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Efficiency of Probiotics in Farm Animals

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1. Introduction

The first concept of probiotics was originally developed by [38]. He suggested that ingested bacteria could have a positive influence on the normal microbial flora of the intestinal tract. Probiotics are considered as growth and health stimulators and are used extensively in animal feeding, especially in pig and poultry production.

Probiotics have been defined also by [6] as “*a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance*”. There is a relatively large volume of literature that supports the use of probiotics to prevent or treat intestinal disorders. Currently, the best studied probiotics are the lactic acid bacteria, particularly *Lactobacillus sp* and *Bifidobacterium sp*.

Therefore, an intensive research work is carrying out in this topic from many researcher groups in different countries. Many years later, probiotics were determined as: viable microbial feed supplements, which are believed to stimulate growth and the health as well as to modify the ecology of the intestine in a beneficial manner for the host [3], [34], [54]. Probiotics should lead to beneficial effects for the host animal due to an improvement of the intestinal microbial balance [12] or of the properties of the indigenous micro-flora [21]. There are also many mechanisms by probiotics enhance intestinal health, including stimulation of immunity, competition for limited nutrients, inhibition of epithelial and mucosal adherence, inhibition of epithelial invasion and production of antimicrobial substances [47].

Possible modes of actions are the modification of the intestinal microorganisms and the nutrient availability with response to the morphology and histology as well as the transport physiology. Significant positive effects of probiotics on performance, health, vitality, gut ecology as well digestibility are observed in many studies, although the mode of action of probiotics is not still completely explained [24], [55], [25], [4]. Efficiency probiotic on a focus of combined preparation have hardly been concluded.

2. Efficiency of probiotic in farm animals

The claims made for probiotics are many and varied but it is not always possible to provide good scientific evidence to support them. However the potential benefits that can arise from applications of the probiotic concept are shown as below:

Potential beneficial effects of probiotics for farm animals by [13].

- Greater resistance to infectious diseases
- Increased growth rate
- Improved feed conversion.
- Improved digestion.
- Better absorption of nutrients
- Provision of essential nutrients
- Improved milk yield
- Improved milk quality.
- Increased egg production.
- Improved egg quality
- Improved carcass quality and less contamination

Since probiotics are discussed as alternatives to antimicrobial growth promoters their impact on performance of farm animals is of prime interest. For authorization of microorganisms as feed additives it is also required to show significant effects on performance data [54]. By far most experiments were performed with piglets. According to a literature review by [61] no significant positive effects could be found from the hitherto results with piglets and fattening pigs. Later, the evaluation of studies conducted with raising piglets drew a different picture [11]. [61] was used the strict criteria of biostatistics and only significant effects were documented. Today, trends without statistical significance are also considered as positive effect by [54]. It is obvious that majority of the experiments show trends toward positive effects, however the significance level of $p \leq 0,05$ was reached only in 5% of experiments. Due to the complexity of the intestine, individual variations of animals to probiotic inclusion may be the rule and not the exception. Considering this concept, the range between no effect and significant effects seem to be reasonable.

In a trial with 90 treated and 90 untreated *Bacillus cereus* –preparation weaned piglets; the probiotic treated animals gained 7% more live weight during 6 weeks after weaning with a reduced feed conversion ratio of 2.4%. Both results were not significant [25]. This point towards a high variation in the response of the individual animals to this type of feed additives [54].

With regard to the evaluation of animal performance, the same conclusion can be draw for experiments with fattening chicken carried out by [53]. This is also reflected by a series of experiments with turkey, poultry under field conditions using three probiotics [34]. Again none of the effects in performance were significant, on average weight gain was improved by 1,5% (+0,1 to + 3,8) and feed conversion by -2% (-7 to -3,5). A further observation was a

more pronounced effect of additive during weeks 1 to 5. However again no significance was seen in the period's week 1 plus 2 and 3 to 5, respectively [54].

Authors in [54] concluded that the inconsistency of the effectiveness of a feed additive is of course not convenient, but on the other hand comprehensible for this type of feed additive. Probiotic do not act like essential nutrients in term of a clear dose response until the requirements are met. Due to the complexity of intestine, individual variations of animals to probiotic inclusion may be the rule and not the exception. Considering this concept the range between no effect and significant effects seem to be reasonable.

3. Mode of action of probiotics

The development of probiotics for farm animals is based on the knowledge that the gut microflora is involved in resistance to disease. The gut microflora has been shown to be involved in protection against a variety of pathogens including *Escherichia coli*, *Salmonella Camylobacter*, *Clostridium* and *Rotavirus*. Hence the probiotic approach may be effective in the prevention and therapy of these infections. No attempt will be made to summarize the evidence available for all of these effects [13].

