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Influence of the probiotic *Enterococcus faecium* DSM 7134 as a feed additive on digestive, microbiological and immunological traits in police, working and sled dogs

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Abbreviations

AAHA	American Animal Hospital Association
Ab	Antibodies
Agg	aggregate substance
AIDS	Acquired immunodeficiency syndrome
ALT	Alanine transaminase
ANOVA	Analysis of Variance
APC	Antigen presenting cell
BHI	Brain-Heart-Infusion
BrdU	5-Bromo-2`-deoxyuridin
CD	Cluster of differentiation
CDV	Canine distemper virus
cfu	colony forming units
cGM-CSF	canine granulocyte macrophage colony stimulating factor
ConA	Concanavalin A
CPV	Canine parvovirus
DC	Dendritic cell
DNase I	Deoxyribonuclease I
DSM	Deutsche Sammlung von Mikroorganismen
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbant Assay
Esp	enterococcal surface protein
FACS	Fluorescence-activated Cell Sorter
FCS	Fetal Calf Serum
FeLV	Feline Leukemia Virus
FITC	fluorescein isothiocyanate
FIV	Feline Immunodeficiency Virus
G	Giga (10^9)
g	Gravitational force
GALT	Gut-associated lymphoid tissue
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IFN	Interferon
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL	Interleukin
IU	International Units
kcal	Kilocalorie
LAB	Lactic acid bacteria
LPS	Lipopolysaccharide
M	Molarity
MadCAM-1	Mucosal cell addressin molecule-1
ME	Metabolic Energy
MHC	Major Histocompatibility Complex
MJ	Megajoule
mM	millimolar
mmol	millimole
mol	mole
MRS	De Man, Rogosa and Sharpe Agar

mV	millivolt
MW	Molecular weight
N	Normality
n	number
NAD^+	Nicotinamide adenine dinucleotide
NFE	Nitrogen free Extracts
OD	Optical density
p	p-value, probability
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pen/Strep	Penicillin/Streptomycin
pH	Potentia hydrogenii
PHA	Phytohemagglutinin
ppm	parts per million
PWM	Pokeweed Mitogen
RAPD	Random Amplification of Polymorphic DNA
RPMI	Medium developed at Roswell Park Memorial Institute
SI	Stimulation Index
spp.	Species pluralis
T	Tera (10^{12})
TEM	transmission electron microscopy
TGF	Tumor growth factor
TLR	Toll-like receptor
TMB	3,3´,5,5´tetramethylbenzidine
TNF	Tumor necrosis factor
TSC	Tryptose sulphite cycloserin
Vit.	Vitamin

1 Introduction

Within hours after birth, the gastrointestinal tract is populated with numerous bacterial species, diversifying after weaning and further on the change to a more complex diet. The established indigenous flora is stable and very important for the development of a healthy gastrointestinal system. Since the largest immune organ is located in the gut, these luminal bacteria can have an influence to the entire host's health (Martineau and Laflamme, 2002).
The equilibrium of the microflora can be manipulated by some dietary and environmental factors. Antibiotic therapy, excessive hygiene and stress cause the most disruption (Fuller, 1989). Probiotics are of potential value in all situations where the balance of the gut microflora is adversely affected, enabling a possible restoration.
Despite a lot of knowledge obtained, the mode of action of probiotics has not been fully explained yet. Their effectiveness may be classified into three modes (Oelschlaeger, 2010):

1. Probiotics can modulate the host's defences, including the innate and acquired immune system.
2. Probiotics may have a direct effect on other microorganisms, commensal and pathogenic.
3. Probiotic effects are based on actions affecting microbial products, for example, inactivation of toxins or the detoxification of food components in the gut.

Enterococcus faecium DSM 7134 has not been extensively tested on the dog to this point in time. To show possible beneficial aspects of this strain, three different groups of active working dogs, confronted with their routine of daily work and training, received the probiotic as supplement in addition to their feeding. The three classified modes of actions were incorporated to investigate possible interactions of the probiotic with the cellular and humoral immune functions, the concentration of selected microorganisms in faeces, and the microbial products affecting faecal parameters.

2 Literature review

2.1 Definition of probiotics

The hypothesis made by Eli Metchnikoff in the early 20th century, that the consumption of fermented milk products was the clue to a long and healthy life of Bulgarian peasants, introduced the concept of probiotics (Culligan et al., 2009; Douglas and Sanders, 2008; Gupta and Garg, 2009).

The Greek derived word *probiotic* (*πρό βίος*), meaning "for life", was first used to describe "substances secreted by one microorganism which stimulates the growth of another" (Lilly and Stillwell, 1965).

The later definition of Fuller (1989), "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance", included not only the animal but also emphasised the importance of live cells.

Currently the FAO and WHO define probiotics likewise as "live microorganisms which when administered in adequate amounts confer a health benefit on the host". This definition applies for both human and animal probiotics.

2.2 Bacteria used as probiotics

Lactic acid bacteria, including the genera *Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Carnobacterium* (Carr et al., 2002), are mainly used in human nutrition. The main probiotic microorganisms in animal nutrition are *Enterococcus* spp., *Saccharomyces* yeast and spore-forming *Bacillus* spp. (Simon et al., 2001). *Enterococcus faecium* is the most important and most frequently used among *Enterococcus* species (Strompfova et al., 2004).

EC Nr. 1831/2003 regulates animal feed additives within the European Union. Currently, *Enterococcus faecium* NCIMB 10415, *Enterococcus faecium* DSM 10663/NCIMB 10415 and *Lactobacillus acidophilus* DSM 13241 are approved to be used as feed additives for dogs.

60% of probiotics include enterococci (Jin et al., 2000). *Enterococcus faecium* is part of the normal gut microbiota. Enterococci isolated from dog faeces ranged from 3.3 to 7.3 $\log_{10}$ cfu/g. Among 40 isolates, 11 were identified as *E. faecium*, 2 as *E. hirae* and 2 as *E. faecalis*. The other 25 strains could not be speciated (Strompfova et al., 2004). Probiotic effects can be strain specific: therefore, the description of a probiotic must include genus, species and strain (Douglas and Sanders, 2008).

2.3 Criteria and characteristics of probiotics

To exert beneficial effects, probiotics should have several desirable properties (Collins and Gibson, 1999; Fuller, 1989; Ouwehand et al., 1999; Simon et al., 2001; Teitelbaum and Walker, 2002). These include the following:

- Isolation from same species as intended host
- Acid and bile stability
- Adhesion to mucosal surfaces
- Be non-pathogenic and non-toxic
- Contain large number of viable cells
- Clinically validated and documented health effects
- Good technological properties, i.e. cultivable at large scale, strain stability or survival of viable bacteria in the preparation

Additional characteristics are required for probiotics, but not all have to be fulfilled, depending on the outcome or effect wanted:

- Lower serum cholesterol
- Increased bioavailability of vitamins and minerals
- Binding of mutagens and decreasing faecal carcinogen levels
- Effect on immune response
- Influence on intestinal motility
- Alleviation of symptoms of lactose malabsorption
- Production of anti-microbial substances
- Co-aggregation with pathogens

2.4 Mechanisms and established effects of probiotics

Probiotics yield many beneficial claims. Unfortunately, the scientific evidence to support these claims has not always been attained. Improvements caused by probiotics are known to be partially due to bacterial antagonism, competitive exclusion and immune stimulation (Zimmermann et al., 2001). The competitive exclusion concept has its origin in the establishment of adult-type resistance in newly hatched chicks by administering adult intestinal microorganisms (Nurmi et al., 1992). The possible modes of action are similar to

the assumptions of Fuller (1989). These include the competition for nutrients and/or adhesion sites, and the production of antimicrobial compounds. Probiotics could also change the microbial metabolism, increase antibody levels and macrophage activity. Others believe that probiotics interact not only with the indigenous microflora, but influence the host directly, which is seen locally on the mucous membrane and on the immune system (Görke and Liebler-Tenorio, 2001). Colonisation resistance is the so-called process where bacteria are inhibited to colonise by other strains. The same probiotic can have different mechanisms to hinder different pathogens (Rolfe, 2000). Different origins of probiotic bacteria also lead to the assumption of dissimilar modes of action, thus not all mechanisms are relevant for each probiotic microorganism (Simon et al., 2001).

2.4.1 Effects of probiotics on the intestinal mucosa

Adhesion to the intestinal mucosa is considered one of the main mechanisms (Rinkinen et al., 2003a) and one of the main selection criteria for probiotics (Ouwehand et al., 1999). Adhesion is vital for transient colonisation (Alander et al., 1999), antagonism against pathogens (Coconnier et al., 1993), as for modulation of the immune system (Schiffrin et al., 1997). The capability to adhere to the intestinal mucosa lengthens the perseverance in the intestine. The beneficial effect of the probiotic can therefore be longer exerted (Apostolou et al., 2001).

A strain dependency of different enterococci was found on *in vitro* adhesion to human, canine and porcine intestinal mucus. Isolates did not bind preferably to mucus from the same host as from which they were collected (Laukova et al., 2004). Similar, probiotics intended for human use (lactobacilli) had good adhesion properties to canine jejunal mucus (Rinkinen et al., 2000). The strain dependency was confirmed through further studies (Rinkinen et al., 2003b), suggesting that the animal model for probiotic adhesion assays may be implied on other species.

Probiotic bacteria compete with pathogenic bacteria, due to their own proliferation in the intestine and adhesion to its wall, forming a so called "protective layer" and therefore, preventing the adhesion, intrusion and proliferation of the pathogenic bacteria (Flachowsky and Daenicke, 1996).

Selected lactic acid bacteria were evaluated for their ability to inhibit adhesion of *Staphylococcus intermedius*, *Salmonella* Typhymurium ATCC 14028, *Clostridium perfringens* and *Campylobacter jejuni* to immobilised mucus isolated from canine jejunal chyme *in vitro*. All tested lactic acid bacteria strains reduced the adherence of *Cl. perfringens*

significantly. The two strains *Enterococcus faecium* M74 and *Enterococcus faecium* SF273 however increased the adhesion of *C. jejuni* (Rinkinen et al., 2003a). Live *E. faecium* 18C23 inhibited, dose dependent, the adhesion of *E. coli* K88ac and K88MB to porcine small intestinal mucus. An inhibition of > 90 % occurred when 10^9 cfu of *E. faecium* 18C23 were added simultaneously with *E. coli* to immobilised mucus (Jin et al., 2000).

Bifidobacterium breve 4 and Bifidobacterium infantis showed also dose dependent inhibition of enterotoxic and enteropathogenic *E. coli*, and *Salmonella* Typhimurium adhesion to Caco-2 cells (Bernet et al., 1993). The adhering number of the same pathogenic bacteria to Caco-2 cells decreased, when *L. acidophilus* was increased from 10^6 to 10^9 cfu/ml (Ostad et al., 2009). *Lactobacillus rhamnosus* GG showed good binding ability to Caco-2 cells and again inhibiting adhesion of pathogens, such as *L. monocytogenes, Salmonella* Typhimurium, *Shigella boydii* and *Staphylococcus aureus* (Xu et al., 2009).

Zentek et al. (1998) demonstrated a transient colonisation of enterococci in the ileum chyme and faeces whilst feeding *E. faecium*. The application stop caused a reduction of the enterococci concentration within a few days. An investigation with transmission electron microscopy (TEM) of *L. casei* in the gut showed that it adhered to the epithelial villi and was present in the intestinal lumen (Galdeano and Perdigon, 2004), but only the bacterial antigen was found in the cytoplasm of enterocytes. The assumption was made, that probiotic bacteria adhere only temporarily, since probiotics are not long-termed colonists of the gastrointestinal tract (Marco et al., 2006). For possible health effects, a constant application has to be warranted.

Bernet et al. (1993) analysed the mechanism of adhesion for *Bifidobacterium breve 4* and found the involvement of a labile surface-associated proteinaceous component. The adhesion of *Lactobacillus fermentum* BCS87 is mediated also by two surface-associated proteins. The expressed mucus- and mucin-binding proteins (*32-Mmubp*) have a relative molecular weight of 29 and 32 kDa (Macias-Rodriguez et al., 2009). Kravtsov et al. (2008) found that the adhesion characteristics of *Lactobacillus* strains are partly due to the lectin-binding adhesin. Again, this cell surface-associated protein has a molecular weight of 25-30 kDa. For enterococci, the so-called aggregation substance (Agg) and enterococcal surface protein (Esp) are known to aid in the colonisation of the host. Unfortunately, these are virulence factors and may be associated with infection (Fisher and Phillips, 2009; Shankar et al., 1999).

2.4.2 Effects in the intestinal lumen

2.4.2.1 Competition for nutrients

Following the theory of chemostat, a possible mode of action for probiotics is the competition for nutrients with pathogenic bacteria. It is assumed that the probiotic bacteria utilise nutrients, rendering them unavailable for pathogenic bacteria (Fuller, 1989; Rolfe, 2000). The *in vivo* evidence is lacking for this assumption.

2.4.2.2 Modification of the intestinal conditions

The consumption of metabolic active probiotic strains can lead to a decrease in the faecal pH, indicating the production of organic acids (Ouwehand et al., 1999), and especially the stimulation of lactic acid producing bacteria (Collins and Gibson, 1999). Zentek et al. (1998) measured a lower pH, whilst administering *E. faecium*, in the ileum chyme of dogs, with a concurrent increase of lactic acid.

Similar, a modification of the intestinal condition took place when *E. faecium* NCIB 10415 was fed to dogs, under different environmental conditions, causing a significant decrease in *Clostridium* spp. count. It was assumed that the probiotic strain enhanced the activity of other lactic acid bacteria and henceforth the production of lactate (Vahjen and Männer, 2003), since earlier examinations with this strain on turkey poults had exactly these effects (Vahjen et al., 2002). Swanson et al. (2002) also encountered a *Clostridium* spp. reduction due to lactic acid increase. Audisio et al. (1999, 2000) investigated the antagonistic compounds of *E. faecium* J96. The antibacterial action (bacteriostatic and bactericidal) was based upon the combination of lactic acid and bacteriocins (see 2.4.2.3).

Probiotic bacteria can also produce enzymes, or suppress and activate already existing enzymes in the gut (Fuller, 1989). These self-produced enzymes assist the digestive breakdown of feed or the detoxification of harmful metabolites (Vanbelle et al., 1990).

One example is 1,3-1,4 endo-β-glucanase (lichenase), produced by an *E. faecium* strain isolated from chickens, aiding in the partial breakdown of plant fibres (Beckmann et al., 2000). Lactose malabsorption, due to a deficiency of β-galactosidase in the intestinal mucosa (Salminen et al., 1998), can be alleviated through bacterial enzymes. Yoghurt consumption enhances the lactose digestion in lactase deficient subjects (Kolars et al., 1984). The amount of lactose is not only lower in yoghurt, but yoghurt is also tolerated better than milk, due to the β-galactosidase produced by probiotic bacteria (Kolars et al., 1984; Marteau et al., 2002).

Their lactase activity is exerted in the gut lumen (Saavedra, 2001) and the modification of the colonic microflora also aids the utilisation of lactose (Marteau et al., 2002; Vesa et al., 1996). The normal intestinal flora also produces enzymes that can convert precarcinogens into active carcinogens (Goldin et al., 1980; Rolfe, 2000). Glycosidase, β-glucuronidase, azoreductase and nitroreductase are such enzymes. *Lactobacillus* GG, *L. casei* and *L. acidophilus* can reduce these enzymes by inhibiting the producing bacteria (Goldin, 1990; Goldin et al., 1980; Hayatsu and Hayatsu, 1993; Lee and Salminen, 1995; Lidbeck et al., 1992; Ling et al., 1994). Other anti-tumour activity of probiotics include the inhibition of tumour cells and the direct destruction of carcinogens (Bomba et al., 2002).

Although only 2 % of neoplasms are located in the gastrointestinal tract of the dog, 40 % of these neoplasms have its origin in the large intestine. These tumours include mainly adenomatous polyps, adenocarcinoma and malignant lymphoma, whilst leiomyoma and –sarcoma are sparse (Suter and Kohn, 2006).

2.4.2.3 Antimicrobial substances

Lactic acid bacteria can produce antimicrobial (inhibitory) substances. Most commonly are organic acids, such as lactic (discussed earlier) and acetic acid, hydrogen peroxide, carbon dioxide and, most important, bacteriocins (Zimmermann et al., 2001).

Bacteriocins are peptides, proteins or protein-carbohydrate complexes, with mainly a narrow spectra of activity (Tannock, 1998).These substances can not only decrease viable cells, but can also alter the bacterial metabolism or toxin production (Rolfe, 2000). Bacteriocins may help to control the autochthonous microflora and may be advantageous to the producing strain for its establishment and competition in the gastrointestinal tract (Du Toit et al., 2000).

Early on, Vincent et al. (1959) already discovered the antibacterial product lactocidin from *L.* acidophilus. A broad-spectrum antimicrobial substance is reuterin, which is produced by *Lactobacillus reuteri* during glycerol fermentation (Talarico et al., 1988). *Bacillus cereus* GN105 produces a bacteriocin called cerein, which is only active against other *Bacillus cereus* strains and not lactic acid bacteria (Naclerio et al., 1993). Lactacin B is produced, pH dependently, by *Lactobacillus acidophilus* N2. Its bactericidal effect is restricted to other members of lactobacilli (Barefoot and Klaenhammer, 1984). The *Enterococcus faecium* strain EK13, isolated from cattle dung water, also produces bacteriocins. Enterocin A and P have an inhibitory effect on different strains of the genera *Enterococcus*, *Leuconostoc*, *Lactobacillus*, S*treptococcus*, *Staphylococcus*, *Bacillus* and *Listeria* (Marekova et al., 2003). Enterocin M is a newly found bacteriocin, which is produced by *Enterococcus faecium* AL41. It showed a

wide spectrum of inhibitory activity and is very similar to Enterocin P, with difference in the molecular weight (Marekova et al., 2007). Out of pig faeces derived *E. faecalis* strains produce bacteriocins with narrow range of bactericidal activity, mainly towards other enterococci. *E. faecium* strains derived from the same faeces, produce bacteriocins, which inhibit a much wider range of bacteria, including *L. monocytogenes*, *L. innocua*, *Cl. sporogenes*, *Cl. tyrobutyricum* and *Propionibacterium* spp. (Du Toit et al., 2000).

2.4.3 Effects of probiotics on the host

2.4.3.1 Gastrointestinal immune system

The gastrointestinal tract is the largest organ of host defence (Ouwehand et al., 2002). Next to the immunological defences, the mechanical barriers in first place, aid to withstand intruding pathogens. These include peristalsis, mucus production of the goblet cells, low pH in the stomach and bile acid, as also the competition of indigenous bacteria with pathogens for nutrients or cell surface attachment. The intestinal epithelium also has a number of specialised adaptations, which are not found elsewhere. These are so called defensins (antimicrobial peptides), mucins and trefoil peptides, secreted by goblet cells for the epithelial protection and repair, as not to forget the secretory immunoglobulin A, which bind luminal antigens at the cell surface to hinder infiltration of the *Lamina propria* (Mostov, 1994; Nagler-Anderson, 2001).

Neutrophiles, basophiles, eosinophiles, macrophages and dendritic cells are all involved in mediating innate immune responses. These cells are equipped with different receptors, e.g. Toll-like receptor or complement receptor, which can enhance phagocytosis, secrete antimicrobial peptides, cytokines and chemokines in response to pathogens.

The GALT (gut-associated lymphocyte tissue) represents the immune system of the intestine. Secondary lymphatic organs found within the intestinal wall are the Peyer's patches in the small intestine and isolated lymphoid follicles in the small and large intestine. The dome like aggregate of the Peyer's patch initiates the immune response, lying in the *Lamina propria* and consisting of a large number of B-cells including the germinal centre, together with smaller T-cell areas found between or directly below the follicles.

A layer of enterocytes separates the lymphoid tissue from the gut lumen. In between these enterocytes, small amount of microfold cells (M-cells) are found. Unlike enterocytes, they do not present microvilli, but have a folded luminal surface and are the route by which the antigen enters the Peyer's patch (Erickson and Hubbard, 2000; Isolauri et al., 2001; Janeway

et al., 2008; Koch and Berg, 1990; Ouwehand et al., 2002). The isolated lymphoid follicles have a similar structure to the Peyer's patch, but these follicles contain mainly B-cells. Antigen-presenting cells (APCs), like macrophages and dendritic cells collect the antigens, transport and represent them to the lymphocytes in the germinal centre, where the immune reaction is initiated (Koch and Berg, 1990). Dendritic cells (DC) choose also an independent M-Cell passage way, by directly sampling luminal antigen through the opening of tight junctions and reaching for bacteria with the dendrites outside of the epithelium (Nagler-Anderson, 2001; Rescigno et al., 2001).

Activated T-lymphocytes differentiate into T-helper cells, Th_1 and Th_2. Differentiation is dependent on the cytokine production and influence (Playfair and Baron, 1995). IL-2, IL-12, IL-18, TNF-α, TNF-β and IFN-γ are dependent on Th_1, whilst IL-4, IL-5, IL-10, IL-13 and TGF-β are associated with Th_2 (Ouwehand et al., 2002; Valli et al., 2009). Th_1 further activates B-cells to the production of IgA. Secretory IgA is in a dimeric form in the gut, resistant to proteolysis and not involved in an inflammatory response. The major function is the exclusion of foreign antigens, by preventing the binding to the epithelial cells and therefore inhibition of penetration (Erickson and Hubbard, 2000).

Intraepithelial lymphocytes (IELs) are located in the epithelial lining of the small intestine and aid in the surveillance, beside the Peyer's patches (Janeway et al., 2008; Stokes and Waly, 2006). IELs are heterogeneous in the dog, including $CD3^+$ and $CD5^+$ lymphocytes, whilst $CD8^+$ cytotoxic T-cells greatly outnumber $CD4^+$ helper T-cells (mainly Th_1) (Stokes and Waly, 2006), whilst in the *Lamina propria* $CD4^+$ T-cells are more abundant rather than $CD8^+$ cytotoxic T-cells (German et al., 1999). A greater number are encountered in the villi, gradually decreasing towards the crypt epithelium. On the other hand, B-cells are mainly presented in the crypt, while few are encountered in the villi (Stokes and Waly, 2006).

In order for an effective mucosal immune response, lymphocytes must traffic between the inductive (Peyer's patch) and the effector site (*Lamina propria*) (Stokes and Waly, 2006). This is possible due to receptors on the lymphocytes, which recognise adhesion molecules expressed on the endothelial cells of mucosal postcapillary venules (Erickson and Hubbard, 2000), also known as mucosal cell addressin molecules, MAdCAM-1 (Stokes and Waly, 2006). Activated T-lymphocytes percolate through the regional (mesenteric) lymph nodes, returning to the *Lamina propria* of the intestinal and other secretory tissues via the thoracic duct and blood vascular system (Erickson and Hubbard, 2000).

Since many antigens (food components, commensal bacteria) are presented in the gastrointestinal tract, oral tolerance prevents the immune system from overresponding

extensively to potential antigens (Erickson and Hubbard, 2000). Oral tolerance is dose dependent. By exposure to low concentration of antigens, Th_1 cells are suppressed through IL-4, whilst high doses lead to clonal anergy, where T-cells are in a state of unresponsiveness (Janeway et al., 2008; Ouwehand et al., 2002).

2.4.3.2 Immune modulation by probiotic bacteria

To influence the immune system, the probiotic microorganisms must activate the lymphoid cells of the GALT. Indirect influences to the immune system can also occur when non-adhesive microorganisms modulate the microflora, or change the intestinal permeability for other antigens (Ouwehand et al., 1999). The effect of a probiotic depends on the disease state and the immunoreactivity of the individual, in which an unfocussed immune response becomes focussed, or an amplified immune response during infection is eased (Salminen et al., 1998).

Not only intact bacteria can lead to the cascade of immune stimulation, but also parts of the cell wall. These components are thought to be the main immunomodulatory aspects for some authors (Hamann et al., 1998; Meydani and Ha, 2000), which include peptidoglycans, lipoteichoic acid and lipopolysaccharides (LPS). A variety of Toll-like receptors (TLR) on epithelial cells and phagocytes, such as macrophages and dendritic cells, recognise molecular patterns called PAMPs (pathogen associated molecular patterns). TLR-2 recognises lipoproteins/lipopeptides from various pathogens, peptidoglycans and lipoteichoic acid from gram-positive bacteria, whilst TLR-4 distinguishes LPS from gram-negative bacteria (Galdeano and Perdigon, 2006; Vinderola et al., 2005). The application of *Lactobacillus casei* CRL 431 influenced the expression of such receptors. TLR-2 and CD-206, a mannose receptor able to recognise microbial proteoglycans, increased in number on mononuclear cells isolated from Peyer's patches. Concurrent to that, an increase of IL-6 was reported, leading to higher numbers of IgA^+ B-cells, independent of $CD3^+$, $CD4^+$ and $CD8^+$ cells (Galdeano and Perdigon, 2006). IL-6 is produced by the mononuclear cells, as an innate immune response to the receptor attachment. It is a multifunctional cytokine, with one task being the terminal differentiation of B-lymphocytes (Vinderola et al., 2005). Similar outcomes were seen in the study of Vinderola et al. (2005) with *Lactobacillus casei* CRL 431 and *Lactobacillus helveticus* R389. IL-6 and therefore IgA^+ B-cells increased, without any change in the $CD4^+$ T-cell population and therefore no induction of the systemic immunity. In other studies with *Lactobacillus helveticus* R389, secretory IgA and macrophage activity was increased significantly (Matar et al., 2001; Vinderola et al., 2007), as were other cytokines such as IL-2

and TNF-α (Vinderola et al., 2007). Earlier studies, with different LAB showed that only the bacterial antigen was found in the cytoplasm of enterocytes (Galdeano and Perdigon, 2004), but an increase of different cytokines (IFN-γ, IL-12, IL-10, and IL-4) was also found here, often accompanied with stimulated IgA^+ B-cells (Galdeano and Perdigon, 2004; Park et al., 2005; Perdigon et al., 2003). In conclusion, the main mechanism of probiotic bacteria seems to be due to the stimulation of the innate immune system (Galdeano and Perdigon, 2006).

2.5 Probiotic application to dogs

The normal composition of the intestinal microflora can be altered by stressful conditions, such as weaning, dietary changes, gastrointestinal infections and oral administration of antibiotics. The feeding of probiotics could be a valuable tool to improve animal health and act in a prophylactic way to reduce the risk of infection by stabilising the gut microflora.

