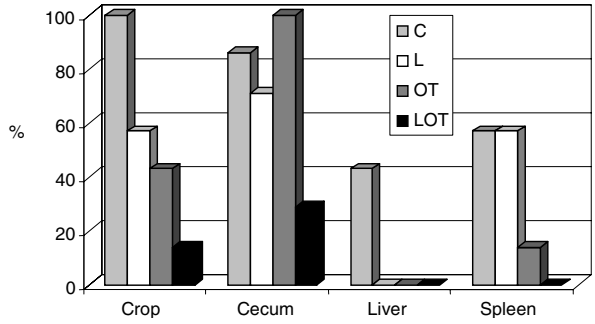

Fig. 1. Percentual incidence of *Salmonella enterica* var. Düsseldorf in examined organs of the chicks 5 days after challenge.

The information obtained from experiments that test a combination of probiotics and essential oils is still very limited. We know, however, that probiotics exert a beneficial effect on animal's health and performance (Cavazzoni et al., 1998; Lan et al., 2003); the same is true for the essential oils (Jamroz & Kamel, 2002; Lee et al., 2003).

Probiotics work through modification of the gastrointestinal microflora (Fuller, 1986). Chateau et al. (1993) showed that probiotics can effectively reduce the infection. Mechanisms that may inhibit the growth of pathogenic microorganisms include the production of organic acids, a decrease in environmental pH, production of H_2O_2, bacteriocins (Servin, 2004), enhancement of animal immunity (Blum & Schiffrin, 2003) and promotion of host-specific adhering activity (Schiffrin & Blum, 2002).

Mechanism of action of essential oils is not very clearly defined yet, but there are suggestions that they alter the permeability of the cell membranes and cause a destruction of the pathogenic bacteria (Skandamis & Nychas, 2001).

Our results demonstrated that orally administration of broiler chicks with lactobacilli decreased the colonization of tested organs of the chickens (except spleen) by *Salmonella enteritidis* var. Düsseldorf. The percentage of *S. enteritidis* var. Düsseldorf in the contents of crops and ceca of the treated chicks in the L group decreased when compared to the control group.

We obtained similar results with application of a blend of essential oils in OT group. The percentage of *S. enterica* var. Düsseldorf in the contents of crop and spleen decreased, while no positive effect was observed in the caeca of treated chicks. The positive effect of essential oils was also observed during *in vivo* trial in newly weaned piglets, where a combination of cinnamon, thyme and oregano extracts was used to investigate their effect on growth performance, gut morphology, microbiota and immune response (Namkung et al., 2004).

In our trial, in group LOT (combination of the *Lactobacillus* strain and a blend of essential oils) we obtained the best results in decrease of the salmonella

strain colonization in all examined organs. Our results showed that the combined administration of *L. fermentum* and essential oils from oregano and thyme seemed to be an effective to inhibit the colonization of gastrointestinal tract by *S. enterica* var. Düsseldorf in chicks.

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