The one area where it is possible to arrive at some scientifically based conclusions is the effect that the probiotics preparations have on resistance to infections.

The stressful conditions experienced by the young animal causes changes in the composition and/or activity of the gut microflora. Probiotic supplementation seeks to repair these deficiencies and provide the type of microflora which exists in feral animals uninfluenced by modern farm rearing methods. The products available are of varying composition and efficacy but the concept is scientifically-based and intellectually sound. Under the right conditions the claims made for probiotic preparations can be realized [13].

Molecule	Defense function	References
Lysozyme	Lyses bacterial cell walls	[2], [46]
Defensins	Form pores in bacterial cell wall	[2], [42]
Mucus	Prevents bacterial adhesion made by goblet cells, a specialized epithelial cell type.	[41]
MHC class I	Presents antigen to cytotoxic T-lymphocytes	[14]
MHC Class II	Presents antigen to helper T-lymphocytes	[14]

Table 1. Defense functions of epithelial cells [37].

There are many proposed mechanisms by which probiotics may protect the host from intestinal disorders. The sum of all processes by which bacteria inhibit colonization by other strains is called colonization resistance. Much work remains to classify the mechanisms of action of particular probiotics against particular pathogens. In addition, the same probiotic

may inhibit different pathogens by different mechanisms. Listed below is a brief description of mechanisms by which probiotics may protect the host against intestinal disease.

Possible mode of action of intestinal bacteria can be summarized as follows by [54]:

- Increase of desired intestinal bacteria;
- Competitive adhesion to epithelial receptors;
- Production of specific substances (bacteriocins, dipicolinic acid, bioactive peptides)
- Competition for nutrients between probiotic and undesired bacteria;
- Micro-environmental pH reduction by production of acid;
- Reduction of bacterial bile salt deconjugation;
- Passive aggregation of probiotics and pathogenic bacteria;

4. Production of inhibitory substances

Probiotic bacteria can produce a variety of substances that are inhibitory to both gram-positive and gram-negative bacteria. These inhibitory substances include organic acids, hydrogen peroxide and bacteriocins. These compounds may reduce not only the number of viable cells but may also affect bacterial metabolism or toxin production.

5. Blocking of adhesion sites

Competitive inhibition for bacterial adhesion sites on intestinal epithelial surfaces is another mechanism of action for probiotics [18]. Consequently, some probiotic strains have been chosen for their ability to adhere to epithelial cells. Gut bacteria prevent intestinal colonization by pathogenic organisms directly by competing more successfully for essential nutrients or for epithelial attachment sites [48].

6. Competition for nutrients

Competition for nutrients has been proposed as a mechanism of probiotics. Probiotics may utilize nutrients otherwise consumed by pathogenic microorganisms. However, the evidence that this occurs in vivo is lacking.

7. Degradation of toxin receptor

The postulated mechanism by which *Saccharomyces boulardii* protects animals against *C. difficile* intestinal disease is through degradation of the toxin receptor on the intestinal mucosa [5].

8. Influence on the immune system

The intestinal micro flora is an important component of host animal. A critical review of the literature indicates that probiotic supplementation of the intestinal micro flora may enhance defense, primarily by preventing colonization by pathogens and by indirect, adjuvant-like

stimulation of innate and acquired immune functions [37]. The role of nonpathogenic bacteria in the development of the intestinal immune system and in protecting the host from pathogenic challenges has been studied.

Intestinal bacteria provide the host with several nutrients, including short-chain fatty acids, vitamin K, some B vitamins and amino acids [49], [67]. Intestinal bacteria also protect the host from pathogens, forming a front line of mucosal defense. The indigenous microflora induces recruitment of lamina propria immune cells, which form a second tier of defense by activation of appropriate inflammatory or immune mechanisms during infection.

Recent evidence suggests that stimulation of specific and nonspecific immunity may be another mechanism by which probiotics can protect against intestinal disease [45]. For example, per oral administration of *Lactobacillus* GG during acute rotavirus diarrhoea is associated with an enhanced immune response to rotavirus [26]. This may account for the shortened course of diarrhoea seen in treated patients. The underlying mechanisms of immune stimulation are not well understood, but specific cell wall components or cell layers may act as adjuvant and increase humoral immune responses.

Reduction of diarrhea by probiotics was studied frequently, because diarrhea is the main problem of piglets during the first weeks after weaning with utmost importance for production [54].