The influence of probiotic bacteria to the canine gut microflora has been reported several times. Lactobacilli strains are well known to alter the indigenous flora. *Lactobacillus acidophilus* DSM 13241, given to adult dogs with a concentration of $1*10^9$ cfu, increased the faecal concentration of lactobacilli, whilst reducing *Cl. perfringens* (Baillon et al., 2004). A numerical increase of lactobacilli and bifidobacteria was also encountered in the study of Pascher et al. (2008) on dogs with non-specific dietary sensitivity using the same bacterial strain. The application also caused a numeric reduction of *Cl. perfringens* and coliform bacteria. The feeding of *Lactobacillus fermentum* AD1 brought also a significant rise of faecal lactobacilli and enterococci about, but coliform bacteria and staphylococci stayed constant (Strompfova et al., 2006). The probiotic cocktail of *L. acidophilus* NCC 2628, *L. acidophilus* NCC 2766 and *L. johnsonii* NCC 2767 given to dogs suffering from chronic diarrhoea, caused also higher lactobacilli and lower enterobacteriaceae concentrations. Here, enterococci concentrations stayed constant over the application period (Sauter et al., 2006). In the *in vitro* studies (Biagi et al., 2007) with fresh faecal samples of adult dogs, *Lactobacillus animalis* LA4 promoted a significant rise of lactobacilli, whilst reducing the concentration of enterococci and after 24 h also *Cl. perfringens*. Additional *in vivo* studies with the same strain over a period of 10 days recorded a significant higher amount of lactobacilli at day 11, compared to the beginning (Biagi et al., 2007). Manninen et al. (2006) fed 5 permanently fistulated beagles, for a time of 7 days, with a mixture of *L. fermentum* LAB8, *L. salivarius* LAB9, *Weissella confusa* LAB10, *L. rhamnosus* LAB11 and *L. mucosae* LAB12. During the feeding period, the diversity of the indigenous microflora changed

towards one dominant *L. acidophilus* strain, not included in the mixture. In 4 out of 5 dogs, this *L. acidophilus* strain remained after the application had ceased.

The application of enterococci as probiotics produced similar effects. Feeding *Enterococcus faecium* to young pups caused temporarily higher concentrations of enterococci in faeces, as well as in the ileum chyme (Molitor, 1996; Weiß, 2003). Parallel outcomes were seen with adult dogs (Molitor, 1996; Zentek et al., 1998). Marcinakova et al. (2006) fed *Enterococcus faecium* EE3 over a period of 7 days and measured different faecal bacteria concentrations for up to 3 month. Enterococci concentration increased not only for the one week of feeding, but a continuous climb was seen for these 3 months. The lactobacilli concentration followed this pattern as well. After the first 7 days, a reduction of staphylococci and pseudomonas-like bacteria was also noticed. *E. coli* concentration remained uninfluenced.

During the probiotic application with *E. faecium* DSM 7134, Mück (2007) too encountered higher number of enterococci during these periods. In addition, after the first probiotic period, a decrease of *Clostridium* spp. was illustrated, which stayed at this low concentration for over the rest of the trial period. A reduction of *Clostridium* spp. is reported frequently (Rinkinen et al., 2003a; Vahjen and Männer, 2003; Weiß, 2003). Vahjen and Männer (2003) applicated the probiotic *E. faecium* NCIMB 10415 to 12 healthy, adult dogs over a period of 18 days. Not only was the *Clostridium* spp. concentration significantly reduced, but also furthermore higher amounts of *Salmonella* spp. and *Campylobacter* spp. were found in the faeces. Similarly reduced *E. faecium* M74 and *E. faecium* SF273 the adhesion ability of *Cl. perfringens* significantly, but additionally enhanced the adhesion of *C. jejuni*, which is considered to be pathogenic for dogs and humans (Rinkinen et al., 2003a).

In the context to probiotics, prebiotics and synbiotics should be mentioned. A prebiotic substance is used as a substrate, which cannot be digested, but can be utilised by the gut microflora, which is mostly a non-digestible fructooligosaccharide. The term synbiotics combines probiotics and prebiotics in a synbiotic relationship, were the prebiotic favours and enhances the probiotic compound (Schrezenmeir and Vrese, 2001).

Non-digestible oligosaccharides can have an influence on the composition of the microflora. *Cl. perfringens* reduction and an increase of bifidobacteria or lactobacilli was found after feeding a non- digestable oligosaccharide (Swanson et al., 2002; Zentek et al., 2003), but the combination of *L. acidophilus* and fructooligosaccharide, for an example, amplified the concentration of total anaerobic bacteria, compared to their use alone (Swanson et al., 2002).

A potential probiotic should colonise the intestinal tract temporarily. The feeding of *Lactobacillus rhamnosus* GG (LGG), at different doses ($1*10^9$, $1*10^{10}$, $5*10^{10}$, $5*10^{11}$ cfu) to

beagles, showed that LGG was detectable at all levels in the faeces. The faecal levels were significantly higher in the group receiving $5*10^{11}$ cfu, but LGG presented itself only up to 72 hours after cessation in the faeces (Weese and Anderson, 2002). *Lactobacillus animalis* LA4 was depicted in all dogs one day after cessation, and in 4 out of 9 dogs after 5 days of application stop (Biagi et al., 2007). Sauter et al. (2006) detected only the strain *Lactobacillus johnsonii* NCC 2767 out of the probiotic cocktail (included also *L. acidophilus* NCC 2628 and *L. acidophilus* NCC 2766) using the RAPD-PCR method. *L. acidophilus* DSM 13241 could be identified in 23 out 62 isolates collected during probiotic application (Baillon et al., 2004). The probiotic bacteria are mainly reported to exist for up to 3-7 days after the application has stopped (Biourge et al., 1998; Manninen et al., 2006; Weese and Anderson, 2002; Zentek et al., 1998). Other examples show, that some probiotic bacteria can last and therefore colonise much longer. *Enterococcus faecium* EE3 persisted over a time of three months, reaching its peak concentration at that time (Marcinakova et al., 2006). *Lactobacillus fermentum* AD1 counted 7.0-8.7 $\log_{10}$ cfu/g at application stop, and even 6 months later counts of 3.0-5.0 $\log_{10}$ cfu/g were still located (Strompfova et al., 2006).

The increase of lactic acid, in combination with a faecal pH decrease, as for a reduction of ammonia concentration is a desired outcome of probiotic substitution. Most of the time, no such changes are reported (Baillon et al., 2004; Biagi et al., 2007; Mück, 2007; Pascher et al., 2008). Tendencies of lactate increases and pH reduction in the ileum chyme were discovered by the application of *E. faecium* to fistulated dogs (Zentek et al., 1998), but faecal concentrations stayed constant. Again the positive outcome of adding a prebiotic substance has to be mentioned. *L. acidophilus* caused significant lower NH_3 concentrations alone (Pascher, 2004), but when combining *L. acidophilus* and fructooligosaccharide, a reduction of ammonia concentration and better faecal pH were found. Lactate concentrations could increase when fructooligosaccharide was fed alone (Swanson et al., 2002). An *in vitro* study with *L. animalis* LA4 with fresh canine faecal samples showed an increase of lactate concentration after 4 hours and a decrease of ammonia after a time of 8 hours (Biagi et al., 2007).

Changes in faecal dry matter, faecal consistency and frequency of defecation are habitually discussed. *L. acidophilus* DSM 13241 fed to dogs with non-specific dietary sensitivity improved the faecal consistency and defecation frequency, and raised the value of faecal dry matter (Pascher et al., 2008). Faecal scores again excelled in combination with fructooligosaccharides (Swanson et al., 2002).

Immunomodulatory effects are another important benefit that probiotics may exert. Few have been reported referring to dogs. One aspect is the change of special cell populations of the innate immune system. The application of *E. faecium* in puppies by Weiß (2003) followed a counted increase of lymphocytes, neutrophiles and monocytes. Baillon et al. (2004) encountered as well a rise of neutrophiles and monocytes after administering *L. acidophilus* DSM 13241. The red blood cells, haematocrit and haemoglobin concentrations were additionally higher. The application of a recombinant *Lactobacillus casei* expressing canine GM-CSF also enhanced monocyte counts. Canine GM-CSF (canine granulocyte macrophage colony stimulating factor) is a cytokine with a unique ability to stimulate the differentiation of haematopoietic progenitor cells into dendritic cells (Chung et al., 2009). On the other hand, Kanasugi et al. (1997) came across higher neutophile phagocytic activity after only one single administration of *Enterococcus faecalis* FK-23.

Benyacoub et al. (2003) measured a higher amount of mature B-cells and therefore an increase of secretory (faecal) IgA concentration, after feeding *Enterococcus faecium* SF68 to puppies. An increase of the $CD21^+$ cells was also detected after *E. faecium* DSM 7134 had been applied (Mück, 2007). Other lymphocyte subset ($CD4^+$ and $CD8^+$) stayed constant in the application of *Enterococcus faecium* SF68 in dogs (Benyacoub et al., 2003), while the same probiotic bacteria increased $CD4^+$ cells in cats (Veir et al., 2007).

Probiotics are reported to boost lymphocyte proliferation. *Enterococcus faecalis* FK-23 stimulated the lymphocyte blast transformation (proliferation) with the mitogens PHA (phytohemagglutinin), ConA (concanavalin A) and PWM (pokeweed mitogen) (Kanasugi et al., 1997), or LPS (lipopolysaccharide) (Hasegawa et al., 1999). Mück (2007) also described the elevation of lymphocyte proliferation with PHA, ConA and PWM during *E. faecium* DSM 7134 administration.

Immunoglobulins are part of the specific immune system. Higher serum IgG levels were found after the *L. acidophilus* DSM 13241 probiotic period (Baillon et al., 2004) and high serum IgA levels after the application of recombinant *L. casei* expressing cGM-CSF (Chung et al., 2009). Chung et al. (2009) applied, in addition, a CCV (canine corona virus) vaccine to the puppies, resulting in significantly higher levels of CCV specific IgG in the serum. Benyacoub et al. (2003) tested the immunomodulatory properties of *E. faecium* SF68 by simultaneously vaccinating 9 week old puppies with a live attenuated canine distemper virus (CDV) vaccine, receiving a booster at 12 weeks of age. Again, CDV vaccine specific IgG and, in this case also IgA, were significantly increased in the serum.

While most reports prove only the immunomodulation of the innate immune system, demonstrated Benyacoub et al. (2003) and Chung et al. (2009), the possible influences of probiotics on the adaptive immune system.

2.6 Conclusion and Outlook

Published clinical effects are still rare, despite the fact that probiotics are increasingly used in companion animals. The use of probiotics in the treatment of different diarrheal diseases and immune stimulation appears promising. Although probiotic bacteria are administered with a low dose of 10^9 bacteria compared to about 10^{14} bacteria colonised in the colon (Oelschlaeger, 2010), the modulation of the immune system can still be observed along with the successful competition for limiting resources, as for inhibition of adhesion and invasion of pathogens.

The current work is focussed on the effectivity of the probiotic *Enterococcus faecium* DSM 7134 on healthy, adult working dogs, with the main attention to different aspects of cellular and humeral immune functions, microbial composition of faeces, as for other faecal parameters and microbial products:

- Are there improvements of the faecal consistency to more formed faeces and a reduction of defecation within the group due to administration, as for each individual dog?
- Does the probiotic have an influence on faecal parameters such as pH and dry matter, as on the microbial products lactic acid and ammonia?
- In which way changes the faecal microbial concentration, and does the probiotic strain *Enterococcus faecium* DSM 7134 survive the gastrointestinal passage?
- Is there an influence on the cellular immune system?
- Can the probiotic stimulate and enhance the adaptive immune system, especially after provocation with prior known and unknown antigens, in this case with vaccines?

3 Material and Methods

3.1 Experimental aim

The objective was to study the effects of the application of the probiotic *Enterococcus faecium* DSM 7134 on two different kinds of working dogs, considering different microbiologic and immunologic parameters.

In addition, another aim was to study the effects of the probiotic *Enterococcus faecium* DSM 7134 appliance on the immune response in a vaccination trial with sled dogs.

3.2 Police dogs

3.2.1 Study design

A double-blinded cross-over study was conducted. Two allotted groups received in addition to their feed, either the probiotic *Enterococcus faecium* DSM 7134 (Bonvital®, dosage: $1*10^9$ cfu/kg of complete diet, supplier: Lactosan, Kapfenberg, Austria), or a placebo with the same texture. After 4 weeks of feeding, two weeks of wash out followed. For the second phase, the preparations were exchanged in between the two groups (Fig. 1).

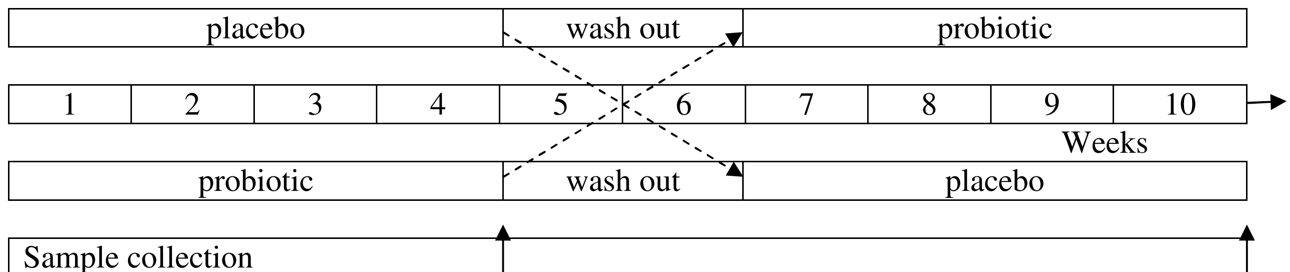

Fig.1: Study design for the double blind cross-over study

3.2.2 Animals, housing and diets

Fourteen adult dogs from the Berlin police force (Dir ZA, 1. BPA ZDhfE, 13597 Berlin, Germany), working/training an average of 22 min a day (during application), each privately owned by police officers and individually fed with commercial dry diets, participated in this study (Table 1). Every dog holder was responsible for giving the feed additive to his/her own dog. The daily preparation was pre-packed, at given dosage, in vessels for easy handling.

All dogs were vaccinated and dewormed as usual by the owners. They undergo regular blood and health checks, to control health and utilisability.

Table 1: Police dogs in the study

Name	Gender	Age (years)	Breed	Feeding
Grete	female	1.75	German shepherd	Aldi Alnutra
Chris	female	1.5	German shepherd	Aldi Alnutra
Rinti	male	8	German shepherd	Happy Dog Lamm & Rice
Boogy	female	3	German shepherd	Happy Dog Balance
Eve	female	6.5	German shepherd	Aldi Alnutra
Rudi	male	3.5	Malinoi	Aldi Alnutra
Askan	male	8	German shepherd	Rinti
Zamb	male	8	German shepherd	Happy Dog Lamm & Rice
Bac	male	4	Black shepherd	Happy Dog Lamm & Rice
Victoria	female	3	Parson Jack Russel	Royal Canin
Julie	female	7.75	German shepherd	Meradog
Woody	male	3.5	Parson Jack Russel	Hills Natures Best
Barry	male	2.5	German shepherd	Eukanuba
Geisha	female	5	Black shepherd	Pet Natur

3.2.3 Traits

An initial health check took place at day 1. All dogs were then inspected immediately after each trial period. The health status and weight was noted. Blood and faecal samples were collected after both trial periods.

Table 2: Examined variables in the study with the police dogs

Owner observation	• Faecal consistency • Frequency of defecation
Faeces	• pH • Faecal dry matter • Ammonium ion concentration • D- and L-lactic acid concentration • Faecal microflora: Coliform bacteria, *Clostridium spp., Lactobacillus spp., Enterococcus spp.*
Whole blood	• Complete blood count • Phenotyping of lymphocytes • Proliferation of lymphocytes
Serum	• Immunoglobulin G • Immunoglobulin A

3.3 Bundeswehr working dogs

3.3.1 Study design

The same double blind cross-over study, as used with the police dogs, was also conducted in a group of Bundeswehr working dogs. The concentration of the probiotic remained the same, as described in 3.2.1.

3.3.2 Animals, housing and diets

Twenty one adult dogs (Table 3) from the German Bundeswehr (SDst-HundeBW-Klinik, 56766 Ulmen, Germany) participated. Each group received a complete dry diet (Bewi Dog® Basic, Bewital® Petfood, Table 4) and either the placebo or the probiotic preparation. The administration was executed and controlled by the animal attendants.

All dogs were tested for suitability by the Bundeswehr, before training started. At beginning, the dogs were examined for their health and body condition by serving veterinarians. The weight was measured once at trial begin. All dogs underwent regular blood checks, vaccination and deworming, to ensure a good and stable health. These dogs were held individually in kennels and received a 45 min daily training.

Table 3: Bundeswehr working dogs in the study

Name	Date of Birth	Gender	Breed	Height (cm)	Weight (kg)
Onex 6628	01.07.06	male	German shepherd	65	30
Pedro 6629	05.01.05	male	Belgian sheepdog	59	31
Adler 6630	01.04.06	male	Belgian sheepdog	55	23
Hedda 5983	01.01.05	female	German shepherd	58	30
Cher 6142	04.12.04	female	German shepherd	61	26
Chimera 5985	01.01.05	female	Belgian sheepdog	55	22
Beppy 6137	01.07.06	female	Belgian sheepdog	52	21
Quat 6192	11.04.06	male	Belgian sheepdog	63	31
Cessy 6639	10.10.05	female	Belgian sheepdog	51	20
Cita 6652	17.06.06	female	Belgian sheepdog	59	23
Nelly 6651	31.08.06	female	Belgian sheepdog	57	24
Balko 6658	21.07.06	male	Belgian sheepdog	53	21
Sem 6659	23.09.06	male	Belgian sheepdog	56	22
Basko 6668	27.05.05	male	German shepherd	60	30
Lucky 4743	17.03.01	male	Belgian sheepdog	61	32
Blacky 2490	14.03.97	male	Belgian sheepdog	60	30
Rocky 2523	10.05.97	male	Belgian sheepdog	64	27
Muc 2862	19.09.99	male	Belgian sheepdog	67	28
Jimmy 2516	25.11.96	male	Belgian sheepdog	61	32
Kevin 2568	02.01.98	male	Belgian sheepdog	62	32
Django 2015	01.01.95	male	Tervueren	59	19

Table 4: Nutrient contents of Bewi Dog® Basic, specified by manufacturer

Crude Protein	23.0 %
Crude fat	10.0 %
Crude fibre	3.0 %
Crude ash	7.0 %
Moisture	10.0 %
NFE	47.0 %
Ca	1.4 %
P	1.0 %
Na	0.3 %
Energy (ME)	14.7 MJ/kg (3520 kcal/kg)

3.3.3 Traits

The working dogs were continually inspected by serving veterinarians. Due to organisatory matters, the weight could not be taken after each trial period, nor was it possible to gain blood samples. The faecal samples were collected after each 4 weeks of trial.

Table 5: Examined variables in the study with the working dogs

Owner observation	• Faecal consistency • Frequency of defecation
Faeces	• pH • Faecal dry matter • Ammonium ion concentration • D- and L-lactic acid concentration • Faecal microflora: Coliform bacteria, *Clostridium spp., Lactobacillus spp., Enterococcus spp.* • RAPD-PCR analysis of *Enterococcus* spp.

3.4 Vaccination trial

3.4.1 Study design

After 4 weeks of adaptation feeding of a placebo or the probiotic *Enterococcus faecium* DSM 7134 (Bonvital®, dosage: $1*10^9$ cfu/kg of complete diet, supplier: Lactosan, Kapfenberg, Austria), 20 dogs were immunised with a polyvalent vaccine (Eurican®, SHPPi2/LT, Merial GmbH, Halbergmoos, Germany), subcutaneously against distemper, hepatitis contagiosa canis, kennel cough, parvovirus, leptospirosis and rabies, and intramuscularly with tetanus toxoid (Equilis Tetanus-Vaccine, Intervet GmbH, Unterschleißheim, Germany). Two weeks after the first injections, the dogs were given a second injection of the tetanus toxoid. Blood samples were drawn on the first day of study (S1), 4 weeks later on the day of vaccination (S2), on the day of the second injection (S3), and 2 weeks after the second injection (S4) (Fig. 2).

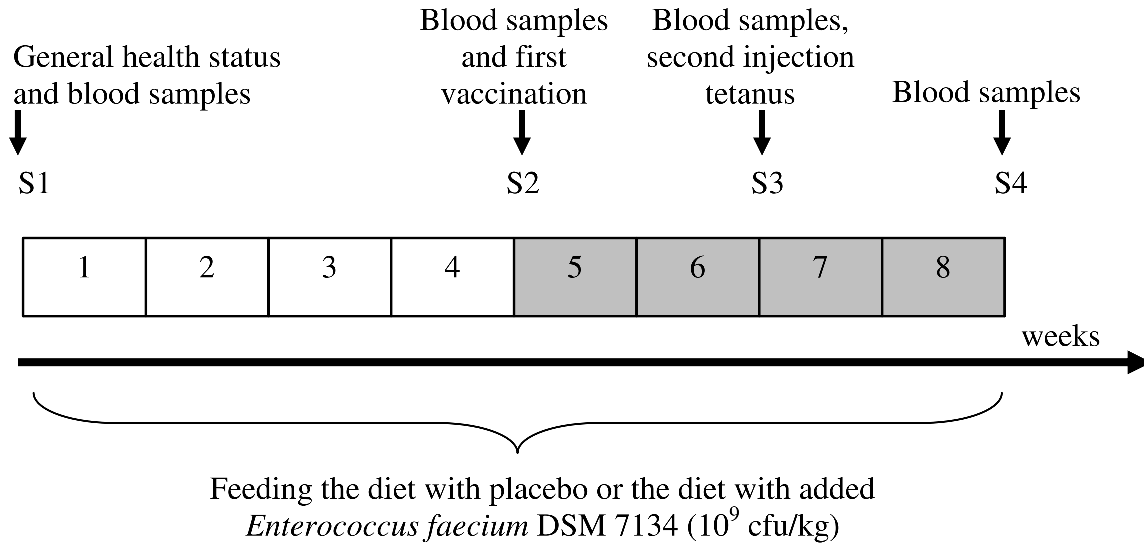

Fig. 2: Study design for the vaccination trial, conducted as a placebo controlled double blind study

3.4.2 Animals, housing and diets

Twenty adult dogs (Table 6), privately owned participated in this study. These were held in groups of 3-4 dogs in large kennels, with free access to an external run.

They were randomly allotted into two equal groups, blocked for age and gender. Each group received a complete dry diet (Dogland® Active, Bewital® Petfood, Table 7) and either a placebo or the probiotic preparation. At the beginning, all dogs were examined on their health and body condition. The weight was measured at each blood sampling.

Table 6: Sled dogs included in the vaccination trial

	Name	Gender	Age (years)	Breed
Placebo	Can	male	10	Husky
	Dustin	male	6	Husky
	Andera	female	12	Husky
	Blizzard	female	2	Husky
	Enyo	male	4	Husky
	Ukki	male	12	Husky
	Gimini	female	3	Husky
	Harley	female	1	Malamute
	Milu	female	11	Malamute
	Elton	male	10	Malamute
Probiotic	Smilla	female	5	Husky
	Eskimo	male	4	Husky
	Bionda	female	12	Husky
	Romo	male	2	Husky
	Lex	male	5	Husky
	Chila	female	10	Husky
	Fenapai	female	6	Husky
	Klecksi	male	8	Malamute
	Chika	female	7	Malamute
	Thea	female	12	Malamute

Table 7: Nutrient contents of Dogland® Active, specified by manufacturer

Crude protein	24 %
Crude fat	11 %
Crude fibre	3 %
Crude ash	7.5 %
Moisture	10 %
Ca	1.4 %
P	1 %
Na	0.3 %

3.4.3 Traits

At each of the 4 times, described in 3.4.1, blood was taken for the following parameters (Table 8). Each dog was examined before vaccination and the weight was noted.

Table 8: Examined variables in the vaccination trial

Whole blood	• Complete blood count • Phenotyping of lymphocytes • Proliferation of lymphocytes
Serum	• Immunoglobulin G • Immunoglobulin A • Titre determination: Canine parvovirus, Canine distemper virus, rabies and tetanus

3.5 Sample collection and storage

3.5.1 Faecal samples

The faecal samples of the police dogs were gathered, shortly before inspection and blood withdrawal, by the owners. These were cooled, until further preparation on the same day. Some parts of the faeces were frozen, at -30 °C, for the determination of dry matter, ammonium ion and D/L-lactic acid concentration.

Since the faecal samples from the working dogs could not be picked up personally, they were collected from the animal attendants and sent cooled, per express delivery, arriving on the same day. After arriving, proceedings were the same as with the faecal samples from the police dogs.

3.5.2 Blood withdrawal

For the antibody and immunoglobulin determination, the blood was drawn into blood clotting tubes (S-Monovette® Serum 4 ml, Sarstedt AG&Co, Nümbrecht, Germany). These were centrifuged at 3345 *g*, 10 min, 4 °C (Megafuge, 1.0R, Thermo Fischer Scientific Inc., Waltham, USA). The supernatant was collected in 2 ml tubes (Eppendorf AG, Hamburg, Germany) and frozen at -30 °C.

Blood was collected in heparinised tubes (S-Monovette® Li.-Heparin 7.5 ml, Sarstedt AG&Co, Nümbrecht, Germany), for the phenotyping and proliferation of lymphocytes. These were processed further shortly after withdrawal.

Into EDTA tubes (2 ml, Sarstedt AG & Co, Nümbrecht, Germany), 1 ml was collected for the blood count. This was kept cool at 4 °C.

3.6 Trait procedure

3.6.1 Faecal consistency and daily defecation frequency

The frequency of defecation and faecal consistency was assessed and recorded daily. Animal owners and attendants received, for this purpose created, a booklet in which they graded the faecal consistency with the aid of a standardised system:

Score 1 = dry crumbly faeces
Score 2 = well formed faeces that leave no mark when picked up
Score 3 = slightly moist, leaving a mark by removal
Score 4 = poorly formed faeces with a very soft consistency
Score 5 = diarrhoea

3.6.2 Faecal dry matter

To determine the faecal dry matter, 1 g of each faecal probe was weighed out into 2 ml tubes and vacuum freeze-dried (Freeze Dryer Alpha 1-4, Christ GmbH, Osterode am Harz, Germany) over a period of 48 h, until weight constancy was reached.

Dry matter (%) = (output weight- tube weight) x (100/ initial weight)

3.6.3 pH in faeces

An amount of 1 g faeces and 9 ml *aqua bidest.* were placed into a 50 ml tube. These were homogenised (Vortex Genie 2, Scientific Industries, West Palm Beach, USA) for about 5 min. The pH was measured using a pH-Electrode (InLab® Routine Pro, Mettler Toledo, Schwerzenbach, Switzerland), which was calibrated using pH buffer solutions of pH 4.00 and 7.00.