Probiotic	Age	Incidence of diarrhoea	Statistical significance	Literature
<i>B. cereus</i>	8 weeks	Reduced	+	[29]
<i>B. cereus</i>	Day 1-85	Reduced	+	[22]
<i>B. cereus</i>	Day 7-21	Reduced	+	[68]
<i>B. cereus</i>	Day 24-66	No effect	-	[10]
<i>B. cereus</i>	25 kg Live weigh	No effect	-	[27]
<i>B. cereus</i>	2 weeks post weaning	Reduced	+	[23]
<i>E. faecium</i>	Day 1-70	Reduced	+	[35]
<i>E. faecium</i>	8 Days before/after weaning	Reduced	+	[51]
<i>P. acidilactici</i>	Day 5-28	Reduced	+	[9]
<i>P. acidilactici</i>	Day 5-28	Reduced	+	[9]
<i>S. cerevisiae</i>	Day 5-28	Reduced	+	[9]

Table 2. Incidence of diarrhoea in piglets fed probiotic supplemented feed (Effects compared to control animals) [54].

The mucosal surface of the intestinal tract represents the largest interface between the body and its environment. An effective local immune is necessary to protect the organism against the invasion of noxious antigens and microbes [54]. No other organ of the body harbours more immune cells than the gut –associated lymphoid tissue (GALT), and a tremendous amount of antibodies is secreted into the intestinal lumen to neutralize and exclude harmful antigens. In numerous studies it has been shown that bacterial colonization influences the

function of immune cells belonging to the GALT and even affects the systemic immune system [60].

Immune suppression has been observed after associating germfree rodents with defined bacterial species [69], [50]. In some studies the inductions of immune suppressive cytokines have been implicated in the so-called “by stander suppression” [7]. Moreover, it has been shown that bacterial colonization contributes to the induction and maintenance of immunological tolerance against nutritional antigens [39]. The mechanisms underlying oral tolerance are largely unknown by [54].

The numerous studies have reported immune stimulating abilities for different bacterial species. For example, *in vitro* cytokine production of macrophages was stimulated by *Bifidobacteria* [36]. *Bifidobacterium longum* as well as several other lactic acid bacteria have been found to increase the total amount of intestinal IgA [57], [65]. *Lactobacillus casei* was reported to have immune adjuvant activity by [43] and *Lactobacillus plantarum* was shown to increase antibody production against *Escherichia coli*. Induction of cytokine profiles by lactobacilli is likely to be strain-dependent [31] and it probably also depends on the host examined, since the autochthonous flora varies between different host species. Most of the animal studies with such probiotic micro organisms have been carried out in rodents with lactic acid bacteria with the goal of designing “functional food” for human consumption. Such studies however, are not necessarily suitable or transferable for the supplementation of animal feed in industrial settings [54]. Studies using swine as model system are few but, seem to be promising.

Probiotic treatment using *Bifidobacterium lactis* HN019 reduced weanling diarrhea associated with rotavirus and *Escherichia coli* infection in a piglet model [52]. Information from studies is also available about the age-dependent development of different immune cells in the intestine of the newborn and adult pigs [62], [55], [56]. Studies on these cells require large amounts of intestinal tissue that can hardly be taken from rodents. The composition of the different immune cells in the GALT is drastically changing during the first the first few weeks of life. For instance, the proliferation rate of B cells in the Peyer’s Patches shows a 15-fold increase between days 1 and 42 [56]. Very few observations have been made concerning the influence of bacteria on the development of these immune cells which are the first line of defense against Intestinal infections [54].

A group of authors [54] found a decrease in CD8⁺ intraepithelial lymphocytes in piglets after treatment of sows and their piglets with *Enterococcus faecium* present in the feed. Neither total IgG or IgA levels in the sera of sow and piglets was affected, nor were the amounts of total IgG or IgA in the milk of the sows influenced by the probiotic treatment. Despite these observations, while the total numbers of coliform bacteria was the same in both probiotic and control herds, there appeared to be at least a 50% reduction in the numbers of pathogenic serovars in piglets from the probiotic group although the rate of isolation of these same serovars in sows was the same for both groups. ELISA-tests to detect specific antibodies against certain pathogenic *Escherichia coli* serovars are still ongoing.

9. Other effects of probiotics

Several studies indicate that in pig's intestinal morphology and function of the epithelium may be modified by probiotics [54]. In two trials significantly longer villi were measured in the jejunum of pigs receiving diets supplemented with *Bacillus cereus* [28] and *Bacillus cereus toyoi* or *Saccharomyces boulardii* respectively [17].