3.6.4 Ammonium concentration in faeces

The ammonium concentration was determined with an ammonium ion-selective electrode (Mettler Toledo, Columbo, USA). A 55.56 mM NH_4Cl solution (1000 ppm) was diluted to 500, 100, 50, 10 and 5 ppm solutions. The electrode determined the mV value for each

dilution and using Excel (Microsoft Office 2003), a straight-line graph with equation was created, using mmol/l and mV on the axes.
The frozen faecal samples thawed at room temperature and were then kept on ice. 500 mg and 1 ml *aqua bidest.* were added to 2 ml tubes. After 5 min of homogenisation, centrifugation at 16200 *g*, 10 min, 4 °C (Heraeus Fresco 17 Microcentrifuge, Thermo Scientific, Karlsruhe, Germany) followed. 500 µl of the supernatant and 9.5 ml *aqua bidest.* were transferred into new tubes. Just before measuring the concentration (in mV) with the electrode, 200µl ISA-solution (ammonium sulphate, 0.9 mol/l) was added and mixed thoroughly. The measured mV was then converted into a concentration of µmol/g, by using the previous calculated equation, including all dilution factors.

3.6.5 D- and L- lactate concentration in faeces

To faecal samples of 1 g, 2 ml of 1 M $HClO_4$ was added and vortexed immediately and kept on ice. This followed an addition of 200 µl of 4 N KOH. After 10 min reaction time, the 15 ml tubes were centrifuged at 3345 *g*, 10 min, 4 °C (Megafuge, 1.0R, Thermo Fischer Scientific Inc., Waltham, USA). The supernatant was collected in smaller tubes and centrifuged again at 16200 *g*, 10 min, 4 °C (Heraeus Fresco 17 Microcentrifuge, Thermo Scientific, Karlsruhe, Germany). To 1 ml of supernatant, KOH was added until pH 7.5-8.5 was reached (pH electrode InLab® Micro, Mettler Toledo, Schwerzenbach, Switzerland). Carrez-I solution (potassium ferrocyanide) and Carrez-II solution (zinc sulphate) were added respectively, to reduce interferences. The concentration of D- and L-lactate was determined photometrically, at pH 8-10, using a D-/L-lactic acid UV test-kit (Cat.No.: 11 112 821 035, R-Biopharm, Darmstadt, Germany). D-lactate and NAD^+ are catalysed, with the enzyme D- lactate dehydrogenase, to form pyruvate and NADH. L- lactate reacts in the same way with L- lactate dehydrogenase. The equilibrium lies on the side of lactate, but can be forced to the side of pyruvate and NADH, by taking out pyruvate with the enzyme ALT, in the presence of L- glutamate. The amount of NADH, determined by the photometer, is proportional to the D- and L- lactate in the faecal sample. The photometric analysis was performed with the program Swift Quantification II (Biochrom 1999, Cambridge, UK).

Table 9: Used materials and solutions for determination of dry matter, pH, ammonium and lactate concentration

Description	Manufacturer	Place, State
pH buffer solution; pH 4.00/ 7.00	Mettler Toledo	Urdorf, Switzerland
NH_4Cl	Riedel-de Haën AG	Seelze, Germany
Aluminium sulphate	Mettler Toledo	Urdorf, Switzerland
$HClO_4$	Riedel-de Haën AG	Seelze, Germany
KOH	Carl Roth GmbH	Karlsruhe, Germany
$K_4[Fe(CN)_6]$	Carl Roth GmbH	Karlsruhe, Germany
$ZnSO_4$	Merck kGaA	Steinheim, Germany
2 ml tube	Eppendorf AG	Hamburg, Germany
50 ml tube	Greiner	Frickenhausen, Germany
15 ml tube	Greiner	Frickenhausen, Germany
Seven Multi dual channel pH/ion	Mettler Toledo	Schwerzenbach, Switzerland
ratiolab® cuvets, semi-micro, PMMA	VWR International bvba	Leuven, Belgium

3.6.6 Faecal microflora

Faecal samples were cultured on commercially available selective agar plates (preparation as manufacturer suggests) for coliform bacteria*, Clostridium* spp.*, Lactobacillus* spp. and *Enterococcus* spp..

The 15 ml tubes were filled with sterile, 2.85-3.3 mm glass beads up to the 2 ml mark. One g faeces and 9 ml of phosphate buffered saline were added and vortexed (Vortex Genie 2, Scientific Industries, West Palm Beach, USA) until homogenised completely. Duplicates of an eightfold dilution, with 100 µl volume and 900 µl PBS, were created in a deep well plate. 100 µl of the dilutions (Table 10) were transferred onto the agar plates and distributed with a Drigalski-spatula, until the complete volume was soaked up.

Incubation (Heraeus Instruments Function Line, Type B20, Hanau, Germany) was specific for each kind of agar plate (Table 10). To create an anaerobic environment, agar plates were stored with Anaerocult® A (Merck kGaA, Darmstadt, Germany) in an anaerobic jar (Merck kGaA, Darmstadt, Germany).

Table 10: Cultured bacteria, agar plates, dilutions, incubation time and environment

Bacteria	Agar plates	Dilutions	Incubation time[1]	Environment[1]
Coliform Bacteria	Mac Conkey agar	$10^{-1} – 10^{-5}$	24 h	aerobic, 37 °C
Clostridium spp.	TSC agar	$10^{-2} – 10^{-6}$	48 h	anaerobic, 37 °C
Lactobacillus spp.	MRS agar	$10^{-4} – 10^{-8}$	48 h	anaerobic, 37 °C
Enterococcus spp.	Slanetz-Bartley agar	$10^{-4} – 10^{-8}$	48 h	aerobic, 37 °C

[1] Heraeus Instruments Function Line, Type B20, Hanau, Germany

3.6.7 Preservation of *Enterococcus* spp.

Brain-Heart-Infusion and glycerine were mixed in a ratio of 2:1 and autoclaved for 15 min at 121 °C. 1.5 ml of the cold, sterile mixture was transferred into 2 ml tubes. Enterococci were collected from the Slanetz-Bartley agar plate with an inoculating loop under a laminar airflow cabinet (Uniflow UVUB 1200 Biohazard, UniEquip, Martinsried, Germany) to ensure sterile conditions and then placed into the filled tubes. 10 CFU were collected for each faecal sample and period. The collected enterococci were stored at -30 °C until further usage.

3.6.8 RAPD-PCR analysis of enterococci

The enterococci isolates of 4 dogs from the Bundeswehr were selected for strain typing. Five colonies were tested for every period and dog. To identify the preserved enterococci, the 2 ml tubes were vortexed and 100 µl volume suspended into 15 ml tubes with 5 ml Brain-Heart-Infusion. Anaerobic incubation for 24 h at 37 °C (Heraeus Instruments Function Line, Type B20, Hanau, Germany) proceeded. For a second passage, the incubated tubes with BHI were vortexed and 100 µl volume transferred into a new 15 ml tube with again 5 ml BHI. Another anaerobic incubation, at 24 h and 37 °C, followed. After incubation of the second passage, centrifugation followed at 3345 *g*, 10 min, 4 °C (Megafuge, 1.0R, Thermo Fischer Scientific Inc., Waltham, USA). The supernatant was discarded and the pellet was transferred into 2 ml tubes. These biomass pellets were sent with dry ice to the BOKU, University of Natural Resources and Applied Life Sciences, Department of Food Chemistry Division, Vienna, Austria, for further RAPD (**R**andom **A**mplification of **P**olymorphic **D**NA) PCR analysis. In this PCR, the target sequence to be amplified is unknown. A designed primer with arbitrary sequence is synthesised to amplify DNA segments, which are then separated electrophoretically in an agarose gel. The bacterial strain is determined by comparing the bands, so called fingerprints, to the reference strains (Table 11).

Table 11: Reference strains for RAPD-PCR

Code	Strain	Origin	
DSM 7134	*Enterococcus faecium* DSM 7134	DSZM[1]	Reference strain (En 24)
En 24 (mcb)	*Enterococcus faecium* DSM 7134	Lactosan	Reference strain (En 24) (master cell bank)

[1] Deutsche Stammsammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany

Table 12: Used materials and solutions for microbiologic analysis

Description	Manufacturer	Place, State
2.85-3.3 mm glass beads	Carl Roth GmbH	Karlsruhe, Germany
PBS	Biochrom AG	Berlin, Germany
MacConkey agar	Merck kGaA	Darmstadt, Germany
TSC agar	Merck kGaA	Darmstadt, Germany
MRS agar	Carl Roth GmbH	Karlsruhe, Germany
Slanetz- Bartley agar	Oxoid Ltd.	Besingstoke, Hants., England
BHI	Carl Roth GmbH	Karlsruhe, Germany
Glycerine	Biomol GmbH	Hamburg, Germany
Ethanol, 96 %, denatured	Carl Roth GmbH	Karlsruhe, Germany
Kanamycin sulphate 750 IU/mg	Carl Roth GmbH	Karlsruhe, Germany
Polymixin B sulphate 6500 IU/mg	Carl Roth GmbH	Karlsruhe, Germany
15 ml tube	Greiner BioOne	Frickenhausen, Germany
1.2 ml storage plate	Thermo Scientific	Surrey, UK
Petri dishes	Greiner BioOne	Kremsmünster, Austria
2 ml tube	Eppendorf AG	Hamburg, Germany

3.6.9 Blood count

The EDTA blood samples were sent to Laboklin (Bad Kissingen, Germany) for the analysis of complete blood count.

3.6.10 Lymphocyte phenotyping

The mononuclear cells were isolated by layering the heparinised blood over Ficoll and centrifuging at 400 *g*, 30 min, room temperature (Megafuge, 1.0R, Thermo Fischer Scientific Inc., Waltham, USA). Thereafter, collection of the interphase (containing lymphocytes and monocytes) followed. These cells were washed using 10 ml PBS, centrifuging at 300 *g*,

10 min, 4 °C (Megafuge, 1.0R, Thermo Fischer Scientific Inc., Waltham, USA), discarding the supernatant and washing the pellet twice more with 10 ml PBS. After the third washing, the pellet was resuspended in 1 ml of cell cultural medium (Table 14).

Each suspension was dyed with Trypan blue, microscopically counted (Neubauer haemocytometer) and adjusted to a concentration of $1x10^7$ cells/ml. 100 µl ($1x10^6$ cells) were transferred into **f**luorescence **a**ctivated **c**ell **s**orting (FACS)-tubes, which already contained the primary antibodies (Table 13) against the cell surface antigens of CD3, CD4, CD8, CD21 and MHC II.

After incubation for 25 min on ice, the cells were washed with 2 ml FACS buffer (Table 14) and centrifuged at 300 *g*, 10 min, 4 °C (Megafuge, 1.0R, Thermo Fischer Scientific Inc., Waltham, USA). The supernatant was discarded. To the pellet, 50 µl secondary antibody (α IgG1 FITC goat anti mouse, Southern Biotech, Birmingham, USA) was added, which interlinked with the primary antibodies, creating fluorescently marked cells after another incubation period of 25 min, on ice, in the dark. This suspension was again washed with FACS buffer and centrifuged as above mentioned. The pellet was immersed in 300 µl FACS buffer. Until further measurements, the cell suspension was kept in the dark on ice.

The marked cells were measured using a flow cytometer, FACS Calibur® (Fluorescence-activated cell sorter, BD Bioscience, San Jose, USA), detecting fluorescence, size and granules of the cell. The software CellQuestPro® (BD Biosciences, San Jose, USA) generated this data into a two dimensional dot-plot. The regions on these plots can be sequentially separated, based on the fluorescence intensity, by creating a series of subset extractions, called “gates”.

Table 13: Primary antibodies used for lymphocyte phenotyping

Primary Antibodies	Description	Clone	Isotype	Manufacturer	Dilution
anti CD3	mouse anti dog CD3	CA17.2A12	IgG1	Serotec, Kidlington, UK	1:50
anti CD4	mouse anti dog CD4	CA13.1E4	IgG1	Serotec, Kidlington, UK	1:10
anti CD8β	mouse anti dog CD8 beta	CA15.4G2	IgG1	Serotec, Kidlington, UK	1:20
anti CD21	mouse anti dog B-cells	CA2.1D6	IgG1	Serotec, Kidlington, UK	1:100
anti MHC II	mouse anti horse MHC II monomorphic	CVS20	IgG1	Serotec, Kidlington, UK	1:1000

3.6.11 Lymphocyte proliferation

The lymphocytes were stimulated with three different mitogens, Pokeweed Mitogen (PWM), ConcanavalinA (ConA) and Phytohemagglutinin (PHA).

A volume of 100 µl ($4x10^5$ cells) of the isolated mononuclear cells, were incubated together with 25 µl diluted mitogen (PWM 1:80; ConA 1:40; PHA 1:20) and culture medium (Table 14) in a microculture plate, having 6 wells per mitogen and animal. Instead of the mitogens, 25 µl culture medium, was used for the negative control. After 48 h, 37 °C in a CO_2-incubator (Biocentre BC170, SalvisLab, Rotkreuz, Switzerland), 12 µl 60 µM BrdU (5-Bromo-2`-deoxyuridin) was added into each well and another 24 h incubation followed.

The cells of the same animal and the same mitogen were harvested into FACS-tubes, 2 ml FACS-buffer (Table 14) was added and then centrifuging at 300 *g*, 5 min, 4 °C (Megafuge, 1.0R, Thermo Fischer Scientific Inc., Waltham, USA). The supernatant was discarded and the pellet was resuspended with 200 µl FACS Perm2, diluted 1:10 in *aqua bidest.*. The tubes were sealed and kept on ice at 4 °C for 18 h.

Again, an addition of 2 ml FACS buffer, centrifugation at 400 *g*, 10 min, 4 °C (Megafuge, 1.0R, Thermo Fischer Scientific Inc., Waltham, USA) and removal of the supernatant followed. DNase I (Deoxyribonuclease I) was diluted 1:10 in DNAase I-buffer (Table 14),

200 µl was added to each tube and incubated 30 min at 37 °C (Heraeus Instruments Function Line, Type B20, Hanau, Germany).
Proceedings with 2 ml FACS-buffer, centrifugation and supernatant were as stated before. The pellets were resuspended in 40 µl FACS buffer and 10 µl FITC conjugated mouse anti-BrdU. One last incubation period of 30 min on ice, in the absence of light proceeded. FACS-buffer addition, centrifugation and removal of the supernatant followed again, as mentioned above. This time, the pellet was only reimmersed in 300 µl of FACS-buffer and kept on ice in the absence of light until measurements on the flow cytometer, FACS Calibur® (Fluorescence-activated cell sorter, BD Bioscience, San Jose, USA) took place.
The thymidine analogue, BrdU, is incorporated into cellular DNA, just like thymidine. The incorporated BrdU can be quantitatively detected, using monoclonal antibodies directed against BrdU (FITC conjugated mouse anti-BrdU). Just like by the lymphocyte phenotyping, the fluorescent cells were counted by the FACS, but in this case, the fluorescence was proportional to cell division.

Table 14: Composition of solutions used in lymphocyte phenotyping and proliferation

Solution	**Amount**	**Substance**
Culture medium	500 ml 50 ml 5 ml 5 ml 5 ml	RPMI 1640 Fetal Calf Serum (FCS) HEPES Pen/Strep L-glutamine
FACS-buffer	700 ml 5 g 100 ml 10 ml → filled up to 1 L with *aqua bidest.*	*aqua bidest.* Albumin Fraction V 10x PBS 10% sodium azide
DNase I-buffer	4.383 g 0.427 g 5 µl →solved and filled up to 500 ml *aqua bidest.*	NaCl (MW=58.44) $MgCl_2$ (MW=203.3) 1 M HCl

Table 15: Materials and solutions used for lymphocyte phenotyping and proliferation

Description	Manufacturer	Place, State
Ficoll	Biochrom AG	Berlin, Germany
PBS	Biochrom AG	Berlin, Germany
RPMI 1640	Biochrom AG	Berlin, Germany
FCS	Biochrom AG	Berlin, Germany
HEPES	Biochrom AG	Berlin, Germany
Pen/Strep	PAA Laboratories GmbH	Pasching, Austria
L-Glutamine	PAA Laboratories GmbH	Pasching, Austria
0.5 % Trypan blue	Biochrom AG	Berlin, Germany
Albumin V Fraction	Carl Roth GmbH	Karlsruhe, Germany
Sodium azide	Merck kGaA	Darmstadt, Germany
PWM	Sigma- Aldrich	Steinheim, Germany
ConA	Sigma- Aldrich	Steinheim, Germany
PHA	Sigma- Aldrich	Steinheim, Germany
BrdU	Sigma- Aldrich	Steinheim, Germany
FACS Perm2	BD Biosciences	Franklin Lake, USA
DNase I	Sigma- Aldrich	Steinheim, Germany
NaCl	Merck kGaA	Darmstadt, Germany
$MgCl_2$	Carl Roth GmbH	Karlsruhe, Germany
1 M HCl	Carl Roth GmbH	Karlsruhe, Germany
FITC conjugated mouse anti-BrdU	BD Biosciences Pharmingen	San Diego, USA
Neubauer haemocytometer	Paul Mariendorf GmbH & Co. KG	Lauda-Königshofen, Germany
FACS-tubes	BD Labware	Franklin Lake, USA
Microculture plate, Cellstar®	Greiner BioOne	Frickenhausen, Germany
15ml tube	Greiner BioOne	Frickenhausen, Germany
2ml tube	Eppendorf AG	Hamburg, Germany

3.6.12 Immunoglobulins in the sera

The, at -30 °C stored, sera were thawed at room temperature. The immunoglobulin concentration was analysed with Dog-ELISA Quantitation kits (Bethyl Laboratories, Inc., West/Montgomery, USA).

ELISA´s detect the unknown amount of antigens in sera samples. Through specific binding on a microtitre plate, antigen is captured and then coupled to form a complex with detecting antibodies. Addition of an enzymatic substrate produces a visible signal. The intensity of colouring determines the concentration of antigen in the sera samples.

The optical density was measured with a microtitre plate reader (Tecan Sunrise, Tecan Austria GmbH, Grödig, Austria), using a wavelength of 450 nm.
The creation of a standard curve and evaluation of the samples, were performed with the software Magellan V 6.4 (Tecan Austria GmbH, Grödig, Austria).

Table 16: Used kits and dilutions for the quantification of IgA and IgG

Immunoglobulin	**Kit**	**Dilution of samples**	**Dilution of enzyme conjugate**
IgA	Dog IgA ELISA Quantitation Kit	1:10000	1:40000
IgG	Dog IgG ELISA Quantitation Kit	1:200000	1:40000

3.6.13 Vaccination titre

3.6.13.1 Canine parvovirus, canine distemper virus and rabies

The sera were stored at -30 °C and thawed at room temperature. Antibodies against CDV (canine distemper virus), CPV (canine parvovirus) and rabies, were measured using ELISA-kits (DRG Instruments GmbH, Marburg, Germany). The sera samples before vaccination (S2) and 2 weeks after the first vaccination (S3) were diluted (Table 17) and proceeded further as manufacturer suggested.
Again, the optical density was measured, immediately after stopping the reaction, at a wavelength of 450 nm, using a microtitre plate reader (SW Magellan V 6.4, Tecan Sunrise, Tecan Austria GmbH, Grödig, Austria).
The titres were found by comparing the positive and negative controls (as manufacturer suggests), to the OD of the samples measured at different dilutions.

Table 17: Used ELISA-kits for titre determination

Antibodies	**Description**	**Kit – No.:**	**Dilutions**
Canine parvovirus	CPV IgG	EIA-2475	1:30, 1:90, 1:270, 1:810
Canine distemper virus	CDV IgG	EIA-2478	1:30, 1:100, 1:300, 1: 1000
Rabies	Rabies Virus IgG Ab	EIA-2486	1:50, 1:150, 1:450, 1:1350

3.6.13.2 Tetanus

The sera samples before vaccination (S2), 2 weeks after the first vaccination (S3) and 2 weeks after the second vaccination (S4) were used for the determination of tetanus antibodies, in a dilution of 1:100 (Table 18). 100 µl of each diluted sample was brought upon a toxoid-antigen coated microtitre plate (Tetanus IgG ELISA, EIA-3514, DRG Instruments GmbH, Marburg, Germany). An incubation of 60 min at 37 °C followed.
After incubation, each well was washed 3 times with 300 µl washing solution (Table 18).
Then 100 µl of the enzyme conjugate (Sheep anti-Dog IgG-h-ch HRP conjugate, Bethyl Laboratories, Inc., West/Montgomery, USA) was added, diluted 1:40000. Another incubation at room temperature for 30 min followed.
Again, the wells were washed (3 times, 300 µl). 100 µl TMB (3,3´,5,5´tetramethylbenzidine) were added and the plate was incubated in the dark for 15 min at room temperature. The enzymatic reaction was stopped with 100 µl 2 M H_2SO_4.
The optical density was measured, immediately after stopping the reaction, with a microtitre plate reader (SW Magellan V 6.4, Tecan Sunrise, Tecan Austria GmbH, Grödig, Austria), using a wavelength of 450 nm and a reference wavelength of 620 nm.

Table 18: Dilution and washing solutions for Ig concentration and tetanus titre determination

Solution	Amount	Substance
Dilution solution (samples and enzyme conjugate)	1 packet in 1 L aqua bidest. 1% 10%	50 mM tris buffered saline: 0.05 M Tris, 0.138 M NaCl, 0.0027 M KCl, pH 8.0 Albumin Fraction V Tween 20
Washing solution	1 packet in 1 L aqua bidest. 0.05%	50 mM tris buffered saline: 0.05 M Tris, 0.138 M NaCl, 0.0027 M KCl, pH 8.0 Tween 20

Table 19: Materials and solutions used for ELISA

Description	Manufacturer	Place, State
0.05 M Sodium carbonate	Sigma- Aldrich	Steinheim, Germany
50 mM tris buffered saline	Sigma- Aldrich	Steinheim, Germany
Albumin V Fraction	Carl Roth GmbH	Karlsruhe, Germany
Tween 20	Merck kGaA	Darmstadt, Germany
TMB	Sigma- Aldrich	Steinheim, Germany
2 M H_2SO_4	Carl Roth GmbH	Karlsruhe, Germany
Nunc Immunoplate	Thermo Fischer Scientific	Roskilde, Denmark
1.5 ml tube	Eppendorf AG	Hamburg, Germany

3.7 Statistical analysis

Statistical analysis was done with SPSS 15.0 for Windows (SPSS Inc., Chicago, Illinois, USA). Probability values of $p \leq 0.05$ were taken as significant and $p \leq 0.10$ tending to be significant. Results were expressed as mean ± standard deviation. Faecal quality and defecation frequency were displayed as frequency distributions and differences tested with a Chi-Square Test. Differences of the faecal quality were also tested for each dog separately. In this case the Mann-Whitney U-Test was applied.

Normal distribution was tested with Kolmogorov-Smirnoff test. The two means in the police- and working dogs were tested with the Paired Sample t-Test between placebo and probiotic period, since every dog went through both trial periods.

In the vaccination trial the Independent Sample t-Test was used to determine significances between the placebo and probiotic group at each sample collection (S1-S4). To compare changes in each group of the vaccination trial separately over the complete time of study, a Scheffé-Post Hoc Multiple Comparison was performed.

4 Results

4.1 Police dogs

4.1.1 General health status and weight

All dogs had a good health without clinical signs of disease and a normal body condition. Any change in behaviour and appetite were observed and noted by the police officers. The preparations were accepted well by all dogs and had no effects to the appetite during the trial periods. The behaviour was normal. The weight of the police dogs (Table 20) remained in normal range between the periods and did not differ significantly.

Table 20: Bodyweight of the police dogs after the placebo and probiotic period

Period	**Weight (kg)**
Placebo	30.0 ± 10.3
Probiotic	30.1 ± 10.4

4.1.2 Faecal quality

The faecal consistency was well formed in general. The booklets of 13 dogs were assessed. The chi-square-test is based on a test statistic that measures the divergence of the observed data from the values that would be expected under the null hypothesis of no association.

During the feeding periods, score 1 (dry crumbly) faeces occurred significantly more, score 2 (well formed) and 4 (poorly formed) faeces occurred significantly less in the probiotic group (Fig. 3)

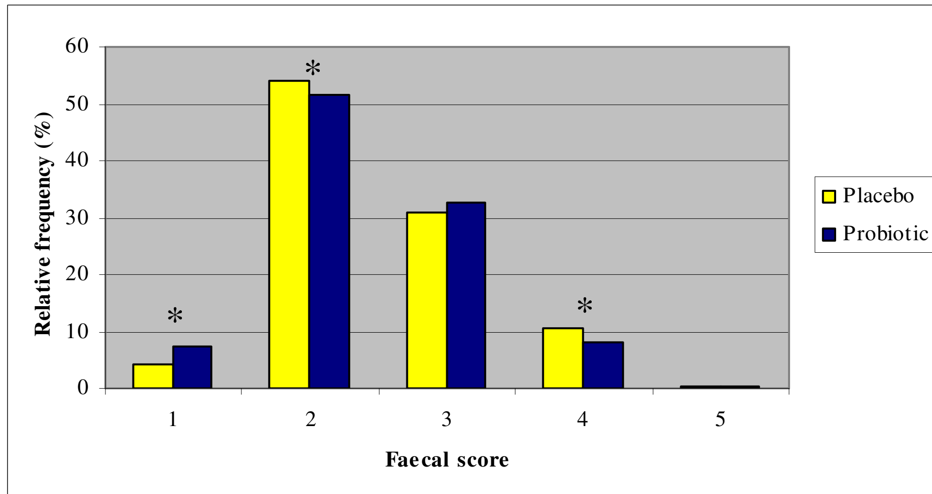

Fig. 3: Relative frequency of faecal scores[a] from the police dogs (placebo period, n=1014; probiotic period, n=1004). *Indicate significant differences (Chi-square-test, $p<0.05$) between the observed and expected relative frequencies.

[a] Faeces were graded on a 1-5 scale. Grade 1 represented dry crumbly faeces. Grade 2 represented well formed faeces that left no marks when picked up. Grade 3 was slightly moist, leaving a mark by removal. Grade 4 represented poorly formed faeces with a very soft consistency. Grade 5 represented diarrhoea.

Of interest was also, if a change occurred in the faecal quality for each individual dog. A mean score was created per day and dog, to compare both 28 days of trial. The Mann-Whitney U-Test was performed to find any significant differences.