The probiotic product	Composition of microorganisms	Utilization
Toyocérine	<i>Bacillus toyoi</i>	In all animals
Paciflor	<i>Bacillus cereus</i> CIP 5832	In all animals
Adjulact standart	<i>Enterococcus spp</i> , <i>Lb. lactis</i> , <i>Lb. helveticus</i> , <i>Lb. acidophilus</i>	Calfs, piglets
Adjulact 1000	<i>Lb. helveticus</i> , <i>Enterococcus spp</i>	Calfs, piglets
Adjulact 2000	<i>Enterococcus spp</i> , <i>Lb. plantarum</i> .	Calfs, piglets
Yea -sacc	<i>Saccharomyces cerevisiae</i>	Ruminants
Lacto-sacc	<i>Saccharomyces cerevisiae</i> <i>Lb. acidophilus</i> <i>Ec. faecium</i>	In all animals
Fermacton	<i>Lactobacillus spp</i> . <i>Ec. faecium</i> SF68 <i>Pediococcus spp</i>	In all animals
Bio-Plus Porc	<i>Lactobacillus spp</i> . <i>Ec. faecium</i> SF68 <i>Pediococcus spp</i>	Pigs
Lyobacter P ₁	<i>Lb. plantarum</i> . <i>Ec. faecium</i> <i>Lb. rhamnosus</i>	In all animals
Lyobacter SFL	<i>Ec. faecium</i> SFL	In all animals
Multigerm	<i>Lb. plantarum</i> . <i>Ec. faecium</i> <i>Lb. acidophilus</i>	Pigs
Biosaf SC 47	<i>Saccharomyces cerevisiae</i> SC 47	In all animals, especially in ruminants
Bio-Plus 2B	<i>B. subtilis</i> <i>B. licheniformis</i>	In all animals
Enteroferm	3 kind of <i>Lactobacillus</i> , <i>Enterococcus spp</i> , <i>Saccharomyces</i>	In all animals
Degeferments	<i>Lb. acidophilus</i> , <i>Lb. lactis</i>	In all animals
Bacteriolact	<i>Lb. casei</i> , <i>Str. thermophilus</i>	Calfs, piglets, lamb

Table 3. Some probiotics used as feed additives in European countries [59]

The microstructure of the epithelium is of great functional importance for nutrient transport (absorption and secretion) as well as maintenance of transcellular and paracellular barrier functions. This structure inhibits uncontrolled passage of substances and provides a barrier against infection with intestinal bacteria. Carbohydrate structures on the mucosal surface are used for adhesion by pathogenic and non pathogenic bacteria. *In vitro* studies also indicate that some probiotics *Lactobacillus plantarum* 299v and *Lactobacillus rhamnosus* GG have the ability to inhibit adherence of attaching and effacing of pathogenic *Escherichia coli* HT 29 to intestinal epithelial cells by increasing expression of the intestinal mucins MUC2 and MUC3, [32].

A group of authors [3], [66] concluded that Intestinal mucosa from pigs which were adopted to diets containing *Bacillus cereus* or *Saccharomyces boulardii* had an increased paracellular barrier function and modified nutrient transport kinetics for glucose and amino acids. For *Lactobacillus plantarum* 299v was shown, that pretreated rats were protected against increase in intestinal permeability induced by *Escherichia coli* [33].

10. Experiments in extensive farm conditions

10.1. Material and methods

Two animal trials were carried out at the same private farm of pigs. Twenty four piglets (White x Duroc) of four litters were transferred after weaning (35 days) to flat decks and randomly allocated to 4 groups with 6 animals (3 male and 3 female). The basal diet (see Table 4 and 5) was also supplemented with 1000mg, 1500mg and 2000mg/kg of the probiotic preparation (three experiment groups) or without supplementation (control group). The diets were offered ad-libidum and animals had free access to water. The probiotic preparation included the following strains: *Lactobacillus plantarum* ATCC 4336 (5×10^9 CFU/kg), *Lactobacillus fermentum* DSM 20016 (5×10^9 CFU/kg) and *Enterococcus faecium* ATCC 19434 (5×10^{10} CFU/kg) (AKRON s.r.l-Milano). During the eight weeks experimental period in the first experiment and six weeks experimental period in the second experiment, body

Diet composition (g/kg feed)		Nutrient concentration (g/kg feed)	
Maize	620	ME (MJ/kg)	12,33
Soya bean meal	280	Crude protein	196,4
Sunflower meal	50	Crude fat	28,70
Fish meal	10	Crude fibre	42,90
Limestone	15	Calcium	10,77
Monocalcium phosphate	15	Phosphorus	6,50
Vitamin -mineral premix ^a	5	Lysine	11,30
L-Lysine	5	Methionine+Cystine	6,70

Table 4. Diet composition and calculated nutrient concentration on the first experiment.