A total of five out of thirteen dogs (Table 21) showed a significant difference between the feeding periods. Three dog's faeces were within normal ranges of scoring. Under the probiotic influence, the faeces of one dog became more firm. Another dog had an unfavourable high scoring to begin with, which showed a significant decrease during the probiotic feeding period.

Table 21: Individual analysis of police dog faecal scoring (Mean for whole period)

Dog	Placebo	Probiotic	*p*
Grete	2.4	2.5	§
Rinti	2.1	2.0	§
Boogy	2.6	2.5	§
Eve	2.6	2.2	< 0.0001
Rudi	3.5	3.2	0.006
Askan	2.9	3.0	§
Zamb	1.7	1.1	0.001
Bac	2.9	3.0	§
Victoria	2.0	2.2	< 0.0001
Julie	2.7	2.8	§
Woody	2.0	2.0	§
Barry	2.4	2.2	§
Geisha	2.1	2.3	0.033

§ Not significant

4.1.3 Frequency of defecation

The frequency of defecation ranged from one to five times per day. A rate of two and three times per day occurred most often (Fig. 4). A significant difference with the Chi-square-test was not determined.

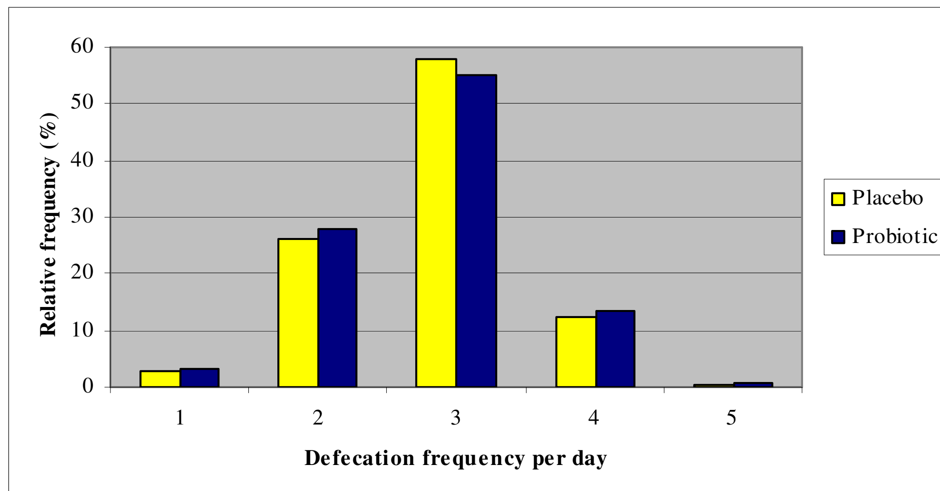

Fig. 4: Relative frequency of defecation per day of the police dogs (placebo period, n=362; probiotic period, n=359).

4.1.4 pH in faeces

Although the pH between the periods was scarcely different, a tendency ($p<0.1$) for a decrease, after the probiotic period was registered (Table 22).

Table 22: pH in police dog faecal samples

Period	pH
Placebo	6.38 ± 0.31
Probiotic	6.16 ± 0.44

4.1.5 Faecal dry matter

The faecal dry matter tended ($p = 0.10$) to be higher after the probiotic period. All values were within normal ranges for dry diets (Table 23).

Table 23: Faecal dry matter of police dog faecal samples

Period	Dry matter (%)
Placebo	27.0 ± 4.17
Probiotic	30.5 ± 7.57

4.1.6 D- and L-lactate concentration in faeces

It was not possible to compare statistically between the placebo and probiotic period, because too many samples (39.3%) were under the detection limit (Table 24). In general, the concentrations of D- and L-lactate were at a very low level (µmol/g).

Table 24: D- and L- lactate concentration in police dog faecal samples

Period	D-Lactate (µmol/g) [1]	L-Lactate (µmol/g) [2]
Placebo	1.04 ± 1.02	1.03 ± 1.16
Probiotic	0.90 ± 0.78	1.00 ± 1.11

[1] Placebo, n=6; Probiotic, n=7
[2] Placebo, n=11; Probiotic, n=10

4.1.7 Ammonium concentration in faeces

There were no significant differences found in the ammonium concentrations between the two periods. A slight numeric decrease of concentration was found in the probiotic period (Table 25).

Table 25: Ammonium concentration in police dog faecal samples

Period	Ammonium (µmol/g)
Placebo	19.6 ± 4.39
Probiotic	18.2 ± 6.01

4.1.8 Faecal microflora

The faecal concentration of coliform bacteria, *Clostridium* spp., *Lactobacillus* spp. and *Enterococcus* spp. continued to be stable through both periods (Table 26). Colony morphology from the Slanetz and Bartley agar for *Enterococcus* spp. had great variations. Larger colonies (up to 5 mm), purple in colour with a lighter rim existed, as did smaller colonies (up to 2 mm), ranging from light to dark red. All colony types were viewed under the microscope and in all cases, chain-forming cocci were found.

Table 26: Bacteria concentrations in police dog faecal samples

Period	Coliform bacteria	*Clostridium* spp.	*Lactobacillus* spp.	*Enterococcus* spp.
	(cfu log_{10}/g faeces)			
Placebo	6.83 ± 1.45	5.92 ± 0.90	8.13 ± 1.86	8.01 ± 1.92
Probiotic	6.83 ± 1.00	5.72 ± 1.05	8.11 ± 0.92	7.65 ± 1.03

4.1.9 Complete blood count

All blood parameters were within normal ranges, except for monocytes and eosinophiles (Table 27). These however, exceeded the upper margin on the same level for both periods. In all, only four dogs did not surpass 6% of eosinophiles. Monocyte values reached most of the time, just over the upper margin, two dogs stayed within ranges.

Table 27: Complete blood count from police dogs

	Period	
	Placebo	**Probiotic**
Erythrocytes (T/l)	7.20 ± 0.35	7.24 ± 0.53
Haematocrit (l/l)	0.48 ± 0.04	0.49 ± 0.03
Haemoglobin (g/l)	178 ± 7.57	180 ± 13.5
Leukocytes (G/l)	10.3 ± 2.34	10.2 ± 2.47
Segmented granulocytes (%)	59.5 ± 8.04	59.6 ± 8.23
Lymphocytes (%)	26.6 ± 5.60	27.1 ± 5.74
Monocytes (%)	5.07 ± 1.44	4.50 ± 1.16
Eosinophiles (%)	7.86 ± 4.31	7.71 ± 6.16
Basophiles (%)	0.29 ± 0.47	0.36 ± 0.84
Banded granulocytes (%)	0.64 ± 0.84	0.79 ± 0.80
Thrombocytes (G/l)	192 ± 54.21	215 ± 75.65

4.1.10 Lymphocyte phenotyping

No significant changes were found in any of the tested subpopulations (Fig. 5). One dog had no expression of MHC II lymphocytes in either period and was therefore excluded from the calculations. The $CD4^+$:$CD8^+$ ratio underwent also no significant changes (Table 28).

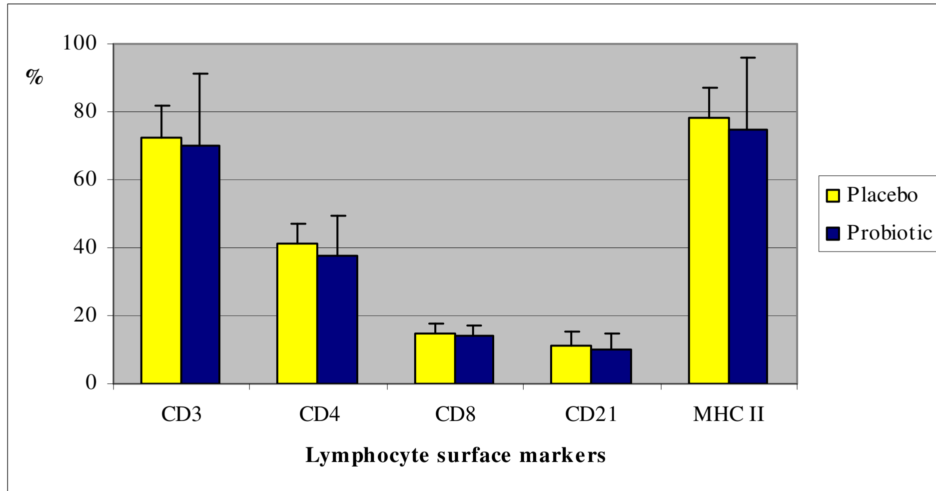

Fig. 5: Lymphocyte surface markers detected in the police dog blood samples

Table 28: $CD4^+$:$CD8^+$ T-lymphocyte ratio of the police dogs

Period	$CD4^+$:$CD8^+$
Placebo	2.91 ± 0.60
Probiotic	2.77 ± 0.69

4.1.11 Lymphocyte proliferation

The stimulation index (SI) indicates the level of proliferation of the parameters measured, when stimulated cells are compared to unstimulated control cells. Thus a SI of 1.0 indicates no proliferation of the cultured cells.

$$SI = \frac{\text{Proliferation of cells treated with mitogens}}{\text{Proliferation of cells cultured without mitogen}}$$

Stimulation with PWM and ConA increased lymphocyte proliferation during the probiotic period. The mitogen PHA caused minimal differences between the periods (Fig. 6).

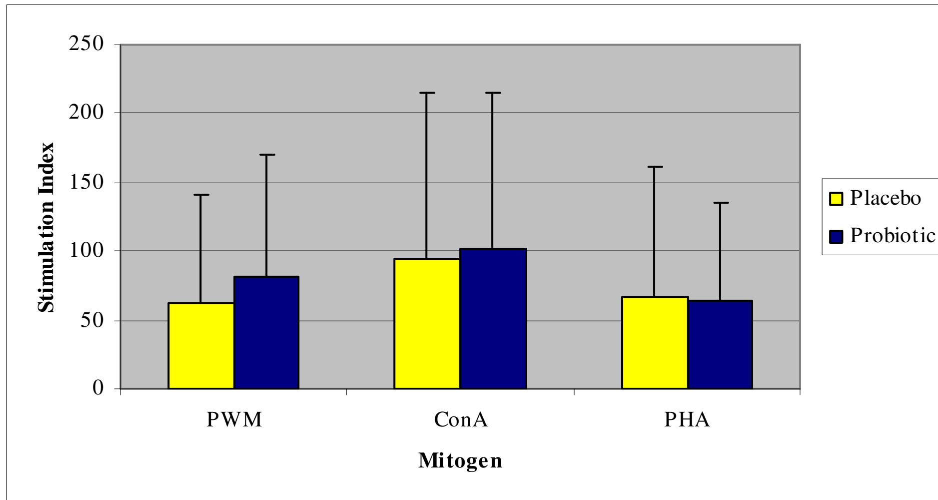

Fig. 6: Stimulation indices of lymphocyte proliferation with the mitogens PWM, ConA and PHA from the police dogs

4.1.12 Immunoglobulins in the sera

Slight differences of IgA and IgG were measured after the periods. No differences were found (Table 29).

Table 29: IgA and IgG concentration in police dog sera samples

Period	IgA (mg/ml)	IgG (mg/ml)
Placebo	1.26 ± 0.48	19.4 ± 5.80
Probiotic	1.36 ± 0.59	18.5 ± 3.41

4.2 Bundeswehr working dogs

4.2.1 General health status

During the experimental period, all dogs remained healthy on the absence of clinical signs. The preparations were accepted well by these dogs and had no effects on the appetite during the trial periods. All dogs showed normal behaviour and no variation was seen during the trial. No change in body condition was reported.

4.2.2 Faecal quality

The chi-square-test measures the divergence of the observed data from the values that would be expected under the null hypothesis of no association.

The working dogs had faecal scores, which were overall higher as the police dog scores. Figure 7 shows clear differences at the score of 3 (slightly moist faeces) and 4 (poorly formed). The high frequency of faecal score 4 is mainly due to one dog.

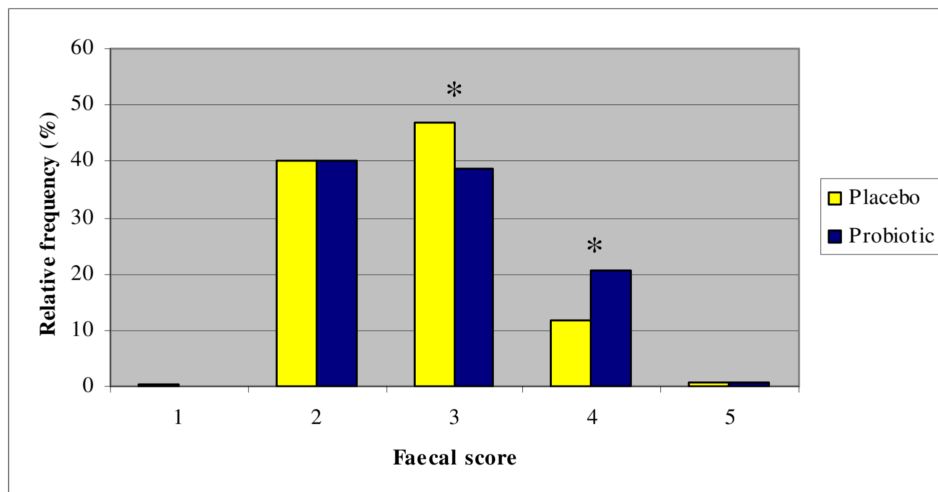

Fig. 7: Relative frequency of faecal scores[a] from the working dogs (placebo period, n=1113; probiotic period, n=1156). Chi-square-test determined a significant difference (*, $p<0.0001$), between the observed and expected relative frequencies.

[a] Faeces were graded on a 1-5 scale. Grade 1 represented dry crumbly faeces. Grade 2 represented well formed faeces that left no marks when picked up. Grade 3 was slightly moist, leaving a mark by removal. Grade 4 represented poorly formed faeces with a very soft consistency. Grade 5 represented diarrhoea.

To individually asses the faecal quality, the mean scoring per day was calculated for each dog. The two periods of placebo and probiotic feeding were tested for significance with the Mann-Whitney-U-Test. A lower significant scoring was determined for three dogs during the probiotic period (Table 12), whilst significant higher faecal scores were found for five dogs.

Table 30: Individual analysis of working dog faecal scoring (Mean for whole period)

Dog	**Placebo**	**Probiotic**	***p***
Onex 6628	2.6	2.6	§
Pedro 6629	2.2	2.0	§
Adler 6630	3.0	3.3	§
Hedda 5983	2.4	2.5	§
Cher 6142	2.7	2.6	§
Chimera 5985	2.5	2.7	§
Beppy 6137	2.3	2.6	0.080
Quat 6192	2.7	2.6	§
Cessy 6639	2.5	2.4	§
Cita 6652	2.6	2.8	§
Nelly 6651	2.5	2.7	§
Balko 6658	3.1	2.6	0.009
Sem 6659	2.2	3.1	< 0.0001
Basko 6668	2.1	2.6	0.001
Lucky 4743	2.7	2.2	< 0.0001
Blacky 2490	3.3	3.9	< 0.0001
Rocky 2523	3.1	2.9	0.012
Muc 2862	2.6	2.6	§
Jimmy 2516	2.7	2.8	§
Kevin 2568	3.0	3.3	< 0.0001
Django 2015	2.9	3.2	0.001

[§] Not significant

4.2.3 Frequency of defecation

The frequency of defecation ranged again from one to five times per day. Rates of one and two times per day occurred primarily. Chi-square-test determined a tendency towards a significant difference (p=0.08) between the observed and expected frequency of three faeces per day (Fig. 8).

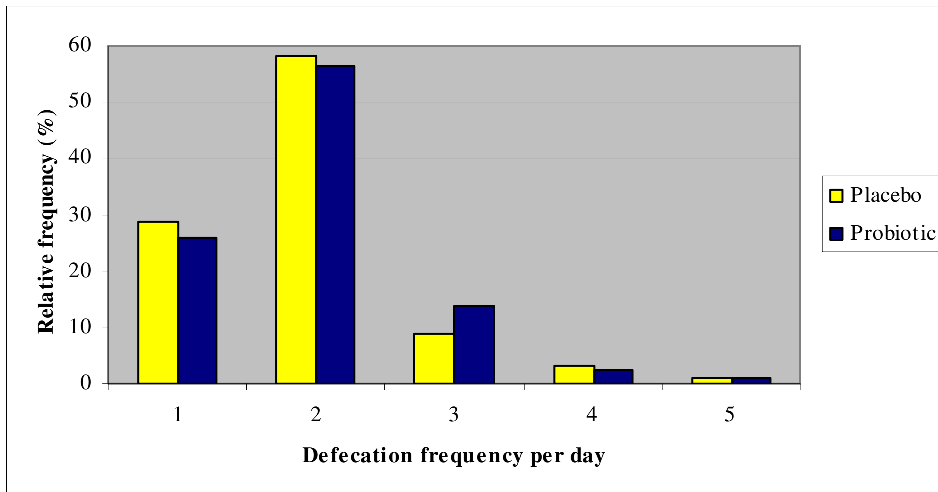

Fig. 8: Relative frequency of defecation per day of the working dogs (placebo period, n=588; probiotic period, n=588).

4.2.4 pH in faeces

The pH tended to be significant (p=0.054) between the feeding periods. During the probiotic period, a marginal higher pH was exposed (Table31).

Table 31: pH in working dog faecal samples

Period	pH
Placebo	6.00 ± 0.38
Probiotic	6.27 ± 0.54

4.2.5 Faecal dry matter

The faecal dry matter showed a tendency towards significance ($p = 0.056$), being higher after the probiotic period (Table 32). These values were within normal ranges for dogs fed dry diets.

Table 32: Faecal dry matter of working dog faecal samples

Period	Dry matter (%)
Placebo	24.7 ± 2.34
Probiotic	26.5 ± 4.70

4.2.6 D- and L-lactate concentration in faeces

Although a numeric increase can be seen during the probiotic period (Table 33), some samples (7.1%) had concentrations below the detection limit. The inconsistent concentrations ranged from 0.47-36.8 µmol/g.

Table 33: D- and L- lactate concentration in working dog faecal samples

Period	D-lactate (µmol/g) [1]	L-lactate (µmol/g) [2]
Placebo	5.57 ± 5.61	6.35 ± 5.51
Probiotic	8.69 ± 11.0	8.94 ± 10.0

[1] Placebo, n=19; Probiotic, n=20
[2] Placebo, n=19; Probiotic, n=20

4.2.7 Ammonium concentration in faeces

A slight numeric, yet not significant increase of the ammonium ion concentration was registered during the probiotic period (Table 34).

Table 34: Ammonium concentration in working dog faecal samples

Period	Ammonium (µmol/g)
Placebo	17.4 ± 6.42
Probiotic	18.6 ± 6.49

4.2.8 Faecal microflora

The concentration of coliform bacteria, *Clostridium* spp., *Lactobacillus* spp. and *Enterococcus* spp. showed no significant differences (Table 35).

Great variations of colony types were found on the Slanetz and Bartley agar for *Enterococcus* spp.. Colony morphologies differed as described in 4.1.8.

Table 35: Bacteria concentrations in working dog faecal samples

Period	**Coliform bacteria**	***Clostridium* spp.**	***Lactobacillus* spp.**	***Enterococcus* spp.**
	(CFU log_{10}/g faeces)			
Placebo	6.60 ± 1.20	5.61 ± 1.33	9.21 ± 0.97	8.63 ± 1.06
Probiotic	7.07 ± 1.10	5.94 ± 1.03	9.18 ± 0.94	8.59 ± 0.81

4.2.9 RAPD-PCR analysis of enterococci

In all, eleven RAPD-primers were used to identify *Enterococcus faecium* DSM 7134. First, a quick screening was performed with four primers to identify the probiotic strain DSM 7134. Another seven primers were used thereafter to confirm these results.

The following table 36 shows the number of identified *Enterococcus faecium* DSM 7134 colonies, for each of the four randomly chosen animals and period. After the first results, more colonies collected from one dog (Pedro 6629) were tested (five colonies from the placebo period and one colony from the probiotic period, results in table are shown in brackets).

Table 36: RAPD-PCR examined and identified *Enterococcus faecium* DSM 7134 colonies in working dog faecal samples

Working dog	Placebo		Probiotic	
	No. of tested colonies	**Positive identified colonies**	**No. of tested colonies**	**Positive identified colonies**
Pedro 6629	5 (+5)	1 (+0)	5(+1)	1 (+1)
Hedda 5983	5	2	5	2
Jimmy 2516	5	0	5	2
Django 2015	5	0	5	2
Total	20 (25)	3	20 (21)	7 (8)

4.3 Vaccination trial

4.3.1. General health status and weight

At begin, all dogs were in good condition without clinical signs of disease. This continued so through the complete duration of study by all except for one dog. This dog (Thea) was taken out of the study at S4, due to an infected bite wound, lethargy and slight fever.
The weight varied within normal ranges and showed no major changes (Table 37).

Tab. 37: Bodyweight of the sled dogs at sample collection

Group	Weight (kg)			
	Application begin	**Before vaccination**	**2 weeks after 1st vaccination**	**2 weeks after 2nd vaccination**
	S1	**S2**	**S3**	**S4**
Placebo	26.3 ± 7.68	26.5 ± 6.84	26.0 ± 6.92	26.4 ± 7.09
Probiotic	25.2 ± 8.08	26.2 ± 8.94	25.5 ± 9.26	25.7 ± 9.79

4.3.2 Complete blood count

No significant differences were seen between the groups at any time of trial period. Although all dogs received regular deworming and were in good health, high values of eosinophiles occurred often and only 26.3 % of the tested samples were within normal ranges (Table 38).

Table 38: Complete blood count from the sled dogs

		Application begin S1	Before vaccination S2	2 weeks after 1st vaccination S3	2 weeks after 2nd vaccination S4
Erythrocytes (T/l)	Placebo	6.32 ± 0.66	6.44 ± 0.49	6.34 ± 0.48	6.66 ± 0.60
	Probiotic	6.30 ± 0.51	6.38 ± 0.47	6.29 ± 0.48	6.38 ± 0.37
Haematocrit (l/l)	Placebo	0.45 ± 0.05	0.45 ± 0.03	0.42 ± 0.03	0.44 ± 0.04
	Probiotic	0.45 ± 0.03	0.44 ± 0.03	0.42 ± 0.03	0.43 ± 0.03
Haemoglobin (g/l)	Placebo	151± 9.64	151 ± 8.81	150 ± 6.65	159 ± 10.4
	Probiotic	151± 7.06	151 ± 10.1	150 ± 8.72	154 ± 10.7
Leukocytes (G/l)	Placebo	9.93 ± 2.39	9.65 ± 2.30	9.39 ± 2.04	10.1 ± 2.62
	Probiotic	10.1 ± 1.93	10.3 ± 1.67	10.5 ± 2.05	10.6 ± 1.32
Segmented granulocytes (%)	Placebo	52.3 ± 11.0	55.6 ± 8.46	57.7 ± 7.23	55.0 ± 6.11
	Probiotic	55.7 ± 5.72	58.1 ± 6.44	58.8 ± 4.42	55.7 ± 5.66
Lymphocytes (%)	Placebo	29.4 ± 8.25	27.9 ± 4.63	29.3 ± 5.74	29.9 ± 5.27
	Probiotic	25.0 ± 9.71	26.6 ± 5.17	26.4 ± 4.79	27.2 ± 3.90
Monocytes (%)	Placebo	5.10 ± 2.73	3.20 ± 0.92	2.33 ± 0.71	2.70 ± 0.82
	Probiotic	5.10 ± 3.38	3.30 ± 1.49	2.20 ± 0.79	2.11 ± 0.93
Eosinophiles (%)	Placebo	12.5 ± 10.2	12.2 ± 5.81	10.0 ± 3.64	11.9 ± 4.31
	Probiotic	13.5 ± 10.3	11.2 ± 5.37	11.8 ± 4.73	14.4 ± 6.91
Basophiles (%)	Placebo	0.00 ± 0.00	0.10 ± 0.32	0.11 ± 0.33	0.30 ± 0.48
	Probiotic	0.00 ± 0.00	0.10 ± 0.32	0.10 ± 0.32	0.11 ± 0.33
Banded granulocytes (%)	Placebo	0.70 ± 1.25	0.00 ± 0.00	0.56 ± 0.73	0.30 ± 0.48
	Probiotic	0.70 ± 0.95	0.70 ± 1.89	0.60 ± 0.52	0.44 ± 0.53
Thrombocytes (G/l)	Placebo	223 ± 74.3	272 ± 68.2	266 ± 91.8	258 ± 85.0
	Probiotic	285 ± 73.5	315 ± 67.4	315 ± 59.0	310 ± 64.8

4.3.3 Lymphocyte phenotyping

The mean percentages of the tested subpopulations showed little variations during the time of trial. The percentage of $CD3^+$ cells remained at a constant level (Figure 9).

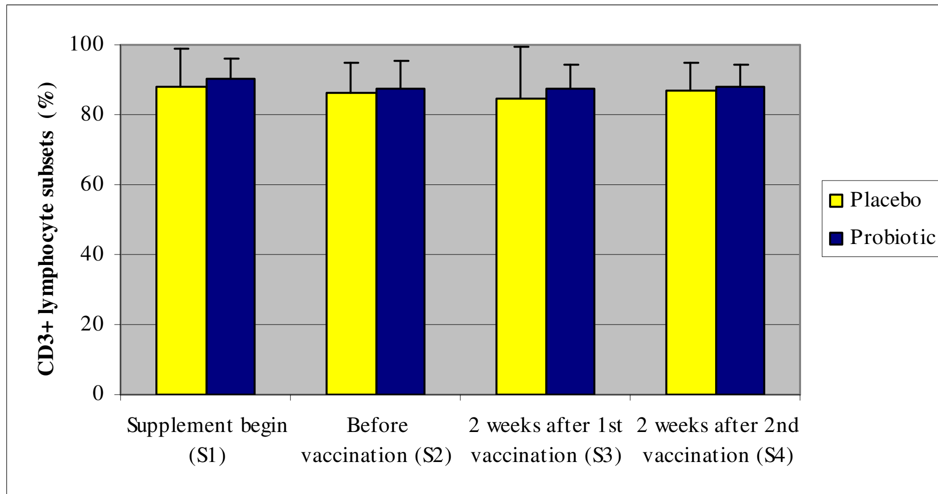

Fig. 9: Comparison of the $CD3^+$ lymphocyte subsets in the vaccination trial during the complete trial period

The concentrations of $CD4^+$ lymphocytes were comparable between the groups and minor time dependent changes were observed (Fig. 10).

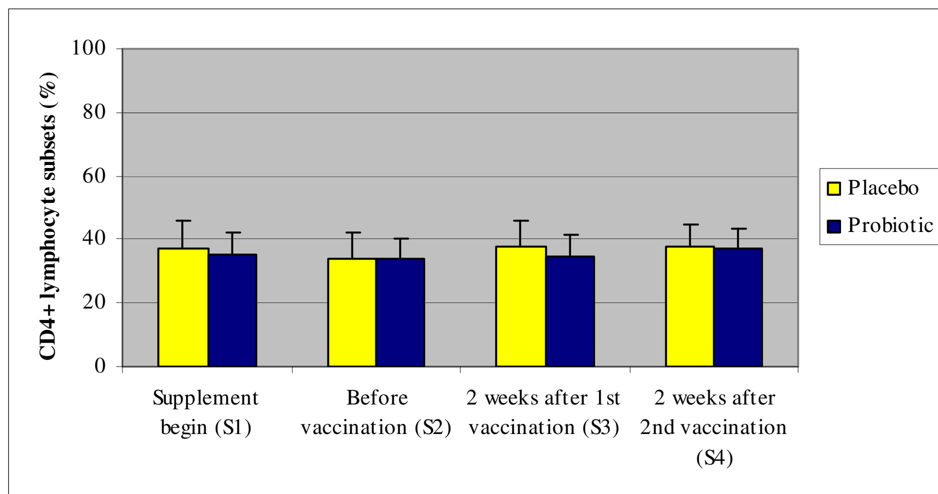

Fig. 10: Comparison of the $CD4^+$ lymphocyte subsets in the vaccination trial during the complete trial period

Group dependent differences were not found for $CD8^+$ lymphocytes (Figure 11). After the vaccinations, slight reduction of $CD8^+$ cells occurred in both groups.

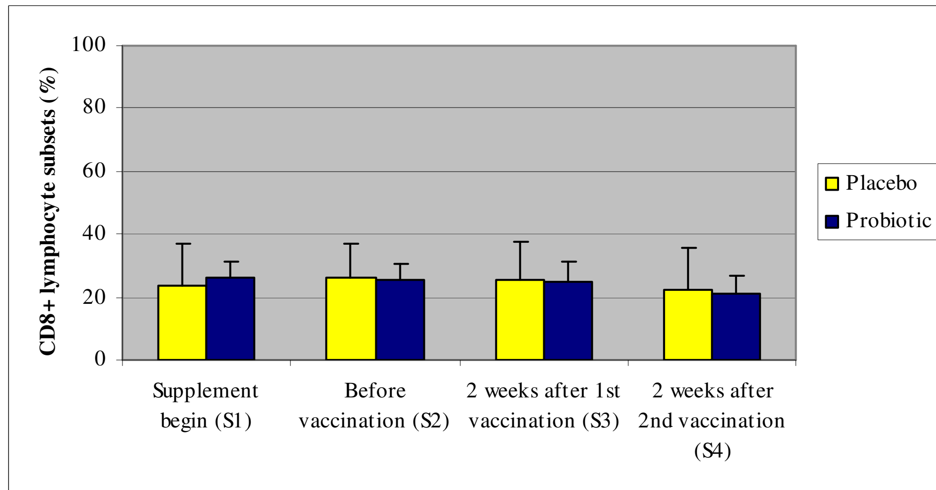

Fig. 11: Comparison of the $CD8^+$ lymphocyte subsets in the vaccination trial during the complete trial period

The $CD4^+$:$CD8^+$ ratio was in all cases higher in the placebo group. The increasing ratio, for both groups over the testing period, is due to the decrease of $CD8^+$ lymphocytes.
No significant differences were found between, or within the groups, over the period of time (Fig. 12).

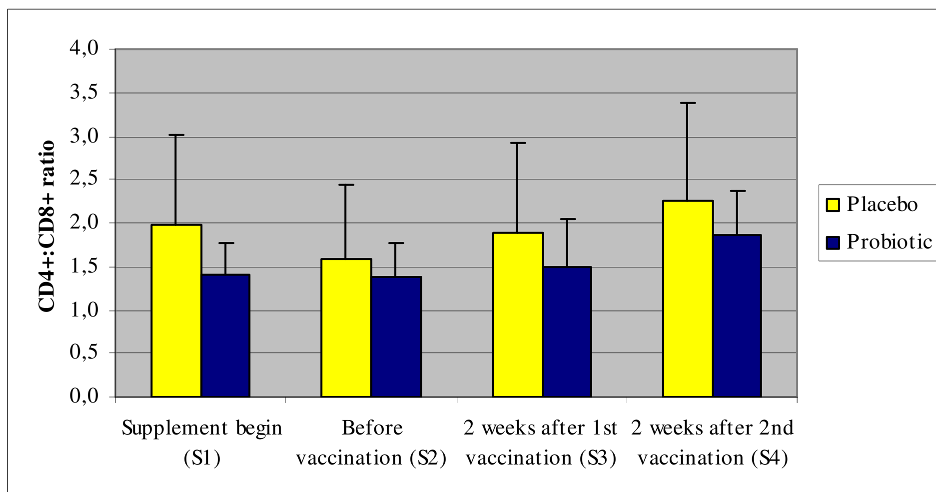

Fig. 12: $CD4^+$:$CD8^+$ T-lymphocyte ratio in the vaccination trial

The CD21$^+$ lymphocyte population (B-lymphocytes) were similar in both groups, but some time related changes were observed (Figure 13). Again, a decreasing number of positive cells was observed over the time.

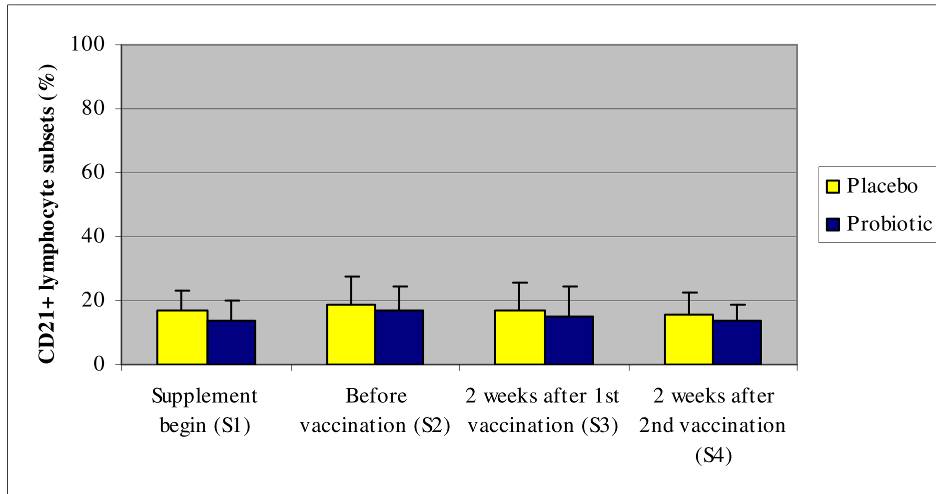

Fig. 13: Comparison of the CD21$^+$ lymphocyte subsets in the vaccination trial during the complete trial period

The expression of MHC II within PBMC´s was high and reached values around 90 % without significant group differences (Figure 14).

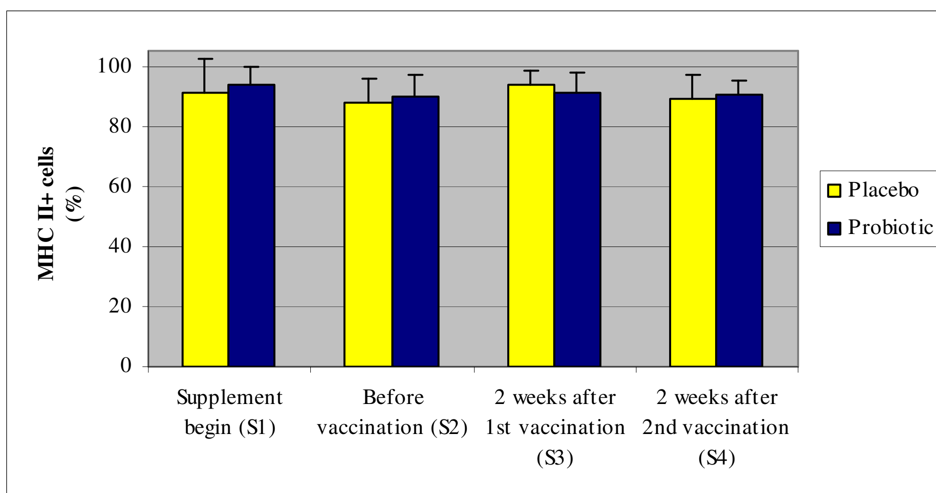

Fig. 14: Comparison of the MHC II$^+$ cells in the vaccination trial during the complete trial period

4.3.4 Lymphocyte proliferation

The stimulation index (SI) is the comparison of stimulated cells to unstimulated control cells (see 4.1.11). To begin with, the lymphocytes proliferated to the same extend in both groups. After the first vaccination (S3), a vast increase of proliferated cells was observed. Although it seems that the placebo group lymphocytes proliferated most at this time, individual values varied strongly due to the vaccination. Of interest are the indices after the booster tetanus injections (S4), which were lower than at S3. Here, the placebo group proliferations showed little difference compared to before the vaccinations (S2). The probiotic group, on the other hand, had a definite increase of indices compared to the beginning (S2). Henceforth, a significant difference ($p<0.05$) was found between the placebo and probiotic group at S4 for the proliferation with ConA. The other two mitogens, PWM and PHA, had tendencies ($p<0.10$) in the same direction (Fig. 15-17).

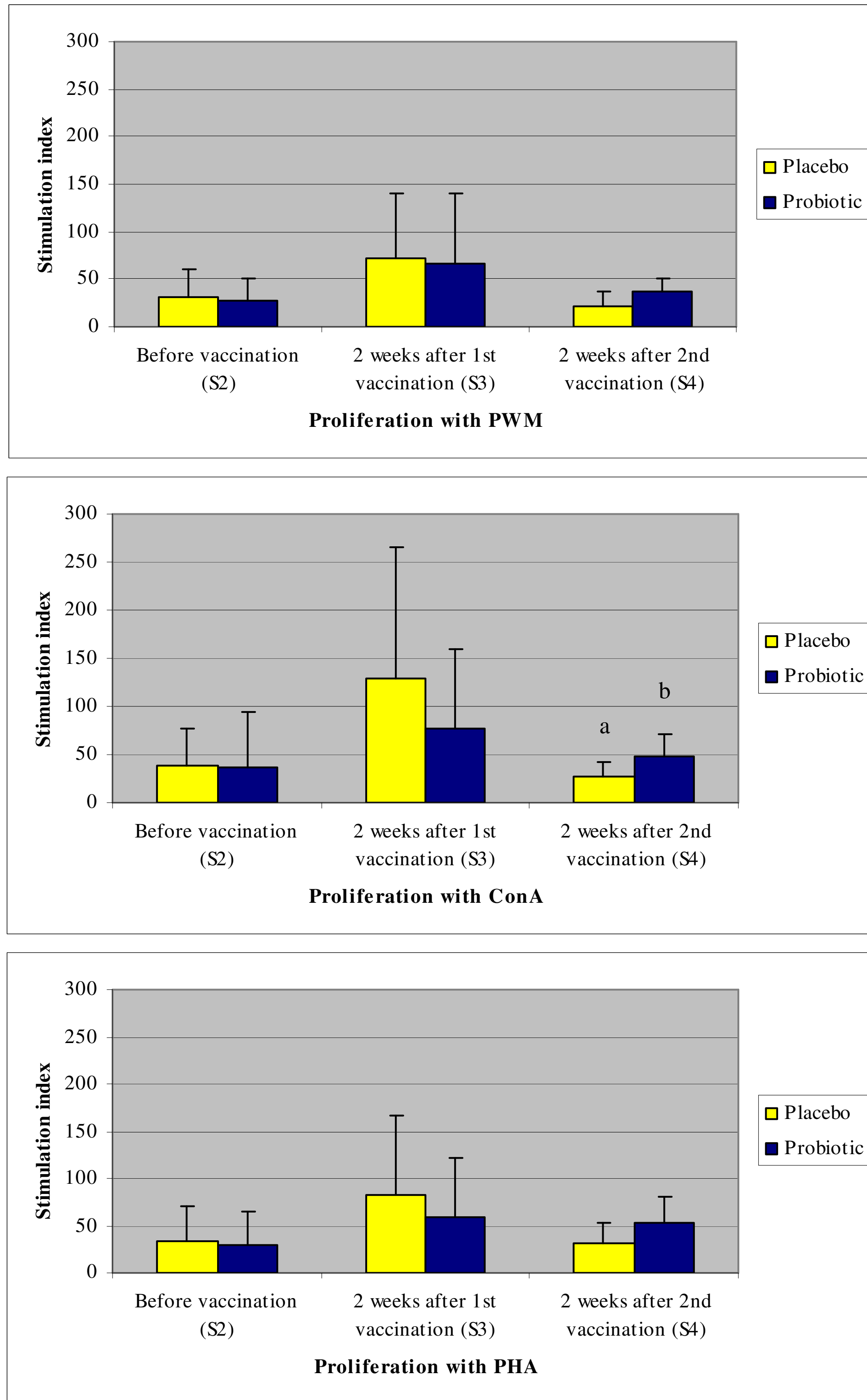

Fig. 15-17: Stimulation indices of lymphocyte proliferation with the mitogens PWM, ConA and PHA, in the vaccination trial. The proliferation with ConA shows a statistical significant difference (p=0.039) between the placebo and probiotic group at the time 2 weeks after the second vaccination (different superscripts are significantly different). The proliferations with PWM and PHA tend to be significant (p=0.059, p=0.095) at the same time (S4).

4.3.5 Immunoglobulins in the sera

4.3.5.1 Immunoglobulin G

Differences between IgG concentrations were not determined between the groups. The rise of serum IgG level at 2 weeks after the first vaccination (S3) in the probiotic group showed a statistical tendency towards significance ($p<0.1$) compared to the default value. No such tendencies were observed in the placebo group (Fig. 18).

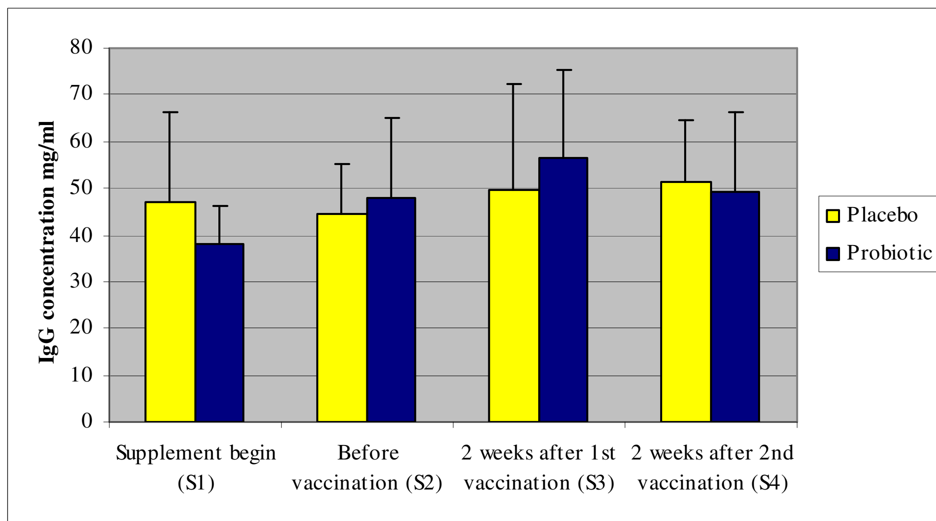

Fig. 18: IgG concentration in the sera samples of the vaccination trial during the trial period

4.3.5.2 Immunoglobulin A

The levels of serum IgA were clearly different from the beginning of the study. Significant differences ($p<0.05$) between the two groups were seen at 2 weeks after the first vaccination (S3), as in 2 weeks after the second vaccination (S4). A tending significant difference ($p<0.1$) between the groups already existed at begin (S1), as after 4 weeks of trial (before vaccination, S2). In all, the placebo group showed higher yields of serum IgA throughout the complete trial period (Fig. 19).

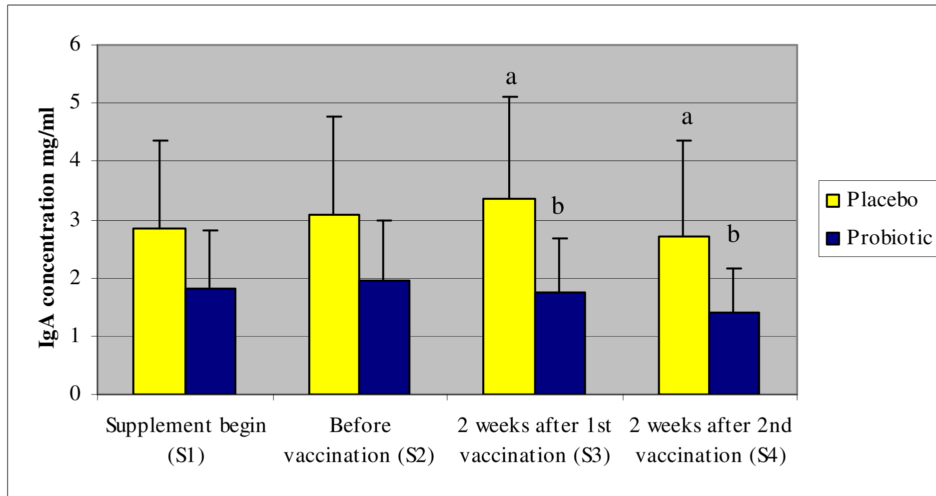

Fig. 19: IgA concentration in the sera samples of the vaccination trial during the trial period. Different superscripts indicate significant differences at $p<0.05$.

4.3.6 Antibody titres

To compare the data of the two groups, a relative ratio correlating with the positive control was formed for CPV, CDV and rabies. A Paired Sample T-Test was used within the placebo or probiotic group, to calculate significant differences before and after the vaccination.

$$\text{Relative ratio}_{\text{(CPV, CDV, rabies)}} = \frac{\text{Optical density sample}}{\text{Optical density positive control}}$$

The same was performed to compare the optical densities measured for tetanus vaccinations. This time optical densities after vaccination (S3) and booster injection (S4) were compared to its default value, since no positive control was available.

$$\text{Relative ratio}_{\text{(Tetanus)}} = \frac{\text{OD sample (S3 or S4)}}{\text{OD before vaccination (S2)}}$$

4.3.6.1 Canine parvovirus titre

Despite vaccination, the titres remained constant for all dogs. The relative ratio showed negligible differences (Fig. 20).

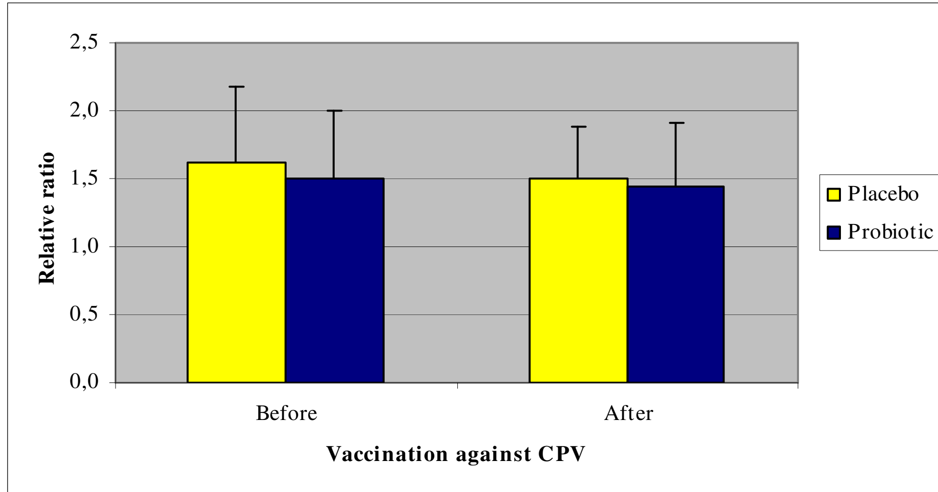

Fig. 20: Comparison of parvovirus titres, before and after vaccination, expressed as relative ratios

4.3.6.2 Canine distemper virus titre

The relative ratio of antibody titres against CDV (Fig.21) was not significantly different between the groups. However the ratio in the probiotic group increased compared to before ($p<0.05$).

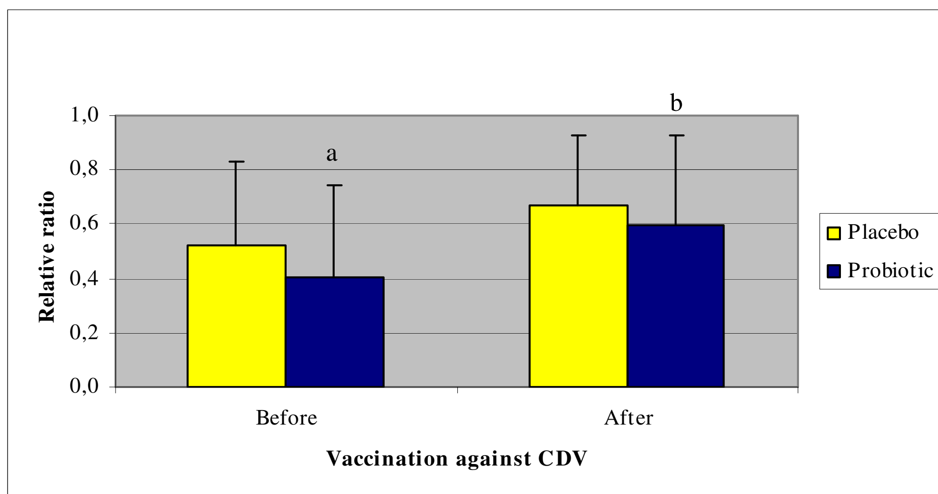

Fig. 21: Comparison of canine distemper virus titres, before and after vaccination, expressed as relative ratios. Different superscripts indicate significant differences at $p<0.05$.

4.3.6.3 Rabies titre

No significant differences where found between the groups, but the relative rabies titres increased significantly within both groups (Fig. 22). The significant increase was clearly more pronounced in the probiotic group ({$p_{Probiotic}$<0.01} < {$p_{Placebo}$<0.05}).

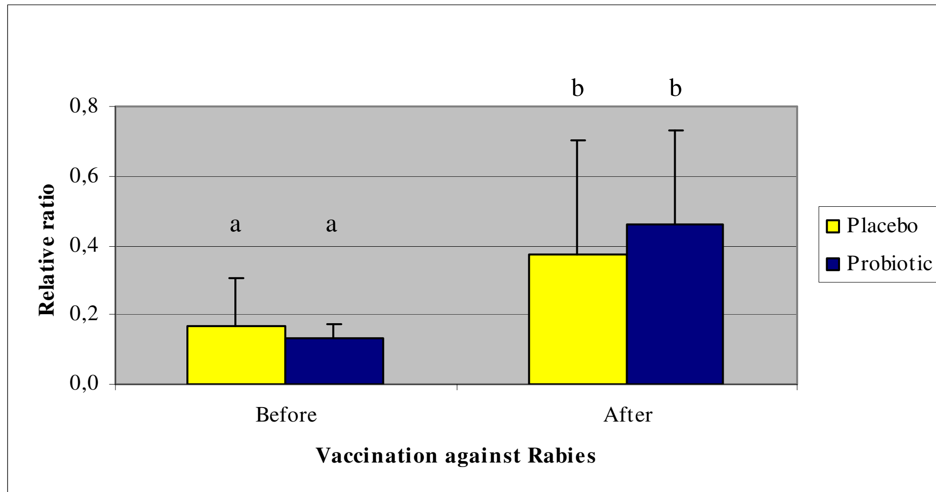

Fig. 22: Comparison of rabies titres, before and after vaccination, expressed as relative ratios. Different superscripts indicate significant differences at p<0.05.

4.3.6.4 Tetanus titre

Group differences were not observed. Both groups experienced similar significant increases of titres (p<0.01) after the first and second vaccination (Fig. 23).

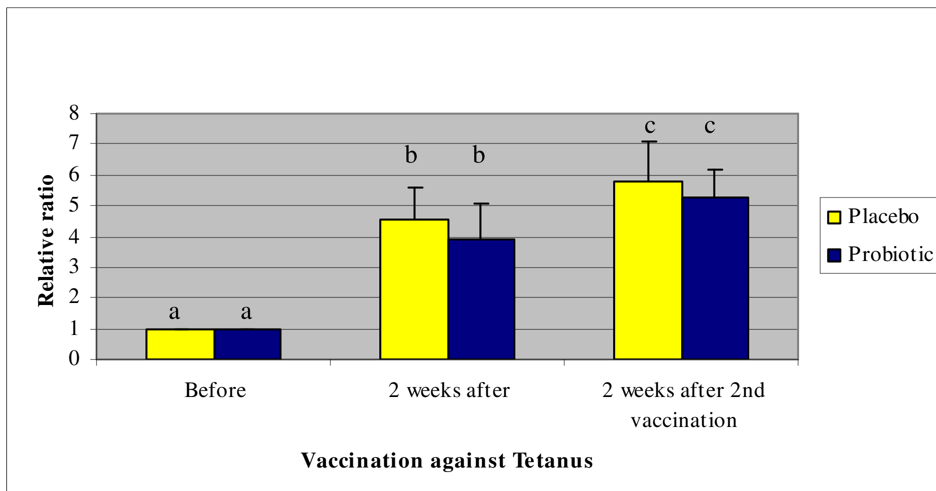

Fig. 23: Comparison of tetanus titres, before and after vaccination, expressed as relative ratios. Different superscripts indicate significant differences at p<0.01.

5 Discussion

5.1 Study design

The police and working dogs both underwent 4 weeks of a placebo and probiotic trial period. Each dog served as its own control in the cross-over study. The period of 2 weeks between the different applications was chosen due to findings of prior studies on probiotics (Biourge et al., 1998; Manninen et al., 2006; Weese and Anderson, 2002). In these cases, the probiotic was no longer detectable after 3-7 days, after the administration had ceased.
In the vaccination trial, the dogs were separated into a placebo or probiotic group. The effect of the vaccines in combination with the probiotic over a longer period of time was of interest. The probiotic was fed 4 weeks prior to vaccination, so the dogs could adjust to their new diets and any differences between the groups were related with a high probability to the probiotic administration.