^a Contents in 1 kg: 1,200,000 IE vit. A, 120,000 IE vit. D₃, 4000 mg vit. E, 200 mg vit. B₁, 600 mg Vit. B₂, 2500 mg Niacin, 400 mg Vit. B₆, 4500 µg Vit. B₁₂, 20,000 µg Biotin, 1800 mg Pantothenic acid, 160 g Na, 50 g Mg, 10,000 mg Zn, 7500 mg Fe, 7500 mg Mn, 150 mg J, 70 mg Co and 40 mg Se.

Diet composition (g/kg feed)		Nutrient concentration (g/kg feed)	
Maize	630	ME (MJ/kg)	12,90
Soya bean meal	320	Crude protein	197,1
Fish meal	10	Crude fat	28,08
Limestone	10	Crude fibre	35,94
Monocalcium phosphate	15	Calcium	8,60
Vitamin-mineral premix	10	Phosphorus	6,72
L-Lysine	5	Lysine	10,65

Table 5. Diet composition and calculated nutrient concentration on the second experiment.

weight (BW), daily weight gain (DWG) and feed conversion ratio (FCR), kg feed/kg body weight gain were measured weekly. Data are presented as arithmetic means with standard deviations (Mean \pm SD). One-way analysis of variance and Student's t-test ($P < 0.05$) were performed to test the differences between levels of the probiotic in the diet.



Figure 1. Piglets in the first and second experiments, in extensive farm condition.

10.2. Results and discussions

Parameters		Probiotic Dose (mg/kg feed)			
		0	1000	1500	2000
Production	n ¹				
Initial BW, kg	6	5.3 \pm 0.65	5.4 \pm 0.77	5.6 \pm 0.37	5.1 \pm 0.17
Fourth weeks ⁴		12.59 \pm 2.63	14.20 \pm 1.62 ^a	13.93 \pm 0.82	10.97 \pm 0.93 ^b
Eighth weeks		19.89 \pm 2.05	23.00 \pm 2.73 ^a	22.26 \pm 2.42	18.84 \pm 1.43 ^b
DWG, g ²		260.7 \pm 33.8	314.3 \pm 62.9 ^a	297.6 \pm 71.6	245.4 \pm 46.5 ^b
FCR ³		3.01 \pm 0.68	2.61 \pm 0.25	2.67 \pm 0.32	2.94 \pm 0.42

Table 6. Effects of probiotic preparation on performance parameters in the first experiment (Mean \pm SD).

¹ Number of animals/every group

² DWG for whole experimental period.

³ FCR for whole experimental period.

⁴ Significant differences, indicated with different superscripts.

Parameters		Probiotic Dose (mg/kg feed)			
		0	1000	1500	2000
Production	n ¹				
Initial BW, kg	6	4.8 \pm 0.65	5.1 \pm 0.77	5.0 \pm 0.37	4.9 \pm 0.17
Sixth weeks		16.37 \pm 3.76	17.37 \pm 4.06	16.98 \pm 3.98	16.25 \pm 3.45
DWG, g ²		275.6 \pm 46.7	292.3 \pm 57.3	285.4 \pm 51.8	270.4 \pm 43.7
FCR ³		3.20 \pm 0.76	2.80 \pm 0.48	2.87 \pm 0.57	2.93 \pm 0.68

Table 7. Effects of probiotic preparation on performance parameters in the second experiment (Mean \pm SD).

In last ten years, most of the experiments were performed with piglets. According to the literature review, in many trials showed positive effects of probiotics on weaned piglets and also there were no significant effects of growing and finishing pigs. In the first trial the body weight gain was improved with graded levels (1000 and 1500 mg/kg feed) of the probiotic preparation respectively 15% to 11%, compare to control group. In the fourth and eighth weeks of this trial, a significant difference was documented. The body weight gain, on the second experiment was improved with graded levels (1000-1500 mg/kg feed) of the probiotic preparation from 3% to 6%, compare to control group, without significance. The FCR (kg feed/kg weight gain) in the first trial was improved with graded levels by up to 13.3%, 11.3% and 0.4% compare to control group and in the second trial respectively 12.5%, 10.4% and 8.5% compare to control group. The tendency for increasing of probiotic dose has not positive effects on performance parameters. Because of the low dose-response between 1000 and 1500 mg/kg feed, the level of 1000 mg/kg feed seems to be the optimal dose [64].

According to [20] on the experiments with weaned pigs and growing-finishing swine, used 1g/kg *Lactobacillus acidophilus*, which contains 4×10^6 viable cells per gram. Supplementation of the diet with 1g/kg *Lactobacillus acidophilus* on weaned pigs did not improve daily gain, feed intake or feed efficiency. Daily weight gain and feed intake of pigs, treated with 500 mg/kg *Lactobacillus acidophilus* showed non significant trends.