5.2 Clinical observations

Probiotics are generally regarded as safe (GRAS) organisms (Culligan et al., 2009; Weese and Anderson, 2002). In this study, all dogs tolerated the probiotic preparation well. The health status of all dogs was good during feeding and no further clinically negative effects, due to the probiotic, were seen. These observations coincide with other authors (Benyacoub et al., 2003; Marcinakova et al., 2006; Zentek et al., 1998).
The weight of the police dogs stayed constant over the whole trial period. In the vaccination trial, the probiotic group had a lower mean weight than the placebo group, from the beginning on. However, the weight gain and loss in each group were of the same notion for the complete trial period, indicating that other external influences were the cause and not the probiotic strain.
The application of *Enterococcus faecium* SF68 in young dogs showed no increase in the body weight (Benyacoub et al., 2003), nor did the application of *E. faecium* in puppies (Weiß, 2003). No significant influence was also seen in adult dogs, which were additionally fed with different *Lactobacillus* spp. (Sauter et al., 2006).
The function of weight gain, due to probiotics, in companion animals has a minor relevance. In the nutrition of production animals, weight gain and feed conversion are often improved, along with a reduction of diarrhoea, during probiotic administration (Bohmer et al., 2006; Zeyner and Boldt, 2006).

5.3 Faecal quality and defecation frequency

The faecal quality was assessed using a 1-5 scale. Placebo and probiotic period were compared, using relative frequencies (%). The dogs were not only pooled to compare placebo and probiotic period overall, but the scoring and defecation frequency were also assessed individually for every dog to compare possible distinct effects. Individual interpretations are important since experienced stress can cause changes in the pH, barrier function and the motility of the intestinal tract. When cessation of the nutrient flow occurs, commensal bacteria can be reduced offering possible opportunistic bacteria to take over, causing loosely formed faeces.

The police dogs faeces were always assessed from the same person, the owner. This was not possible for the faeces of the Bundeswehr dogs. Here, the animal attendant on duty assessed the faeces, creating the possibility of variations.

The police dogs showed a significant reduction of poorly formed faeces (score 4) and an increase of dry faeces (score 1) during the probiotic feeding period. The reduction of the unacceptable consistency score 4 assumes that a positive effect has taken place during probiotic application with *E. faecium* DSM 7134. In regard to each individual dog, a positive significant outcome was achieved by 3 dogs. Another 3 dogs experienced a numeric reduction of their scoring. All mean scorings resembled acceptable consistencies. Only one dog had higher faecal scorings, but with a definite significant reduction during the probiotic period.

The Bundeswehr dogs had a significant increase of the unacceptable score 4, but a decrease in score 3 during the probiotic period. When viewing each dog individually, this increase of score 4 can be traced back to one individual dog. Three dogs showed a significant reduction in their faecal scores and another 4 dogs a numeric score decrease during the probiotic period, although not significant. In all, 12 dogs had an increase in their faecal scorings, where 6 dogs had significant results.

The large differences between the scoring of the police and Bundeswehr dogs are probably due to the individual judgement of the faecal quality. Baillon et al. (2004) found no influence to the faecal quality when feeding the strain *Lactobacillus acidophilus* DSM 13241. Neither could any effect be described during the administration of a strain of *Enterococcus faecium* to adult dogs and pups, to the faecal consistency (Molitor, 1996).

In contrast to these studies, Pascher et al. (2008) found a positive effect on the faecal consistencies, when administering *L. acidophilus* DSM 13241 to 6 German Shorthair Pointers with non-specific dietary sensitivity. In another study, the administration of *L. acidophilus* in combination with fructooligosaccharides caused a trend to improvement of the faecal quality

(Swanson et al., 2002). The probiotic cocktail *Lactobacillus acidophilus*, *Pediococcus acidilactici*, *Bacillus subtilis*, *Bacillus licheniformis* and *Lactobacillus farciminis*, given three times a day to dogs with acute self-limiting gastroenteritis, shortened the time from start of treatment until the last observed abnormal formed faeces (Herstad et al., 2010).

The defecation frequency was not influenced by the application of the probiotic in the police dogs. Most of the police dogs had a defecation frequency of 3 times a day, whilst the Bundeswehr dogs mainly had a frequency of 2 faeces a day. A slight numerous increase, to 3 defecations a day, was noted in the probiotic group of the Bundeswehr dogs.

The defecation frequency and the faecal quality are dependent of the whole gut transit time. An increased rate of transit reduces water absorption in the colon, resulting in loose faeces (Rolfe et al., 2002b). The reduction of crypt length and density, which was seen in dogs with non-specific dietary sensitivity, causes a decrease in the absorptive surface area, resulting also in loose faeces (Rolfe et al., 2002a). Similar observations can be seen when comparing different breeds. The digestive tract of large breeds is in relation to their size shorter and has a relative lower weight, thus resulting in a shorter transit time, predisposing for loose and watery faeces (Zentek and Meyer, 1993). Another cause can be the fed diets. In small breeds, no difference was found in the faecal quality, when feeding a dry or canned diet. In larger breeds, canned diets increased the defecation frequency (Meyer et al., 1999). Temper, activity and mobility, as well as stress can provoke individual differences in the faecal quality and defecation rate. To minimise these dog-related stimuli, each dog represented its own control.

5.4 Faecal pH

A low pH is inhibitory to the growth of many pathogenic bacteria and reduces the absorption in the intestine of potentially toxic compounds, like ammonia (Martineau and Laflamme, 2002). During probiotic feeding, the pH of the faeces was not affected in other studies (Baillon et al., 2004; Mück, 2007; Pascher, 2004; Swanson et al., 2002). In this study, the faecal pH from the police dogs showed a tendency to decrease during the probiotic period, compared to the Bundeswehr dogs, which experienced a minimal rise of pH. These negligible effects are situated in such a minimal margin that possible probiotic influences need to be discharged.

5.5 Dry matter of faeces

The normal range of the faecal dry matter, when fed with a dry diet, is 25-35 % and for a canned diet 20-30 % (Meyer and Zentek, 2005). Both, the police and the Bundeswehr dogs,

showed an increasing trend of the dry matter in faeces after the probiotic period, but all mean values stayed within normal ranges. Pascher (2004) observed also an increase of the faecal dry matter after *Lactobacillus acidophilus* application, but encountered no decrease in the faecal unbound water. The amount of unbound faecal water was not investigated in this study. The faecal dry matter and faecal unbound water have a direct influence on the faecal consistency.

5.6 D- and L-lactate in faeces

Lactic acid bacteria have the property of producing lactic acid because of carbohydrate fermentation. An increased lactate concentration can decrease luminal pH, and thereby inhibits the growth of bacteria sensitive to acidic conditions (Swanson et al., 2002; Wohlgemuth et al., 2010).

In this study, although the faecal lactate concentration of the Bundeswehr dogs increased slightly after the probiotic feeding, the concentrations of lactic acid were too low to affect the pH in a declining manner.

An addition of *E. faecium* to ileum chyme under in vitro fermentation increased the lactate concentrations. The amount of increase was dependent on the bacteria concentrations (Zentek et al., 1998). The same authors also conducted in vivo studies with dogs applying *E. faecium*. Lactate concentration tended to increase in the ileum chyme. Simultaneously, a pH reduction took place. No such effect was seen in the faeces, since lactate is mainly produced in the small intestine.

In a study conducted with puppies (Weiß, 2003), the addition of *E. faecium* DSM 7134 tended to decrease the lactate concentration in the faeces, whilst feeding milk-replacer. During the period of additional canned food, the lactate concentration increased in the probiotic group. After weaning, lactate concentrations were similar in both groups.

An increase of the intestinal lactate concentration was seen in growing turkey poults, which received *Enterococcus faecium* SF68 (Vahjen et al., 2002). An increase of counted lactobacilli and enterococci was not encountered, as in the own studies. The same *Enterococcus faecium* strain, applied to dogs, caused a reduction of *Clostridium perfringens* count, which was assumed to be initiated by the lactate increase (Vahjen and Männer, 2003). This phenomenon was not encountered in the own studies.

5.7 Ammonium concentration in faeces

A lower bacteria ammonia production in the intestine is desirable to relieve the metabolism especially in older and diseased dogs. In the intestine, urease-producing bacteria synthesise ammonia from proteins and urea. This toxic metabolic product is normally detoxified in the liver to urea and then excreted via the kidney (Kraft and Dürr, 1999).

In all, no significant changes in the faecal ammonium ion concentration were found in either of the group. In agreement, Zentek et al. (1998) found no impact on the ammonia concentration of isolated ileum chyme under in vitro conditions with *E. faecium*. Feeding *Lactobacillus acidophilus* alone had no effect on the ammonia concentration in faeces. Only the combination with fructooligosaccharides reduced faecal ammonia levels (Swanson et al., 2002).

5.8 Faecal microflora

Coliform bacteria, *Clostridium* ssp., *Lactobacillus* spp. and *Enterococcus* spp. were chosen to monitor the composition of the intestine microbiota. No significant changes were encountered when *Enterococcus faecium* DSM 7134 was fed.

In other studies, the addition of *E. faecium* strains had also no effect on the count of faecal *E. coli* (Marcinakova et al., 2006; Vahjen et al., 2002), neither did the application of *Lactobacillus* spp. (Baillon et al., 2004; Pascher et al., 2008; Strompfova et al., 2006; Swanson et al., 2002).

Clostridium perfringens is a common bacterium in the gut of carnivores, since the protein-rich diet favours its growth (Werdeling et al., 1991; Zentek et al., 2003). Yet it is more often found in dogs with diarrhoea, than in healthy dogs. The enterotoxin of *Clostridium perfringens* type A is known to cause severe diarrhoea due to food intoxication in humans and the same enterotoxin can also be found in dog faeces (Werdeling et al., 1991). A lower level of clostridia is therefore considered as favourable.

The addition of *E. faecium* strains caused a reduction of faecal *Clostridium perfringens* in several studies (Mück, 2007; Rinkinen et al., 2003a; Vahjen and Männer, 2003; Weiß, 2003). The same effect caused *L. acidophilus* strains (Baillon et al., 2004; Swanson et al., 2002). Vahjen and Männer (2003) concluded that the decrease of clostridia is due to general modification of intestinal conditions by *E. faecium,* since the same probiotic strain enhanced the activity of lactic acid bacteria and lactate concentration in the intestine of poultry (Vahjen et al., 2002). Rinkinen et al. (2003a) proved that the in vitro mucus adhesion of

Cl. perfringens was significantly reduced by lactic acid bacteria, which was believed to lead to lower levels of colonisation.

The amount of lactobacilli varies with age, breed and feeding (Simpson et al., 2002). The dogs of the Bundeswehr had about 10 times more lactobacilli than the police dogs, but all staying within normal ranges. Feed dependent effects were also encountered in the study of Weiß (2003). The lactobacilli concentrations dropped greatly after the puppies received canned food instead of the milk-replacer, but the counts of lactobacilli were still greater in the *E. faecium* fed group. The combined addition of *L. fermentum, L. salivarius, Weissella confusa, L. rhamnosus and L. mucosae* had only a minor effect on jejunal lactic acid bacteria concentration, but changed the indigenous microflora in such a way, that *L. acidophilus* became dominant in the jejunal chyme after administration had ended (Manninen et al., 2006). Since in the own study no change in the enterococcus concentration was encountered, the existence of a shift of bacterial diversity and of possible dominant strains would have been interesting.

The use of Slanetz-Bartley agar seemed to be good and reliable for counting enterococci, when grown aerobically at 37°C (Mück, 2007). The manufacturer (Oxoid Ltd., England) implies that red and violet colonies represent enterococci. In practice, many colonies did not resemble a red or violet, but were rather pink in colour. These colonies were only counted, if microscopically proven to be cocci. *Lactobacillus* spp. also seem to grow well on this agar (Macha, 2007). The manufacturer also suggests the incubation at 44°C, to reduce possible accompanying bacteria. The wrong interpretation of the colonies may also be the cause for the high counts found in the own study (10^8 – 10^9 cfu/g faeces), without differences in the feeding periods. The abundance of enterococci, as part of the intestinal microflora, is also dependent of age, breed and feeding (Simpson et al., 2002) and has a concentration of circa 10^7 cfu/g faeces. Molitor (1996) described a range of about 10^4 – 10^5 cfu/g faeces, when no additional *E. faecium* is added, and 10^8 cfu/g faeces when the probiotic is added. Mück (2007) found enterococci concentrations of about 10^7 cfu/g faeces during probiotic feeding. Both, Molitor (1996) and Mück (2007), observed a definite concentration increase, after adding an *E. faecium* probiotic strain, indicating the survival in the gastrointestinal tract.

5.9 RAPD-PCR analysis

Although no higher concentration of enterococci was found after the probiotic period, the survival of the probiotic in the gastrointestinal tract was of interest. The best method for the identification of lactic acid bacteria (strain typing) is the RAPD-PCR (Domig et al., 2003).
A clear identification was made of *Enterococcus faecium* DSM 7134 of at least two colonies during the probiotic period, proving the passage of the intestinal tract. One dog also had one positive colony in the placebo period. Since he started of with the placebo application, no explanation for this finding can be given. More colonies were tested for this dog and no further positive results occurred. Another dog on the other hand, had two positive colonies during the placebo period, but started with the probiotic administration. In this case, it is likely that the probiotic strain has survived over a period of six weeks and colonised the digestive tract.
Enterococcus faecium EE3 survived almost three months after cessation (Marcinakova et al., 2006) and *Lactobacillus fermentum* AD1 survived even six months in the digestive tract of dogs (Strompfova et al., 2006), clearly demonstrating the colonisation and replication of these strains. The probiotic strain should persist in the digestive tract during administration at least transiently (Strompfova et al., 2006).
The probiotic administration to dogs and the following RAPD-PCR analysis for the fed *L. acidophilus* NCC2628, *L. acidophilus* NCC2766 and *L. johnsonii* NCC2767, identified only a few *L. johnsonii* NCC2767 strains (Sauter et al., 2006). The concentration of the *L. acidophilus* strains may have been to low to be grasped by colony picking.
In the own study, all positive identified colonies were of violet colour, but not all violet coloured colonies represented *Enterococcus faecium* DSM 7134. Randomly picking colonies for the RAPD-PCR off the agar plate, disregards the possibility of finding all *E. faecium* DSM 7134 colonies especially if their concentration is low.

5.10 Lymphocyte phenotyping

Flow cytometry is a standard method for specifically determining the percentage of lymphocytes with particular cell surface proteins (Byrne et al., 2000). It is a typical tool to assess peripheral blood on the effects of disease external factors, the population of lymphocytes and especially the immunocompetence of the host (Tarrant, 2005). In addition, it is applied to define a diseases state, such as the Acquired Immunodeficiency Syndrome, by the changes in subsets. Similarly, in veterinary medicine it is used to asses the progression of

Feline Immunodeficiency Virus and Feline Leukaemia Virus infections (Barlough et al., 1991; Hoffmann-Fezer et al., 1996; Torten et al., 1991).

CD3 antigens are expressed on T-lymphocytes and are often revered to as Pan T markers (Byrne et al., 2000), being an integral part of the T-cell receptor complex (Williams, 1997).

Lymphocytes expressing CD4 are known as T-helper cells. The special case in the dog, compared to other species, is that CD4 is also expressed on neutrophiles in a high density. The reason for this is still unknown (Moore et al., 1992; Williams, 1997). CD4 is also a co-receptor. It stabilises the interactions of the T-cell receptor with the MHC class II molecule (Janeway et al., 2008).

The CD8 antigen is carried by cytotoxic T-cells. It also acts as a co-receptor, but recognises only the MHC class I molecules (Garcia et al., 1996; Janeway et al., 2008).

CD21 is expressed on B-lymphocytes and is part of the B-cell co-receptor (with CD19 and CD81). Antigen binding, just like in the T-cell receptor, is enhanced when the co-receptor is simultaneously bound to the B-cell receptor. CD21 is also known as complement receptor 2 (CR2) and binds to the inactive form of C3b fragment of complement, that remains attached to the pathogen surface. The fragment is cleaved into iC3b, which binds to CR3 and stimulates phagocytosis, and C3dg, binding only to CR2, causing an augmented signal and contributing to producing a strong antibody response (Janeway et al., 2008).

MHC class II antigens are expressed by both resting B- and T-cells, in contradiction to men and mice, where only activated T-cells do this. They are involved in the presentation of exogenous antigen (peptide fragments of pathogens) to CD4 T-cell co-receptor (Janeway et al., 2008; Williams, 1997).

The following table 39 gives an overview of possible values for the different lymphocyte subsets.

Table 39: Literature values (%) for investigated lymphocyte subsets in dogs

	O'Neill et al. (2009)	Faldyna et al. (2005)	Gauthier et al. (2005)	Faldyna et al. (2001)	Byrne et al. (2000)	Kim et al. (2000)
$CD3^+$		83.9 ± 1.7	75.0 ± 8.6	80.0 ± 7.4	80.6	
$CD4^+$	39.8 ± 7.3	58.6 ± 3.7	43.7 ± 6.4	43.2 ± 6.9	45.0	40.9 ± 2.4
$CD8^+$	26.3 ± 14.0	15.1 ± 4.4	20.1 ± 4.4	19.1 ± 4	28.8	21.6 ± 1.8
$CD21^+$	14.8 ± 8.9	15.7 ± 1.8	13.4 ± 7.1	14.4 ± 5.9	12.9	11.3 ± 1.2
MHC II			93.6 ± 6.8		98	53.6 ± 4.6

In the own study, $CD3^+$ lymphocyte subsets had a mean range of 70.3-72.9 % in the police dogs and a higher range in the sled dogs, being 84.5-90.2 %. $CD4^+$ lymphocytes tended to be a bit lower than the given literature values, ranging from 33.7-41.3 % in both groups of dogs. The values in the vaccination trial coincide with literature values, ranging from 21.1-26.0 % for $CD8^+$ lymphocytes. Again, the police dog values are lower (13.9-14.7 %). For $CD21^+$ lymphocyte subsets, the police dogs ranged at a lower level with 10.3-11.3 %, but a higher value range was reported in the sled dogs with 13.7-18.5 %. The MHC II positive lymphocytes also displayed a variety of values. The police dogs expressed 74.5-79.2 % positive cells and in the vaccination trial high values of 88.0-93.8 % were found.

The amount of $CD4^+$ and $CD8^+$ cells in peripheral blood lymphocytes alters with age. $CD4^+$ lymphocytes decrease with age (Byrne et al., 2000; HogenEsch et al., 2004; Toman et al., 2002), whereas $CD8^+$ lymphocytes increase (Byrne et al., 2000; Faldyna et al., 2001; Greeley et al., 1996; HogenEsch et al., 2004; Toman et al., 2002), which therefore causes a diminution to the $CD4^+$:$CD8^+$ ratio. On the other hand, $CD3^+$ lymphocytes (Blount et al., 2005; Faldyna et al., 2001; HogenEsch et al., 2004) and $CD21^+$ lymphocytes (Blount et al., 2005) show no such effects.

Literature notes that the value for the $CD4^+$:$CD8^+$ ratio can be from 1.87 (Blount et al., 2005), 2.2 (Discherl et al., 1995) to 2.5 (Faldyna et al., 2001). The police dogs had high mean values of 2.77-2.91, whilst in the vaccination trial values ranged from 1.37-2.25. The differences occur due to the relatively low percentage of $CD8^+$ lymphocytes in the peripheral blood of the police dogs.

In the own application, no significant changes were found in any of the lymphocyte subsets due to the probiotic or the vaccination. The application of *Enterococcus faecium* SF68 to young dogs (Benyacoub et al., 2003) caused also no effect in the percentages of $CD4^+$ and $CD8^+$ lymphocytes, but did enhance the amount of $CD21^+$ cells. Same results were achieved by Mück (2007) by orally administrating *Enterococcus faecium* DSM 7134. The addition of *Lactobacillus acidophilus* DSM 13241 (Baillon et al., 2004) brought also no change in $CD4^+$ and $CD8^+$ subsets about.

5.11 Lymphocyte proliferation

Substances referred to as polyclonal mitogens induce mitosis in lymphocytes. This proliferation is used to study the adaptive immunity, since polyclonal mitogens seem to trigger the same growth response mechanisms as antigens (Janeway et al., 2008).

Most studies of lymphocyte proliferation in dogs are based on the incorporation of radiolabelled ^{3}H-thymidine into DNA (De Luna et al., 1999; Krakowka and Ringler, 1986). This incorporation into newly synthesised DNA in lymphocytes correlates with cell growth (Lijnen et al., 1997). Although it is still the method of choice, radioactive waste is produced. The substitution of BrdU as a pyrimidine analogue in place of thymidine into newly synthesised DNA during lymphocyte proliferation produces no such waste. The recognition of these proliferated cells is detected by a monoclonal anti-BrdU-antibody. A highly significant, but nonlinear relationship between BrdU and ^{3}H-thymidine incorporation exists, making the BrdU-proliferation assay to an excellent alternative for the evaluation of canine lymphocytes (Wagner et al., 1999).

Classical polyclonal activators are concanavalinA (ConA), phytohemagglutinin (PHA) and poke weed mitogen (PWM) (De Luna et al., 1999). In the stimulation of canine lymphocytes is ConA a restricted T-cell mitogen, PWM stimulates only B-cells and PHA is not limited to either T- or B-cells (Krakowka and Ringler, 1986).

The addition of *Enterococcus faecium* DSM 7134 in the own study produced numerously higher stimulation indices in the proliferations with ConA and PWM for the police dogs. The vaccinations of the sled dogs caused a clear reaction of higher indices for all three mitogens, showing a definite alteration of the adaptive immunity two weeks after the first vaccination. Of interest was that after the second vaccination of tetanus or four weeks after the first vaccination, the placebo group fell back to its initial indices, whilst all in the probiotic group experienced higher stimulations with all three mitogens, compared to the beginning.

It is reported that immunisation causes immunosupression in dogs. Polyvalent vaccines provoked suppressed lymphocyte response to mitogens (Phillips et al., 1989), but this suppression was of relative short time. At day 14 of post inoculation, lymphocyte response to mitogens returned to normal levels. This suppression was not encountered in the own study, nor in other studies with vaccinated dogs (Phillips and Schultz, 1987; Strasser et al., 2003).

Mück (2007) experienced similar effects due to the application of *Enterococcus faecium* DSM 7134. Higher proliferative activities were seen for the same tested mitogens during probiotic feeding. A single administration of *Enterococcus faecalis* FK-23 to mice caused stimulations of twice that of the control levels for PHA and PWM and 1.5 times more for ConA (Kanasugi et al., 1997).

On the other hand, *L. rhamnosus, L. gasseri* and *L. casei* have been reported to inhibit lymphocyte proliferation, whilst *L. acidophilus* had no significant influence (Kirjavainen et al., 1999). *L. plantarum* caused also no increase in the proliferative activity of spleen cells

compared to the control group, but did partially restore the ability of spleen cells from mice treated with the immunosuppressive agent cyclophosphamide, to proliferate in response to LPS (Bujalance et al., 2007). A significant higher stimulation of spleen cells was found with LPS in mice given *L. fermentum* PL9005. The stimulation with ConA produced a numerically higher proliferation (Park et al., 2005).
The effect of probiotics on canines to lymphocyte proliferation has not been examined often, but with the few data available the assumption seems to be justified, that the probiotic *Enterococcus faecium* DSM 7134 has an effect on the immune system.

5.12 Immunoglobulins in sera

A measurement of serum immunoglobulin concentration is one of the most commonly used assessments of immune competence in dogs (German et al., 1998).
Immunoglobulin G is the prevailing antibody in the serum and colostrum of dogs and cats. Its functions include activation of complement, neutralisation of bacteria and viruses, assistance of antibody-dependent cellular cytotoxicity, and opsonisation for phagocytosis (Janeway et al., 2008).
The most predominant antibody present in the lumen of the gut is secretory IgA. It acts as an antigen-specific barrier to pathogens and toxins in the gut lumen (Janeway et al., 2008). Secretory IgA is dimeric and so is, in contrast to humans, almost all serum IgA in the dog. The conclusion followed that since the dimeric serum IgA is synthesised in gut associated lymphoid tissue and secretory IgA from plasma cells in the lamina propria of the duodenum, the serum IgA concentration reflects the amount of secretory IgA produced (Batt et al., 1991; Day and Penhale, 1988; Vaerman and Heremans, 1970). Recent studies proved no such correlation between serum and secretory IgA levels (German et al., 1998; Tress et al., 2006). Uneven distribution of intestinal IgA producing plasma cells causes a high variability of faecal IgA concentration (Hart, 1979). Faecal IgA levels can vary with diet, environment, level of stress, antigenic stimulation, gastrointestinal transit time and vaccination status. Therefore, the importance lies within the collection of several faecal samples of one dog, for reliable interpretation of results (Tress et al., 2006). In the own study, serum IgA was determined due to the fact that no (sled dogs) or only one faecal sample per period (police dogs) were possible to be collected.
The concentration of IgG and IgA did not differ greatly between placebo and probiotic period for the police dogs. IgG concentration had mean values of 18.9 mg/ml and 18.4 mg/ml

respectively for placebo and probiotic period. IgA concentration had means of 1.23 mg/ml and 1.30 mg/ml.

In the vaccination trial, IgG concentrations were located overall at much higher levels. The means ranged from 38.0 mg/ml to 56.5 mg/ml, showing a concentration of 2-3 times higher than in the police dog study. The same phenomena occurred with the IgA concentration. Means ranging from 1.39 mg/ml to 3.36 mg/ml were also clearly up to 3 times higher.

Serum concentrations of IgA are dependent on age (German et al., 1998), sex (Glickman et al., 1988) and breed (Griot-Wenk et al., 1999), which alone could be the explanation for the difference in values of police- and sled dogs. German shepherds are reported to have a relative deficiency in serum IgA values compared to other breeds (Whitbread et al., 1984) and also lower values of faecal IgA (Littler et al., 2006), without clinical signs of disease. Low levels of IgA also occurred in parasitic or atopic dogs and in contrast IgG concentrations were higher in such dogs (Hill et al., 1995). No age relationship exists with IgG concentrations in sera (Blount et al., 2005). For healthy dogs, IgA values of 0.19-3.9 mg/ml (Hill et al., 1995), 1.0 mg/ml (German et al., 1998) and 0.61 mg/ml (Day, 1996) have been found. IgG ranges in the literature are 7.1-21.0 mg/ml (Hill et al., 1995), 17 mg/ml (Day, 1996; German et al., 1998), or up to 28.8 mg/ml (Blount et al., 2005). Mück (2007) obtained similar high values for IgG as in the own study.

In the vaccination trial, IgG concentration rose predominantly in the probiotic group 2 weeks after the immunisation, tending to be significant, indicating a possible influence of the probiotic as an adjuvant for the vaccination. Immunisation causes changes in the humoral immunity. IgG rose in several studies in the sera of dogs after vaccination (HogenEsch et al., 2002; Strasser et al., 2003). IgA concentrations were significantly lower in the probiotic group at 2 weeks after vaccination, as in 2 weeks after the booster injection. These significant differences should not be evaluated as an effect of the probiotic, since IgA concentrations were clearly lower in the probiotic group from beginning on.