Reduction of diarrhoea by probiotics and vitality of piglets is one of the second topics in this study, because diarrhoea is the main problem for weaned piglets, especially during the first week after weaning. After two weeks of probiotic supplementation, we showed a reduction of diarrhoea on three treated groups. Reduction of diarrhoea by probiotic supplementation was study frequently by many scientist groups. Some of the trials showed significant effects, but the others have collected not significant data. A group of authors [29], [22], [68], [23] have used the same probiotic *Bacillus cereus* in different age of piglets, respectively 8 weeks piglets, 1-85 day after birth, 7-21 day after birth and 2 weeks post weaning. They showed statistical significance of diarrhoea reduction. [10] showed non significant effects, while they used *Bacillus cereus* in pigs 24-66 days of life.

11. Experiment in intensive farm condition

11.1. Material and methods

The animal trials were carried out at the experimental station of the Institute of Animal Nutrition of the Free University of Berlin, Germany. Thirty two piglets (White x Duroc) of three litters were transferred after weaning (28 days) to flat-decks and randomly allocated to 4 groups with 8 animals (4 male and 4 female). The basal diet was either supplemented with 1000, 1500 and 2000 mg/kg of the probiotic preparation or without supplementation (control).

The diets were offered ad libitum and animals had free access to water. The probiotic preparation included the following strains: *Lactobacillus plantarum* ATCC 14917 1×10^{11} CFU/kg, *Lactobacillus fermentum* DSM 20016 1×10^{11} CFU/kg and *Enterococcus faecium* ATCC 19434 1×10^{11} CFU/kg. During the six weeks period body weight (BW), daily weight gain (DWG) and feed conversion ratio (FCR), kg feed/kg body weight gain were measured weekly. Three piglets from each trial group were euthanized one week after probiotic administration by intracardial injection of T61 (Fa. Hoechst) after sedation with Stresnil*. Immediately after death, the abdomen was opened and ligatures were applied to collect digesta samples for pH measurement in defined segments of the duodenum, jejunum, ileum, caecum and colon. This operation was finished between 12-14 hours after death.

Diet composition (g/kg feed)		Nutrient concentration (g/kg feed)	
Maize	620	ME (MJ/kg)	12.82
Soybean meal	275	Crude protein	197.8
Soya oil	50	Crude fat	34.3
Fish meal	30	Crude fibre	31.4
Limestone	10	Calcium	9.10
Monocalcium phosphate	15	Posphorus	7.68
Vitamin -mineral premix ^a	12	Lysine	11.77
L-Lysine	10	Methionine+Cystine	7.64
Methionine+cystine	10	Threonine	8.04
Threonine	10	Tryptophane	2.37
Tryptophane	3		

Table 8. Diet composition and calculated nutrient concentration.

^a Contents in 1 kg: 1,200,000 IE vit. A, 120,000 IE vit. D₃, 4000 mg vit. E, 200 mg vit. B₁, 600 mg Vit. B₂, 2500 mg Niacin, 400 mg Vit. B₆, 4500 µg Vit. B₁₂, 20,000 µg Biotin, 1800 mg Pantothenic acid, 160 g Na, 50 g Mg, 10,000 mg Zn, 7500 mg Fe, 7500 mg Mn, 150 mg J, 70 mg Co and 40 mg Se.

For determination of intestinal bacteria, the "Selective Media" method was used (CATC-agar (Citrat Acid Tween Carbonate - agar base) for *Enterococci spp*, MRS-agar (*Lactobacillus* agar acc to Man Rogosa and Sharp) for *Lactobacilli spp* and Mac Conkey for *Enterobacteria spp*). The colony of *aerobe and anaerobe* micro organisms by visual numbering were measured on agar plate.

The apparent nutrient digestibility was determined by the indicator method during the last week of the experiment using chromium (III) oxide (0.5%).

$$\text{Coefficient of digestibility} = 100 - \left(\frac{\% \text{ e indicator in feed} \times \% \text{ e nutrient in faeces}}{\% \text{ e indicator in faeces} \times \% \text{ e nutrient in feed}} \times 100 \right)$$

Data are presented as arithmetic means with standard deviations (Mean \pm SD). One-way analysis of variance and Student's t-test ($P < 0.05$) were performed to test the differences between levels of the probiotic in the diet.

12. The methodology for determination of microbiological charge of faeces

Microbiological analyzes of faeces were performed in two periods:

- Week 1-3
- Week 5-7

In the first period, such analysis aim to consistently follow microbiological changes due to the "probiotics" effect.

In the second period, such analysis aim to compare the microbiological changes in the beginning and in the end of the experiment, as well as to judge on the duration of the "probiotics" effect after its termination.

Microbiological analyses were carried out of as follows:

3-4 hours after the feed, fresh faeces was collected in plastic boxes. Faeces of all boxes were gathered and placed in a separate box. 1 g of faeces was taken for each box, in three parallel tests A, A₁, A₂.

9 ml Ringer solution was added to it, and the following dilutions were prepared:

10⁻¹-10⁻⁹, MRS for identification of *Lactobacillus spp*

10⁻⁴-10⁻⁸, CATC for identification of *Enterococcus spp*

10⁻³-10⁻⁸, McK for identification of *Enterobacteriaceae*

Its cultivation in Agar plates and incubation at a temperature of 37°C was conducted within 24 hours.

13. The physiological and microbiological parameters of intestinal mucosa and digesta

A week after administration of probiotics, a total of 12 piglets were slaughtered, 3 piglets for every group.

The slaughtering of pigs a week after administration of probiotics aimed at:

- monitoring of the changes occurring in the pH digesta in the intestines.
- monitoring of all microbiological changes in digesta and mucosa, reflecting *Lactobacillus spp*, *Enterococcus spp* and *Escherichia coli* microbiological load as well as the total number of anaerobic bacteria in the jejunum, ileum, caecum and colon.

The preparation of samples for microbiological analysis was carried out as follows:

A 2x10cm area from all parts of intestine and colon is taken. Then, it is washed away with 0.9% NaCl solution, is measured its length, is thorn with a fine scalpel, is weighed and

finally is placed in plastic tubes. Since jejunum is relatively long, it is divided into three parts for more convenience: jejunum 1, jejunum 2 and jejunum 3.

Measuring and weighing was done for the following parts:

Duodenum	Ileum
Jejunum 1	Caecum
Jejunum 2	Colon
Jejunum 3	

Microbiological load was estimated at:

Middle of jejunum, ileum, caecum, beginning of colon

14. The determination of anaerobic bacteria (*Lactobacillus spp*).

Method of samples in ice

15 ml digesta is taken, is squeezed, and after is being cast into sterile plastic tubes and it is placed in ice.

0.5 g of this digesta is taken, 500 ml Ringer solution is added, and then is placed on ice.

Dilutions are prepared by mixing what is taken from both beakers up to 100 μ l.

20 μ l is taken by pipette and is dripped in Agar plates prepared based on the following dilutions:

MRS: 10^{-6} to 10^{-10} **Columbia - Blut:** 10^{-6} to 10^{-10}

15. Methods of samples in ice

Parts of the intestines are cut and placed in 50ml tubes together Ringer solution. Later solution is shaken and changed until no more digesta remains. The prepared solution is put into a bottle and placed in ice. Intestine is placed on a plate, mucosa is thorn and mixed. 0.5 g mucosa is taken; 500 μ l Ringer solution is added and placed on ice.

Dilutions are prepares as in the first case and are placed on ice.

20 μ l is taken by pipette and transferred to Agar plates prepared according to the following dilutions:

MRS: 10^{-5} to 10^{-9} **Columbia -Blut:** 10^{-5} to 10^{-10}

16. The determination of aerobic bacteria (*Enterobacteriaceae* and *Enterococcus spp*)

Digesta dilutions are prepared as above. 20 μ l solution is taken and transferred to Agar plates prepared according to the following dilutions:

Mac Conkey: 10^{-6} to 10^{-10} **CATC :** 10^{-3} to 10^{-7}

Mucosa dilutions are prepared. 20 μ l solution is taken and transferred to Agar plates prepared according to the following dilutions:

Mac Conkey : 10^{-3} to 10^{-7} **CATC :** 10^{-2} to 10^{-6}

Microbiological load was estimated: Middle of jejunum, ileum, caecum, beginning of colon



Figure 2. Institute of Animal Nutrition, Free University, Berlin



Figure 3. The animal trial at the experimental station of the Institute of Animal Nutrition.

17. Data about probiotic "*Seberini suini*"

17.1. Microbiological composition of probiotic

<i>Lactobacillus plantarum</i>	ATCC 14917 (LMG – S 16691) cfu 1×10^{11}
<i>Lactobacillus fermentum</i>	DSM 20016 (LMG- S 16517) cfu 1×10^{11}
<i>Enterococcus faecium</i>	ATCC 19434 (LMG- S 16690) cfu 1×10^{11}

Composition of the probiotic "Seb Suini"

Lactobacillus plantarum	25 %
Enterococcus faecium	10 %
Lactobacillus fermentum	15 %
Micronized soya extraction meal	50 %

Chemical composition %	Amino acids g/kg
Dry matter 88	Lysine 17
Crude protein 35	Leucine 17
Crude fat 1	Threonine 11
Crude fibre 5	Arginine 10
Crude ash 28	Tryptophan 3
	Izoleucine 11
	Hystidine 6
	Glycine 9
	Cystine 2
	Valine 13

Table 9. Chemical composition of the probiotic "Seb Suini" used in the experiment.