The application of *E. faecium* in puppies caused IgA concentrations to rise and IgG to decrease, at the age interval of 2-4 weeks (Weiß, 2003). Mück (2007) achieved no difference in IgG concentration, but did encounter a numeric increase in serum IgA during both probiotic periods. For the administration of *E. faecium* SF68, higher faecal and serum IgA levels were yielded, whilst IgG levels experienced no influence (Benyacoub et al., 2003).

Similar effects were observed with *L. acidophilus*. Faecal IgA concentrations were clearly higher and IgG lower, during probiotic feeding (Pascher, 2004). In contrast, significantly higher concentrations of IgG were obtained in the study of Baillon et al. (2004).

Although IgG concentrations increased in the probiotic group of the vaccination trial, the assumption that *E. faecium* DSM 7134 has a direct influence on the humoral immune system is far fetched, due to the fact that also no such effects occurred in the police dog trial.

5.13 Antibody titres

The AAHA canine vaccine guidelines (2006) categorise vaccines into core, noncore (or optional) and not recommended. The polyvalent vaccine used, including rabies, is a core vaccine. Core vaccines are meant to be administered to all dogs in one form or another. Noncore vaccines should only be applied selectively based on geographic and lifestyle exposure. The tetanus vaccine is not recommended, due to the few cases experienced. Meanwhile, vaccines against canine distemper virus, parvovirus and rabies have durations of 3 years, yet suggestions exist that titre values should be measured before further vaccination takes place (Horzinek, 2006).

All titres were expressed as relative ratios for better comparisons between the placebo and probiotic group.

Parvovirus titres remained after vaccination at the same level as before, for both groups. A failure to respond to vaccines may be due to host- or vaccine related causes. Host related factors include the blocking effect of maternal antibodies, immunosuppression, nutrition, stress, age, hormones, drugs and vaccine interference. Vaccine related factors are improper handling or administration, the virus strain and excessive attenuation (Dhein and Gorham, 1986). A vaccination failure due to vaccine related factors can be ruled out, since the vaccine was stored cool until application and the parvovirus antigen was administered within the polyvalent vaccine with distemper and rabies, which showed a distinct rise in titres. The response in the animal population to vaccines follows a Gaussian distribution, where the majority of animals produce an average respond. A small fraction will produce an excellent response and another small fraction will produce a poor response, which therefore have no protection (Tizard, 2009).

Canine distemper virus titres experienced a significant rise after the vaccination in the probiotic group. The titres of the placebo group also increased after vaccination, but this was not found to be significant. Similar, the feeding of *E. faecium* SF68 led to higher CDV vaccine-specific IgG concentrations, although no change was observed in the amount of total IgG in plasma (Benyacoub et al., 2003).

Rabies titres rose significantly in both groups, but the probiotic group was more pronounced. This was confirmed, when summarising all titres and comparing the difference. Here, the

difference between before and after the vaccination was vastly greater in the probiotic group. Antibodies against rabies have the highest titre level at day 14 after the vaccination (Minke et al., 2009). This was also the time span used in the own study.
Since tetanus vaccination is not standard practice, no ELISA-kit is yet available for the dog. A tetanus IgG ELISA-kit for human diagnostic was combined with Sheep anti-Dog IgG-h-ch HRP conjugate. Since no positive control was available, comparisons were drawn using the first unvaccinated value. A possible positive control could have been created by pooling all sera taken after the booster vaccination (Roßkopf, 2007), and comparing the OD to this value. The constant rise of the relative ratio of tetanus titres was observed in all dogs. In some dogs even, the rise of titre from beginning on, until 2 weeks after the booster injection was strictly linear. This indicated that the varied ELISA-kit worked well for canine tetanus titres, but a difference between the groups was not recorded.
Few studies have been performed in animals using probiotics together with vaccinations. Pigs receiving *Bacillus cereus* var. *toyoi* had significant higher antibody titres against Influenza virus H1N1 after vaccination. Titres against H3N2 Influenza and *Mycoplasma hyopneumoniae* were also higher in the probiotic group (Schierack et al., 2007). On the other hand, oral administration of *L. casei* to pigs and a simultaneous vaccination against PRRS provided no higher titres (Kritas and Morrison, 2007).
More studies have been performed in human medicine. Children fed with a fermented infant formula had better responses to antipoliovirus IgA titres after vaccination (Mullie et al., 2004). Similar effects were found due to the application of *L. rhamnosus* and *L. acidophilus* strains, when orally vaccinated against poliovirus. Poliovirus neutralising antibody titres were higher, so were the formation of poliovirus specific IgG and IgA (de Vrese et al., 2005). A better response to an influenza vaccination caused the oral application of *L. fermentum.* A significant increase of antigen specific IgA was detected (Olivares et al., 2007). Probiotics also promoted the response to Haemophilus influenza type b (Hib) vaccination (Kukkonen et al., 2006).
Immersed studies need to be made on possible adjuvant effects of probiotics to dogs. Young puppies have the advantage to older dogs, that no or only one previous vaccination has taken place. Older dogs, which have continuously received annual vaccinations, may not show a distinct hoped for respond. An emphasis should not only lie on the post-vaccination titre, but also the possibility of sustaining high titre levels over a longer period of time, regarding that many vaccines are only repeated every 3 years.

6 Summary

Influence of the probiotic Enterococcus faecium DSM 7134 as a feed additive on digestive, microbiological and immunological traits in police, working and sled dogs

The study incorporated 3 different groups of dogs, all receiving the probiotic *Enterococcus faecium* DSM 7134. A cross-over study was conducted with police and Bundeswehr working dogs, each dog having a placebo and probiotic period. The probiotic was added to the daily ration at a concentration of $1*10^9$ cfu/kg of complete diet by owners or animal attendants. Parameters included faecal consistency, frequency of defecation (daily protocol recorded), pH and faecal dry matter, ammonium ion concentration, D- and L-lactic acid concentration, and selective faecal microorganisms, including coliform bacteria, *Clostridium* spp., *Lactobacillus* spp. and *Enterococcus* spp.. Species differentiation of enterococci was performed with RAPD-PCR analysis, using colonies from randomly picked faecal samples of the working dogs. Immunologic parameters, such as lymphocyte phenotyping, lymphocyte proliferation, serum IgA and IgG, also including a differential blood count, were examined in the police dogs.

In addition, a vaccination trial was conducted using a group of privately owned sled dogs, divided into a placebo and probiotic group. The probiotic group received in addition to their feed *Enterococcus faecium* DSM 7134 ($1*10^9$ cfu/kg of complete diet), by the owner. After accommodation, all dogs were vaccinated with a polyvalent vaccine against distemper, hepatitis contagiosa canis, kennel cough, parvovirosis, leptospirosis and rabies. An injection of tetanus toxoid was administered on the same day. Blood was collected on the day of vaccination and 14 days after, for titre determination and other immunologic parameters. An additional injection of tetanus toxoid followed 14 days after the first, where titres were again determined 14 days after. Immunologic parameters included lymphocyte phenotyping, lymphocyte proliferation, IgA, IgG, differential blood count and determination of CPV, CDV, rabies and tetanus titres.

Individual improvements of the faecal consistency were achieved, due to the probiotic. No significant changes were observed in the faecal bacteria concentration, although RAPD-PCR analysis clearly identified *Enterococcus faecium* DSM 7134, indicating the colonisation and possible amplification of the probiotic bacteria.

Lymphocyte proliferation was significantly higher in the probiotic group of the sled dogs, at 4 weeks after the first vaccination, confirmed also by the numerically higher values encountered during the probiotic phase of the police dogs.
The probiotic had distinct effects on the rabies titre. Similar potential effects were also seen with the CDV titre.
Companion animals may benefit from the application of probiotics. Changes in faecal consistency, persistence of *E. faecium* DSM 7134 and an immune stimulation are of importance from this perspective.
Further studies with *Enterococcus faecium* DSM 7134 need to be conducted. Young puppies during the time of first immunisation to investigate immune stimulation are of special interesst. Since no prior contacts to vaccine-antigens exist, the possibility is given that higher vaccination titres are yielded and the probiotic influence can therefore be better demonstrated under these conditions.

7 Zusammenfassung

Einfluss des Probiotikums Enterococcus faecium DSM 7134 als Futterzusatzstoff auf verdauungsphysiologische, mikrobiologische und immunologische Parameter bei Polizei-, Arbeits- und Schlittenhunden

Die Studie zum Einsatz von *Enterococcus faecium* DSM 7134 beinhaltete drei verschiedene Versuchsansätze mit jeweils unterschiedlichen Hundegruppen.

Eine Cross-over Studie wurde mit Hunden von der Polizei und Bundeswehr durchgeführt. Jeder Hund durchlief eine Placebo- und Probiotikum-Phase. Das Probiotikum wurde zusätzlich zur täglichen Ration mit einer Konzentration von $1*10^9$ KbE/kg Futter durch den Besitzer oder Tierpfleger zugefüttert.

Fäkale Konsistenz und Absatzfrequenz (täglich protokolliert), pH, Trockensubstanz, Ammoniumkonzentration, sowie D- und L-Laktatkonzentrationen wurden aus gesammelten Fäzes am Ende jeder Phase bestimmt. Die mikrobiologischen Untersuchungen beinhalteten coliforme Bakterien, Clostridien, Laktobazillen und Enterokokken. Die Enterokokken Speziesdifferenzierung wurde mittels RAPD-PCR von Kolonien der zufällig ausgewählten Kotproben der Bundeswehrhunde durchgeführt. Blutproben der Polizeihunde wurden für die Analyse der immunologischen Parameter gewonnen. Dazu gehörten die Lymphozytenphänotypisierung, Lymphozytenproliferation und die Bestimmung der Serumkonzentration von Immunglobulin A und G.

In einer zusätzlichen Impfstudie wurden Schlittenhunde ein Placebo oder das Probiotikum *Enterococcus faecium* DSM 7134 ($1*10^9$ KbE/kg Futter) vom Besitzer mit der Ration verabreicht. Nach einer Anpassungszeit wurden alle Hunde mit einer polyvalenten Vakzine (Staupe, Parvovirose, HCC, Zwingerhusten, Leptospirose und Tollwut) geimpft. Gleichzeitig erfolgte eine Verabreichung von Tetanustoxoid. Für die verschiedenen getesteten Parameter wurde Blut entnommen zu Beginn der Studie, am Tag der Impfung sowie 14 Tage danach. Tetanus wurde ein zweites Mal geimpft, 14 Tage nach der ersten Vakzinierung. Eine weitere Blutentnahme folgte 14 Tage später. Neben der Impftiterbestimmung von Parvovirose, Staupe, Tollwut und Tetanus beinhalteten die immunologischen Parameter die Lymphozytenphänotypisierung, Lymphozytenproliferation, Immunglobulin A und G im Serum sowie ein komplettes Blutbild.

Individuelle Verbesserungen der Fäzeskonsistenz konnten während der Probiotikumgabe festgestellt werden. Die Konzentration der fäkalen Bakterien zeigten keine signifikanten

Unterschiede zwischen den Versuchsphasen, obwohl die RAPD-PCR Analyse eindeutig *E. faecium* DSM 7134 identifizierte. Dies ist ein Hinweis auf das Überleben des Probiotikums während der Darmpassage. Eine temporäre Besiedelung und Vermehrung des Keims ist im Darm daher möglich.

Die Proliferation der Lymphozyten war signifikant höher in der Probiotikumgruppe bei den Schlitttenhunden vier Wochen nach der ersten Vakzinierung. Es lag auch eine numerische höhere Proliferation in der Probiotikumphase der Polizeihunde vor.

Eindeutige Effekte des Probiotikums wurden auch am Tollwuttiter bemerkt. Der Titer nach Impfung war deutlich höher in der Probiotikumgruppe. Ähnliche Resultate konnten in der Staupetiterbestimmung festgestellt werden.

Ein positiver gesundheitsfördernder Effekt wird als Indikation einer Verabreichung von Probiotika bei Begleittieren angesehen. Veränderungen der fäkalen Konsistenz, Anreicherung von *E. faecium* DSM 7134 im Darm und die mögliche Immunstimulation sind aus dieser Perspektive von Interesse.

Weitere Studien sind erforderlich, um diese Ergebnisse zu bestätigen. Junge Hunde vor der ersten Immunisierung wären zur Überprüfung der immunstimulierenden Eigenschaften von besonderem Interesse. Da kein früherer Kontakt zu Impf-Antigenen existiert, ist die Reaktion des Immunsystems auf Impfungen möglicherweise stärker und der Einfluss des Probiotikums könnte unter diesen Bedingungen besser dargestellt werden.

8 References

Alander, M.; Satokari, R.; Korpela, R.; Saxelin, M.; Vilpponen-Salmela, T.; Mattila-Sandholm, T.; von Wright, A. (1999):
Persistence of colonization of human colonic mucosa by a probiotic strain, Lactobacillus rhamnosus GG, after oral consumption.
Appl Environ Microbiol. 65(1), 351-354.

Apostolou, E.; Kirjavainen, P. V.; Saxelin, M.; Rautelin, H.; Valtonen, V.; Salminen, S. J.; Ouwehand, A. C. (2001):
Good adhesion properties of probiotics: a potential risk for bacteremia?
FEMS Immunol Med Microbiol. 31(1), 35-39.

Audisio, M. C.; Oliver, G.; Apella, M. C. (1999):
Antagonistic effect of Enterococcus faecium J96 against human and poultry pathogenic Salmonella spp.
J Food Prot. 62(7), 751-755.

Audisio, M. C.; Oliver, G.; Apella, M. C. (2000):
Protective Effect of Enterococcus faecium J96, a Potential Probiotic Strain, on Chicks Infected with Salmonella Pullorum.
J Food Prot. 63(10), 1333-1337.

Baillon, M. L.; Marshall-Jones, Z. V.; Butterwick, R. F. (2004):
Effects of probiotic Lactobacillus acidophilus strain DSM13241 in healthy adult dogs.
Am J Vet Res. 65(3), 338-343.

Barefoot, S. F.; Klaenhammer, T. R. (1984):
Purification and characterization of the Lactobacillus acidophilus bacteriocin lactacin B.
Antimicrob Agents Chemother. 26(3), 328-334.

Barlough, J. E.; Ackley, C. D.; George, J. W.; Levy, N.; Acevedo, R.; Moore, P. F.; Rideout, B. A.; Cooper, M. D.; Pedersen, N. C. (1991):
Acquired immune dysfunction in cats with experimentally induced feline immunodeficiency virus infection: comparison of short-term and long-term infections.
J Acquir Immune Defic Syndr. 4(3), 219-227.

Batt, R. M.; Barnes, A.; Rutgers, H. C.; Carter, S. D. (1991):
Relative IgA deficiency and small intestinal bacterial overgrowth in German shepherd dogs.
Res Vet Sci. 50(1), 106-111.

Beckmann, L.; Vahjen, W.; Simon, O. (2000):
Isolation of an 1,3-1,4-beta-glucan degrading Enterococcus faecium strain from the intestinal tract of chicken and partial characterization of its novel 1,3-1,4-beta-glucanase.
J Basic Microbiol. 40(5-6), 303-310.

Benyacoub, J.; Czarnecki-Maulden, G. L.; Cavadini, C.; Sauthier, T.; Anderson, R. E.; Schiffrin, E. J.; von der Weid, T. (2003):
Supplementation of food with Enterococcus faecium (SF68) stimulates immune functions in young dogs.
J Nutr. 133(4), 1158-1162.

Bernet, M. F.; Brassart, D.; Neeser, J. R.; Servin, A. L. (1993):
Adhesion of human bifidobacterial strains to cultured human intestinal epithelial cells and inhibition of enteropathogen-cell interactions.
Appl Environ Microbiol. 59(12), 4121-4128.

Biagi, G.; Cipollini, I.; Pompei, A.; Zaghini, G.; Matteuzzi, D. (2007):
Effect of a Lactobacillus animalis strain on composition and metabolism of the intestinal microflora in adult dogs.
Vet Microbiol, 124(1-2), 160-165.

Biourge, V.; Vallet, C.; Levesque, A.; Sergheraert, R.; Chevalier, S.; Roberton, J. L. (1998):
The use of probiotics in the diet of dogs.
J Nutr. 128(12 Suppl), 2730S-2732S.

Blount, D. G.; Pritchard, D. I.; Heaton, P. R. (2005):
Age-related alterations to immune parameters in Labrador retriever dogs.
Vet Immunol Immunopathol. 108(3-4), 399-407.

Bohmer, B. M.; Kramer, W.; Roth-Maier, D. A. (2006):
Dietary probiotic supplementation and resulting effects on performance, health status, and microbial characteristics of primiparous sows.
J Anim Physiol Anim Nutr (Berl). 90(7-8), 309-315.

Bomba, A.; Nemcova, R.; Mudronova, D.; Guba, P. (2002):
The possibilities of potentiating the efficacy of probiotics.
Trends Food Sci Tech. 13, 121-126.

Bujalance, C.; Moreno, E.; Jimenez-Valera, M.; Ruiz-Bravo, A. (2007):
A probiotic strain of Lactobacillus plantarum stimulates lymphocyte responses in immunologically intact and immunocompromised mice.
Int J Food Microbiol. 113(1), 28-34.

Byrne, K. M.; Kim, H. W.; Chew, B. P.; Reinhart, G. A.; Hayek, M. G. (2000):
A standardized gating technique for the generation of flow cytometry data for normal canine and normal feline blood lymphocytes.
Vet Immunol Immunopathol. 73(2), 167-182.

Carr, F. J.; Chill, D.; Maida, N. (2002):
The lactic acid bacteria: a literature survey.
Crit Rev Microbiol. 28(4), 281-370.

Chung, J. Y.; Sung, E. J.; Cho, C. G.; Seo, K. W.; Lee, J. S.; Bhang, D. H.; Lee, H. W.; Hwang, C. Y.; Lee, W. K.; Youn, H. Y.; Kim, C. J. (2009):
Effect of Recombinant Lactobacillus Expressing Canine GM-CSF on Immune Function in Dogs.
J Microbiol Biotechnol. 19(11), 1401-1407.

Coconnier, M. H.; Bernet, M. F.; Kerneis, S.; Chauviere, G.; Fourniat, J.; Servin, A. L. (1993):
Inhibition of adhesion of enteroinvasive pathogens to human intestinal Caco-2 cells by Lactobacillus acidophilus strain LB decreases bacterial invasion.
FEMS Microbiol Lett. 110(3), 299-305.

Collins, M. D.; Gibson, G. R. (1999):
Probiotics, prebiotics, and synbiotics: approaches for modulating the microbial ecology of the gut.
Am J Clin Nutr. 69(5), 1052S-1057S.

Culligan, E. P.; Hill, C.; Sleator, R. D. (2009):
Probiotics and gastrointestinal disease: successes, problems and future prospects.
Gut Pathog. 1(1), 19.

Day, M. J. (1996):
Inheritance of serum autoantibody, reduced serum IgA and autoimmune disease in a canine breeding colony.
Vet Immunol Immunopathol. 53(3-4), 207-219.

Day, M. J.; Penhale, W. J. (1988):
Serum immunoglobulin A concentrations in normal and diseased dogs.
Res Vet Sci. 45, 360-363.

De Luna, R.; Vuotto, M. L.; Ielpo, M. T. L.; Ambrosio, R.; Piantedosi, D.; Moscatiello, V.; Ciaramella, P.; Scalone, A.; Gradoni, L.; Mancino, D. (1999):
Early suppression of lymphocyteproliferative response in dogs with natural infections by Leishmania infantum.
Vet Immunol Immunopathol. 70, 95-103.

de Vrese, M.; Rautenberg, P.; Laue, C.; Koopmans, M.; Herremans, T.; Schrezenmeir, J. (2005):
Probiotic bacteria stimulate virus-specific neutralizing antibodies following a booster polio vaccination.
Eur J Nutr. 44(7), 406-413.

Dhein, C. R.; Gorham, J. R. (1986):
Host response to vaccination.
Vet Clin North Am Small Anim Pract. 16(6), 1227-1245.

Discherl, P.; Beisker, W.; Kremmer, E.; Mihalkov, A.; Voss, C.; Ziesenis, A. (1995):
Immunophenotyping of canine bronchoalveolar and peripheral blood lymphocytes.
Vet Immunol Immunopathol. 48, 1-10.

Domig, K. J.; Mayer, H. K.; Kneifel, W. (2003):
Methods used for the isolation, enumeration, characterisation and identification of Enterococcus spp. 2. Pheno- and genotypic criteria.
Int J Food Microbiol. 88(2-3), 165-188.

Douglas, L. C.; Sanders, M. E. (2008):
Probiotics and prebiotics in dietetics practice.
J Am Diet Assoc. 108(3), 510-521.

Du Toit, M.; Franz, C. M. A. P.; Dicks, L. M. T.; Holzapfel, W. H. (2000):
Preliminary characterization of bacteriocins produced by Enterococcus faecium and Enterococcus faecalis isolated from pig faeces.
J Appl Microbiology. 88, 482-494.

Erickson, K. L.; Hubbard, N. E. (2000):
Probiotic Immunomodulation in Health and Disease.
J Nutr. 130, 403S-409S.

Faldyna, M.; Leva, L.; Knotigova, P.; Toman, M. (2001):
Lymphocyte subsets in peripheral blood of dogs- a flow cytometric study.
Vet Immunol Immunopathol. 82(1-2), 23-37.

Faldyna, M.; Sinkora, J.; Knotigova, P.; Leva, L.; Toman, M. (2005):
Lymphatic organ development in dogs: major lymphocyte subsets and activity.
Vet Immunol Immunopathol. 104(3-4), 239-247.

Fisher, K.; Phillips, C. (2009):
The ecology, epidemiology and virulence of Enterococcus.
Microbiology. 155(Pt 6), 1749-1757.

Flachowsky, G.; Daenicke, R. (1996):
Probiotika in der Rinderfütterung.
Übers Tierernähr. 24, 62-68.

Fuller, R. (1989):
Probiotics in man and animals.
J Appl Bacteriol. 66(5), 365-378.

Galdeano, C. M.; Perdigon, G. (2004):
Role of viability of probiotic strains in their persistence in the gut and in mucosal immune stimulation.
J Appl Microbiol. 97(4), 673-681.

Galdeano, C. M.; Perdigon, G. (2006):
The probiotic bacterium Lactobacillus casei induces activation of the gut mucosal immune system through innate immunity.
Clin Vaccine Immunol. 13(2), 219-226.

Garcia, K. C.; Scott, C. A.; Brunmark, A.; Carbone, F. R.; Peterson, P. A.; Wilson, I. A.; Teyton, L. (1996):
CD8 enhances formation of stable T-cell receptor/MHC class I molecule complexes.
Nature. 384(6609), 577-581.

Gauthier, M. J.; Aubert, I.; Abrams-Ogg, A.; Woods, J. P.; Bienzle, D. (2005):
The immunophenotype of peripheral blood lymphocytes in clinically healthy dogs and dogs with lymphoma in remission.
J Vet Intern Med. 19(2), 193-199.

German, A. J.; Hall, E. J.; Day, M. J. (1998):
Measurement of IgG, IgM and IgA concentrations in canine serum, saliva, tears and bile.
Vet Immunol Immunopathol. 64(2), 107-121.

German, A. J.; Hall, E. J.; Day, M. J. (1999):
Analysis of leucocyte subsets in the canine intestine.
J Comp Pathol. 120(2), 129-145.

Glickman, L. T.; Shofer, F. S.; Payton, A. J.; Laster, L. L.; Felsburg, P. J. (1988):
Survey of serum IgA, IgG, and IgM concentrations in a large beagle population in which IgA deficiency had been identified.
Am J Vet Res. 49(8), 1240-1245.

Goldin, B. R. (1990):
Intestinal microflora: metabolism of drugs and carcinogens.
Ann Med. 22(1), 43-48.

Goldin, B. R.; Swenson, L.; Dwyer, J.; Sexton, M.; Gorbach, S. L. (1980):
Effect of diet and Lactobacillus acidophilus supplements on human fecal bacterial enzymes.
J Natl Cancer Inst. 64(2), 255-261.

Görke, B.; Liebler-Tenorio, E. (2001):
Probiotika: Gibt es wissenschaftliche Grundlagen für ihre Wirkung?
Dtsch Tierarztl Wochenschr. 108(6), 249-251.

Greeley, E. H.; Kealy, R. D.; Ballam, J. M.; Lawler, D. F.; Segre, M. (1996):
The influence of age on the canine immune system.
Vet Immunol Immunopathol. 55(1-3), 1-10.

Griot-Wenk, M. E.; Busato, A.; Welle, M.; Racine, B. P.; Weilenmann, R.; Tschudi, P.; Tipold, A. (1999):
Total serum IgE and IgA antibody levels in healthy dogs of different breeds and exposed to different environments.
Res Vet Sci. 67(3), 239-243.

Gupta, V.; Garg, R. (2009):
Probiotics.
Indian J Med Microbiol. 27(3), 202-209.

Hamann, L.; EL-Samalouti, V.; Ulmer, A. J.; Flad, H. D.; Rietschel, E. T. (1998):
Components of gut bacteria as immunomodulators.
Int J Food Microbiol. 41, 141-154.

Hart, I. R. (1979):
The distribution of immunoglobulin-containing cells in canine small intestine.
Res Vet Sci. 27(3), 269-274.

Hasegawa, T.; Shimazu, N.; Inomata, T.; Yamamoto, T.; Abe, S.; Yamaguchi, H. (1999):
Evaluation of risk and benefit of oral immunomodulation using heat-killed enterococcus faecalis FK-23 preparation in healthy dogs.
Int J Immunopathol Pharmacol. 12(2), 81-87.

Hayatsu, H.; Hayatsu, T. (1993):
Suppressing effect of Lactobacillus casei administration on the urinary mutagenicity arising from ingestion of fried ground beef in the human.
Cancer Lett. 73(2-3), 173-179.

Herstad, H. K.; Nesheim, B. B.; L'Abee-Lund, T.; Larsen, S.; Skancke, E. (2010):
Effects of a probiotic intervention in acute canine gastroenteritis--a controlled clinical trial.
J Small Anim Pract. 51(1), 34-38.

Hill, P. B.; Moriello, K. A.; DeBoer, D. J. (1995):
Concentrations of total serum IgE, IgA, and IgG in atopic and parasitized dogs.
Vet Immunol Immunopathol. 44(2), 105-113.

Hoffmann-Fezer, G.; Mortelbauer, W.; Hartmann, K.; Mysliwietz, J.; Thefeld, S.; Beer, B.; Thum, I.; Kraft, W. (1996):
Comparison of T-cell subpopulations in cats naturally infected with feline leukaemia virus or feline immunodeficiency virus.
Res Vet Sci. 61(3), 222-226.

HogenEsch, H.; Dunham, A. D.; Scott-Moncrieff, C.; Glickman, L. T.; DeBoer, D. J. (2002):
Effect of vaccination on serum concentrations of total and antigen-specific immunoglobulin E in dogs.
Am J Vet Res. 63(4), 611-616.

HogenEsch, H.; Thompson, S.; Dunham, A.; Ceddia, M.; Hayek, M. (2004):
Effect of age on immune parameters and the immune response of dogs to vaccines: a cross-sectional study.
Vet Immunol Immunopathol. 97(1-2), 77-85.

Horzinek, M. C. (2006):
Vaccine use and disease prevalence in dogs and cats.
Vet Microbiol. 117(1), 2-8.

Isolauri, E.; Sütas, Y.; Kankaanpää, P.; Arvilommi, H.; Salminen, S. (2001):
Probiotics: effects on immunity.
Am J Clin Nutr. 73(suppl), 444S-450S.

Janeway, C.; Murphy, K.; Travers, P.; Walport, M. (2008):
Janeway´s Immunobiology. 7th Ed. -
New York: Garland Science.

Jin, L. Z.; Marquardt, R. R.; Zhao, X. (2000):
A strain of Enterococcus faecium (18C23) inhibits adhesion of enterotoxigenic Escherichia coli K88 to porcine small intestine mucus.
Appl Environ Microbiol. 66(10), 4200-4204.

Kanasugi, H.; Hasegawa, T.; Goto, Y.; Ohtsuka, H.; Makimura, S.; Yamamoto, T. (1997):
Single administration of enterococcal preparation (FK-23) augments non-specific immune responses in healthy dogs.
Int J Immunopharmacol. 19(11-12), 655-659.

Kim, H. W.; Chew, B. P.; Wong, T. S.; Park, J. S.; Weng, B. B.; Byrne, K. M.; Hayek, M. G.; Reinhart, G. A. (2000):
Dietary lutein stimulates immune response in the canine.
Vet Immunol Immunopathol. 74(3-4), 315-327.

Kirjavainen, P. V.; El-Nezami, H. S.; Salminen, S. J.; Ahokas, J. T.; Wright, P. F. (1999):
The effect of orally administered viable probiotic and dairy lactobacilli on mouse lymphocyte proliferation.
FEMS Immunol Med Microbiol. 26(2), 131-135.

Koch, T.; Berg, R. (1990):
Lehrbuch der Veterinär-Anatomie. 4. Aufl.
Jena: Gustav Fischer Verlag.

Kolars, J. C.; Levitt, M. D.; Aouji, M.; Savaiano, D. A. (1984):
Yogurt- an autodigesting source of lactose.
N Engl J Med. 310(1), 1-3.

Kraft, W.; Dürr, M. (1999):
Klinische Labordiagnostik in der Tiermedizin.
Stuttgart: Schattauer Verlag.

Krakowka, S.; Ringler, S. S. (1986):
Activation specificity of commonly employed mitogens for canine B- and T-lymphocytes.
Vet Immunol Immunopathol. 11(3), 281-289.

Kravtsov, E. G.; Yermolayev, A. V.; Anokhina, I. V.; Yashina, N. V.; Chesnokova, V. L.; Dalin, M. V. (2008):
Adhesion characteristics of lactobacillus is a criterion of the probiotic choice.
Bull Exp Biol Med. 145(2), 232-234.

Kritas, S. K.; Morrison, R. B. (2007):
Effect of orally administered Lactobacillus casei on porcine reproductive and respiratory syndrome (PRRS) virus vaccination in pigs.
Vet Microbiol. 119(2-4), 248-255.

Kukkonen, K.; Nieminen, T.; Poussa, T.; Savilahti, E.; Kuitunen, M. (2006):
Effect of probiotics on vaccine antibody responses in infancy--a randomized placebo-controlled double-blind trial.
Pediatr Allergy Immunol. 17(6), 416-421.

Laukova, A.; Strompfova, V.; Ouwehand, A. (2004):
Adhesion properties of enterococci to intestinal mucus of different hosts.
Vet Res Commun. 28(8), 647-655.

Lee, Y.-K.; Salminen, S. (1995):
The coming age of probiotics.
Trends Food Sci Tech. 6, 241-245.

Lidbeck, A.; Nord, C. E.; Gustafsson, J. A.; Rafter, J. (1992):
Lactobacilli, anticarcinogenic activities and human intestinal microflora.
Eur J Cancer Prev. 1(5), 341-353.

Lijnen, P.; Saavedra, A.; Petrov, V. (1997):
In vitro proliferative response of human peripheral blood mononuclear cells to concanavalin A.
Clin Chim Acta. 264(1), 91-101.

Lilly, D. M.; Stillwell, R. H. (1965):
Probiotics: Growth-Promoting Factors Produced by Microorganisms.
Science. 147, 747-748.

Ling, W. H.; Korpela, R.; Mykkanen, H.; Salminen, S.; Hanninen, O. (1994):
Lactobacillus strain GG supplementation decreases colonic hydrolytic and reductive enzyme activities in healthy female adults.
J Nutr. 124(1), 18-23.

Littler, R. M.; Batt, R. M.; Lloyd, D. H. (2006):
Total and relative deficiency of gut mucosal IgA in German shepherd dogs demonstrated by faecal analysis.
Vet Rec. 158(10), 334-341.

Macha, M. (2007):
Auswirkungen eines Enterococcus faecium- Probiotikums auf Leistungsdaten und ausgewählte Parameter der intestinalen Mikrobiota beim Schwein.
Inaugural-Diss., Freie Universität Berlin.

Macias-Rodriguez, M. E.; Zagorec, M.; Ascencio, F.; Vazquez-Juarez, R.; Rojas, M. (2009):
Lactobacillus fermentum BCS87 expresses mucus- and mucin-binding proteins on the cell surface.
J Appl Microbiol. 107(6), 1866-1874.

Manninen, T. J.; Rinkinen, M. L.; Beasley, S. S.; Saris, P. E. (2006):
Alteration of the canine small-intestinal lactic acid bacterium microbiota by feeding of potential probiotics.
Appl Environ Microbiol. 72(10), 6539-6543.

Marcinakova, M.; Simonova, M.; Strompfova, V.; Laukova, A. (2006):
Oral application of Enterococcus faecium strain EE3 in healthy dogs.
Folia Microbiol (Praha). 51(3), 239-242.

Marco, M. L.; Pavan, S.; Kleerebezem, M. (2006):
Towards understanding molecular modes of probiotic action.
Curr Opin Biotechnol. 17(2), 204-210.

Marekova, M.; Laukova, A.; DeVuyst, L.; Skaugen, M.; Nes, I. F. (2003):
Partial characterization of bacteriocins produced by environmental strain Enterococcus faecium EK13.
J Appl Microbiol. 94, 523-530.

Marekova, M.; Laukova, A.; Skaugen, M.; Nes, I. (2007):
Isolation and characterization of a new bacteriocin, termed enterocin M, produced by environmental isolate Enterococcus faecium AL41.
J Ind Microbiol Biotechnol. 34(8), 533-537.

Marteau, P.; Seksik, P.; Jian, R. (2002):
Probiotics and intestinal health effects: a clinical perspective.
Br J Nutr. 88 Suppl 1, S51-57.

Martineau, B.; Laflamme, D. P. (2002):
Effect of diet on markers of intestinal health in dogs.
Res Vet Sci. 72(3), 223-227.

Matar, C.; Valdez, J. C.; Medina, M.; Rachid, M.; Perdigon, G. (2001):
Immunomodulating effects of milk fermented by Lactobacillus helveticus and its non-proteolytic variant.
J Dairy Res. 68(4), 601-609.

Meydani, S. N.; Ha, W. K. (2000):
Immunologic effect of yogurt.
Am J Clin Nutr. 71, 861-872.

Meyer, H.; Zentek, J. (2005):
Ernährung des Hundes. 5. Aufl.
Stuttgart: Parey Verlag.

Meyer, H.; Zentek, J.; Habernoll, H.; Maskell, I. (1999):
Digestibility and Compatibility of Mixed Diets and Faecal Consistency in Different Breeds of Dogs.
J. Vet. Med. A. 46, 155-165.

Minke, J. M.; Bouvet, J.; Cliquet, F.; Wasniewski, M.; Guiot, A. L.; Lemaitre, L.; Cariou, C.; Cozette, V.; Vergne, L.; Guigal, P. M. (2009):
Comparison of antibody responses after vaccination with two inactivated rabies vaccines.
Vet Microbiol. 133(3), 283-286.

Molitor, D. (1996):
In vitro- und in vivo- Effekte eines Probiotikums (Enterococcus faecium) als Futterzusatz bei Hunden.
Inaugural-Diss., Tierärztliche Hochschule Hannover.

Moore, P. F.; Rossitto, P. V.; Danilenko, D. M.; Wielenga, J. J.; Raff, R. F.; Severns, E. (1992):
Monoclonal antibodies specific for canine CD4 and CD8 define functional T-lymphocyte subsets and high-density expression of CD4 by canine neutrophils.
Tissue Antigens. 40(2), 75-85.

Mostov, K. E. (1994):
Transepithelial transport of immunoglobulins.
Annu Rev Immunol. 12, 63-84.

Mück, S. (2007):
Einsatz von Enterococcus faecium DSM 7134 beim adulten Hund.
Inaugural-Diss., Veterinärmedizinische Universität Wien.

Mullie, C.; Yazourh, A.; Thibault, H.; Odou, M. F.; Singer, E.; Kalach, N.; Kremp, O.; Romond, M. B. (2004):
Increased poliovirus-specific intestinal antibody response coincides with promotion of Bifidobacterium longum-infantis and Bifidobacterium breve in infants: a randomized, double-blind, placebo-controlled trial.
Pediatr Res. 56(5), 791-795.

Naclerio, G.; Ricca, E.; Sacco, M.; De Felice, M. (1993):
Antimicrobial activity of a newly identified bacteriocin of Bacillus cereus.
Appl Environ Microbiol. 59(12), 4313-4316.

Nagler-Anderson, C. (2001):
Man the barrier! Strategic defences in the intestinal mucosa.
Nat Rev Immunol. 1(1), 59-67.

Nurmi, E.; Nuotio, L.; Schneitz, C. (1992):
The competitive exclusion concept: development and future.
Int J Food Microbiol. 15, 237-240.

O'Neill, K.; Guth, A.; Biller, B.; Elmslie, R.; Dow, S. (2009):
Changes in regulatory T cells in dogs with cancer and associations with tumor type.
J Vet Intern Med. 23(4), 875-881.

Oelschlaeger, T. A. (2010):
Mechanisms of probiotic actions - A review.
Int J Med Microbiol. 300(1), 57-62.

Olivares, M.; Diaz-Ropero, M. P.; Sierra, S.; Lara-Villoslada, F.; Fonolla, J.; Navas, M.; Rodriguez, J. M.; Xaus, J. (2007):
Oral intake of Lactobacillus fermentum CECT5716 enhances the effects of influenza vaccination.
Nutrition. 23(3), 254-260.

Ostad, S. N.; Salarian, A. A.; Ghahramani, M. H.; Fazeli, M. R.; Samadi, N.; Jamalifar, H. (2009):
Live and heat-inactivated lactobacilli from feces inhibit Salmonella typhi and Escherichia coli adherence to Caco-2 cells.
Folia Microbiol (Praha). 54(2), 157-160.

Ouwehand, A.; Isolauri, E.; Salminen, S. (2002):
The role of the intestinal microflora for the development of the immune system in early childhood.
Eur J Nutr. 41 Suppl 1, I32-37.

Ouwehand, A. C.; Kirjavainen, P. V.; Shortt, C.; Salminen, S. (1999):
Probiotics: mechanisms and established effects.
Int Dairy J. 9, 43-52.

Park, J. H.; Lee, Y.; Moon, E.; Seok, S. H.; Cho, S. A.; Baek, M. W.; Lee, H. Y.; Kim, D. J. (2005):
Immunoenhancing effects of a new probiotic strain, Lactobacillus fermentum PL9005.
J Food Prot. 68(3), 571-576.

Pascher, M. (2004):
Effecte eines probiotischen Laktobazillus-Stammes (Lactobacillus acidophilus) auf verdauungsphysiologische Parameter von Hunden.
Inaugral-Diss., Veterinärmedizinische Universität Wien.

Pascher, M.; Hellweg, P.; Khol-Parisini, A.; Zentek, J. (2008):
Effects of a probiotic Lactobacillus acidophilus strain on feed tolerance in dogs with non-specific dietary sensitivity.
Arch Anim Nutr. 62(2), 107-116.

Paul, M. A.; Carmichael, L. E.; Childers, H.; Cotter, S.; Davidson, A.; Ford, R.; Hurley, K. F.; Roth, J. A.; Schultz, R. D.; Thacker, E.; Welborn, L. (2006):
AAHA Canine Vaccine Guidelines.

Perdigon, G.; Locascio, M.; Medici, M.; De Ruiz Holgado, A. P.; Oliver, G. (2003):
Interaction of bifidobacteria with the gut and their influence in the immune function.
Biocell. 27(1), 1-9.

Phillips, T. R.; Jensen, J. L.; Rubino, M. J.; Yang, W. C.; Schultz, R. D. (1989):
Effects of vaccines on the canine immune system.
Can J Vet Res. 53(2), 154-160.

Phillips, T. R.; Schultz, R. D. (1987):
Failure of vaccine or virulent strains of canine parvovirus to induce immunosuppressive effects on the immune system of the dog.
Viral Immunol. 1(2), 135-144.

Playfair, J. H.; Baron, D. (1995):
Immunologie.
Blackwell Wissenschafts-Verlag.

Rescigno, M.; Urbano, M.; Valzasina, B.; Francolini, M.; Rotta, G.; Bonasio, R.; Granucci, F.; Kraehenbuhl, J. P.; Ricciardi-Castagnoli, P. (2001):
Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria.
Nat Immunol. 2(4), 361-367.

Rinkinen, M.; Jalava, K.; Westermarck, E.; Salminen, S.; Ouwehand, A. C. (2003a):
Interaction between probiotic lactic acid bacteria and canine enteric pathogens: a risk factor for intestinal Enterococcus faecium colonization?
Vet Microbiol. 92(1-2), 111-119.

Rinkinen, M.; Mättö, J.; Salminen, S.; Westermarck, E.; Ouwehand, A. C. (2000):
In vitro adhesion of lactic acid bacteria to canine small intestinal mucus.
J Anim Physiol Anim Nutr. 84, 43-47.

Rinkinen, M.; Westermarck, E.; Salminen, S.; Ouwehand, A. C. (2003b):
Absence of host specificity for in vitro adhesion of probiotic lactic acid bacteria to intestinal mucus.
Vet Microbiol. 97(1-2), 55-61.

Rolfe, R. D. (2000):
The role of probiotic cultures in the control of gastrointestinal health.
J Nutr. 130(2S Suppl), 396S-402S.

Rolfe, V. E.; Adams, C. A.; Butterwick, R. E.; Batt, R. M. (2002a):
Relationships between fecal consistency and colonic microstructure and absorptive function in dogs with and without nonspecific dietary sensitivity.
Am J Vet Res. 63(4), 617-622.

Rolfe, V. E.; Adams, C. A.; Butterwick, R. F.; Batt, R. M. (2002b):
Relationship between faecal character and intestinal transit time in normal dogs and diet-sensitive dogs.
J Small Anim Pract. 43(7), 290-294.

Roßkopf, U. (2007):
Validierung der Wirksamkeitsprüfung für Clostridium tetani Impfstoffe ad usum veterinarium durch den direkten Nachweis von Tetanus-Antitoxin im Zieltier mittels ELISA.
Inaugural Diss., Justus-Liebig-Universität, Gießen.

Saavedra, J. M. (2001):
Clinical applications of probiotic agents.
Am J Clin Nutr. 73(6), 1147S-1151S.

Salminen, S.; Ouwehand, A. C.; Isolauri, E. (1998):
Clinical Applications of Probiotic Bacteria.
Int Dairy J. 8, 563-572.

Sauter, S. N.; Benyacoub, J.; Allenspach, K.; Gaschen, F.; Ontsouka, E.; Reuteler, G.; Cavadini, C.; Knorr, R.; Blum, J. W. (2006):
Effects of probiotic bacteria in dogs with food responsive diarrhoea treated with an elimination diet.
J Anim Physiol Anim Nutr (Berl). 90(7-8), 269-277.

Schierack, P.; Wieler, L. H.; Taras, D.; Herwig, V.; Tachu, B.; Hlinak, A.; Schmidt, M. F.; Scharek, L. (2007):
Bacillus cereus var. toyoi enhanced systemic immune response in piglets.
Vet Immunol Immunopathol. 118(1-2), 1-11.

Schiffrin, E. J.; Brassart, D.; Servin, A. L.; Rochat, F.; Donnet-Hughes, A. (1997):
Immune modulation of blood leukocytes in humans by lactic acid bacteria: criteria for strain selection.
Am J Clin Nutr. 66(2), 515S-520S.

Schrezenmeir, J.; Vrese, M. d. (2001):
Probiotics, prebiotics, and synbiotics- approaching a definition.
Am J Clin Nutr. 73 (suppl), 361S-364S.

Shankar, V.; Baghdayan, A. S.; Huycke, M. M.; Lindahl, G.; Gilmore, M. S. (1999):
Infection-derived Enterococcus faecalis strains are enriched in esp, a gene encoding a novel surface protein.
Infect Immun. 67, 193-200.

Simon, O.; Jadamus, A.; Vahjen, W. (2001):
Probiotic feed additives - effectiveness and expected modes of action.
J Anim Feed Sci. 10, 51-67.

Simpson, J. M.; Martineau, B.; Jones, W. E.; Ballam, J. M.; Mackie, R. I. (2002):
Characterization of fecal bacterial populations in canines: effects of age, breed and dietary fiber.
Microb Ecol. 44(2), 186-197.

Stokes, C.; Waly, N. (2006):
Mucosal defence along the gastrointestinal tract of cats and dogs.
Vet Res. 37, 281-293.

Strasser, A.; May, B.; Teltscher, A.; Wistrela, E.; Niedermuller, H. (2003):
Immune modulation following immunization with polyvalent vaccines in dogs.
Vet Immunol Immunopathol. 94(3-4), 113-121.

Strompfova, V.; Laukova, A.; Ouwehand, A. C. (2004):
Selection of enterococci for potential canine probiotic additives.
Vet Microbiol. 100(1-2), 107-114.

Strompfova, V.; Marcinakova, M.; Simonova, M.; Bogovic-Matijasic, B.; Laukova, A. (2006):
Application of potential probiotic Lactobacillus fermentum AD1 strain in healthy dogs.
Anaerobe. 12(2), 75-79.

Suter, P. F.; Kohn, B. (2006):
Praktikum der Hundeklinik. 10. Aufl.
Stuttgart: Parey MVS Medizinverlage.

Swanson, K. S.; Grieshop, C. M.; Flickinger, E. A.; Bauer, L. L.; Chow, J.; Wolf, B. W.; Garleb, K. A.; Fahey, G. C., Jr. (2002):
Fructooligosaccharides and Lactobacillus acidophilus modify gut microbial populations, total tract nutrient digestibilities and fecal protein catabolite concentrations in healthy adult dogs.
J Nutr. 132(12), 3721-3731.

Talarico, T. L.; Casas, I. A.; Chung, T. C.; Dobrogosz, W. J. (1988):
Production and isolation of reuterin, a growth inhibitor produced by Lactobacillus reuteri.
Antimicrob Agents Chemother. 32(12), 1854-1858.

Tannock, G. W. (1998):
Studies of the Intestinal Microflora: A Prerequisite for the Development of Probiotics.
Int Dairy J. 8, 527-533.

Tarrant, J. M. (2005):
The role of flow cytometry in companion animal diagnostic medicine.
Vet J. 170(3), 278-288.

Teitelbaum, J. E.; Walker, W. A. (2002):
Nutritional impact of pre- and probiotics as protective gastrointestinal organisms.
Annu Rev Nutr. 22, 107-138.

Tizard, I. (2009):
Veterinary Immunology: An Introduction. 8th ed.
Philadelphia: Saunders.

Toman, M.; Faldyna, M.; Knotigova, P.; Pokorova, D.; Sinkora, J. (2002):
Postnatal development of leukocyte subset composition and activity in dogs.
Vet Immunol Immunopathol. 87(3-4), 321-326.

Torten, M.; Franchini, M.; Barlough, J. E.; George, J. W.; Mozes, E.; Lutz, H.; Pedersen, N. C. (1991):
Progressive immune dysfunction in cats experimentally infected with feline immunodeficiency virus.
J Virol. 65(5), 2225-2230.

Tress, U.; Suchodolski, J. S.; Williams, D. A.; Steiner, J. M. (2006):
Development of a fecal sample collection strategy for extraction and quantification of fecal immunoglobulin A in dogs.
Am J Vet Res. 67(10), 1756-1759.

Vaerman, J. P.; Heremans, J. F. (1970):
Origin and molecular size of immunoglobulin-A in the mesenteric lymph of the dog.
Immunology. 18(1), 27-38.

Vahjen, W.; Jadamus, A.; Simon, O. (2002):
Influence of a probiotic Enterococcus faecium strain on selected bacterial groups in the small intestine of growing turkey poults.
Arch Tierernähr. 56(6), 419-429.

Vahjen, W.; Männer, K. (2003):
The effect of a probiotic Enterococcus faecium product in diets of healthy dogs on bacteriological counts of Salmonella spp., Campylobacter spp. and Clostridium spp. in faeces.
Arch Tierernähr. 57(3), 229-233.

Valli, J. L.; Williamson, A.; Sharif, S.; Rice, J.; Shewen, P. E. (2009):
In vitro cytokine responses of peripheral blood mononuclear cells from healthy dogs to distemper virus, Malassezia and Toxocara.
Vet Immunol Immunopathol. 134(3-4), 218-229.

Vanbelle, M.; Teller, E.; Focant, M. (1990):
Probiotics in animal nutrition: a review.
Arch Tierernähr. 40(7), 543-567.

Veir, J.; Knorr, R.; Cavadini, C.; Sherill, S. J.; Benyacoub, J.; Satyaraj, E.; Lappin, M. R. (2007):
Effect of Supplementation with Enterococcus faecium (SF68) on Immune Functions in Cats.
Vet Ther. 8(4), 229-238.

Vesa, T. H.; Marteau, P.; Zidi, S.; Briet, F.; Pochart, P.; Rambaud, J. C. (1996):
Digestion and tolerance of lactose from yoghurt and different semi-solid fermented dairy products containing Lactobacillus acidophilus and bifidobacteria in lactose maldigesters--is bacterial lactase important?
Eur J Clin Nutr. 50(11), 730-733.

Vincent, J. G.; Veomett, R. C.; Riley, R. F. (1959):
Antibacterial activity associated with Lactobacillus acidophilus.
J Bacteriol. 78, 477-484.

Vinderola, G.; Matar, C.; Perdigon, G. (2005):
Role of Intestinal epithelial Cells in Immune Effects Mediated by Gram-Positive Probiotic Bacteria: Involvement of Toll-Like Receptors.
Clin Diagn Lab Immunol. 12(9), 1075-1084.

Vinderola, G.; Matar, C.; Perdigon, G. (2007):
Milk fermented products of L. helveticus R389 activate calcineurin as a signal to promote gut mucosal immunity.
BMC Immunol. 8, 19.

Wagner, U.; Burkhardt, E.; Failing, K. (1999):
Evaluation of canine lymphocyte proliferation: comparison of three different colorimetric methods with the 3H-thymidine incorporation assay.
Vet Immunol Immunopathol. 70(3-4), 151-159.

Weese, J. S.; Anderson, M. E. (2002):
Preliminary evaluation of Lactobacillus rhamnosus strain GG, a potential probiotic in dogs.
Can Vet J. 43(10), 771-774.

Weiß, M. (2003):
Wirkung von Enterococcus faecium auf den Organismus neonataler Hundewelpen.
Inaugural-Diss., Ludwig-Maximillian-Universität München.

Werdeling, F.; Amtsberg, G.; Tewes, S. (1991):
Zum Vorkommen enterotoxinbildender Clostridium-perfringens-Stämme im Kot von Hunden und Katzen.
Berl Münch Tierärztl Wochenschr. 104(7), 228-233.

Whitbread, T. J.; Batt, R. M.; Garthwaite, G. (1984):
Relative deficiency of serum IgA in the german shepherd dog: a breed abnormality.
Res Vet Sci. 37(3), 350-352.

Williams, D. L. (1997):
Studies of Canine Leukocyte Antigens: A Significant Advance in Canine Immunology.
Vet J. 153, 31-39.

Wohlgemuth, S.; Loh, G.; Blaut, M. (2010):
Recent developments and perspectives in the investigation of probiotic effects.
Int J Med Microbiol. 300(1), 3-10.

Xu, H.; Jeong, H. S.; Lee, H. Y.; Ahn, J. (2009):
Assessment of cell surface properties and adhesion potential of selected probiotic strains.
Lett Appl Microbiol. 49(4), 434-442.

Zentek, J.; Marquart, B.; Pietrzak, T.; Ballevre, O.; Rochat, F. (2003):
Dietary effects on bifidobacteria and Clostridium perfringens in the canine intestinal tract.
J Anim Physiol Anim Nutr (Berl). 87(11-12), 397-407.

Zentek, J.; Meyer, H. (1993):
Verdaulichkeit und fäkale Wasserausscheidung- ein vergleich zwischen Doggen und Beagles.
Kleintierpraxis. 38, 311-318.

Zentek, J.; Molitor, D.; Kamphues, J. (1998):
Prüfung intestinaler Effekte eines Probiotikums (Enterococcus faecium) bei Hunden.
Kleintierpraxis. 43, 187-197.

Zeyner, A.; Boldt, E. (2006):
Effects of a probiotic Enterococcus faecium strain supplemented from birth to weaning on diarrhoea patterns and performance of piglets.
J Anim Physiol Anim Nutr (Berl). 90(1-2), 25-31.

Zimmermann, B.; Bauer, E.; Mosenthin, R. (2001):
Pro- and prebiotics in pig nutrition- potential modulators of gut health?
J Anim Feed Sci. 10, 47-56.

9 Publication

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11 Declaration of authorship

I hereby confirm that the present work was solely composed by my own. I certify that I have used only the specified sources and aids.

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfe in Anspruch genommen habe.

Berlin, 15.06.2010

Linda Koiou