18. Physical -chemical characteristics of the probiotic

Smell	tipical, not bad
Apparent densities after shaking	0,45 kg/liter.
Point of degradability	> 250°C
Density	450 gr/liter
Water solubility	non digestible, hydrodispersible.
Granulometry	90% e grimcave kalojnë sitën 200 micron.
Value of pH	6,5 (10 gr on 100 ml in temperature 20°C)

Microbiological characteristics

Total not lactic flora	maximum 5×10^3 UFC/gr
Enterobacteriaceae	absent
Coliformes	absent
Enterococcus	maximum 5×10^2 UFC/gr
Yeasts and moulds	maximum 1×10^2 UFC/gr

According to the analyzes made in the Institute of Soil Chemistry, "Università Cattolica del Sacro Cuore"- Piacenza, results heavy metal contain

Pb	<0,6	ppm
Cd	94	ppm
Ni	11	ppm
Cr	15	ppm
As	1, 18	ppm
Hg	112	ppm

It does not contain Alfa-toxine B₁, B₂, G₁, G₂, Zearalenone, Ocratoxine, Fumosine B₁,

Deossinivalenolo 122,0g / Kg tq

19. Results and discussions

Parameters		Probiotic Dose (mg/kg feed)			
		Control	1000	1500	2000
Production	n ¹				
-Initial BW, kg	8	5.6 ± 1.11	5.5 ± 1.07	5.6 ± 1.17	5.6 ± 1.02
-BW 6 th week ²	5	19.5 ± 5.10	19.8 ± 5.83	23.1 ± 3.17	22.3 ± 7.01
Feed intake, kg		24.5 ± 7.49	25.4 ± 6.44	29.79 ± 5.42	30.4 ± 7.47
DWG, g ³		325 ± 153	341 ± 128	427 ± 71	436 ± 123
FCR ⁴		1.79 ± 0.48	1.78 ± 0.31	1.65 ± 0.05	1.66 ± 0.15

Table 10. Effects of probiotic preparation on performance parameters (Mean ± SD).

¹Number of animals, (8 piglets/ every group, at the beginning of the experiment)

²Number of animals, (5 piglets/every group, one week after probiotic supplementation). n = 4 at treatment 1500 mg/kg in 6th week.

³DWG for whole experimental period.

⁴FCR for whole experimental period.

The body weight gain was improved with graded levels of the probiotic preparation from 4.9 up to 31.7%. Caused by the high coefficient of variation the differences were not significant. The FCR (kg feed/kg weight gain) was improved with graded levels by 0.6 up to 7.3%. The differences were not significant. Because of the low dose-response between 1500 and 2000 mg/kg feed, the level of 1500 mg/kg feed seems to be the optimal dose.

The same results showed [30] on the experiments with weaned piglets, used LFP-*Lactobacillus-Fermentation-Product*. This probiotic contents *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Streptococcus thermophilus*, produced in Quebec, Canada. The basal diet was supplemented with 100 mg LFP/kg feed.

The feed intake and the daily weight gain (DWG) were increased respectively 11.8% and 10.4%, compared with the control group. The feed conversion ratio (FCR) was in the same level.

Two authors [19] used the same probiotic LFP (*Lactobacillus-fermentation-product*) on the weaned piglets. Pigs fed a diet with 0.36 ml/kg LFP required nearly 10% less feed per unit of weight gain than the control group. Also the incidence of scouring decreased ($P < 0.05$) in pigs fed with different levels of LFP. Overall improvement occurred up through the addition of 0.36 ml/kg LFP with no additional benefit from greater amounts. Another group of authors [44] showed the effects of microbial feed additives on performance of starter and growing-finishing pigs. One of the experimental group with weaned piglets was fed with 750 mg *Lactobacillus acidophilus*/kg feed. The second experimental group was supplemented with 1250 mg *Streptococcus faecium*/kg feed.

The addition of *Lactobacillus acidophilus* to the feed of young pigs improved average daily weight gain by 9.7 % and the feed conversion ratio by 21.4%, whereas the addition of *Streptococcus faecium* decreased average daily weight gain. The addition of acid lactic improved feed conversion, suggesting that lactic acid as a metabolite produced during fermentation might be the reason for the improvement in performance. The probiotics had no effect on growing-finishing pigs.

In a trial with 90 untreated and 90 treated (*Bacillus cereus*-preparation) weaned piglets, the probiotic treated animals gained 7% more live weight during 6 weeks after weaning with a reduced feed conversion ratio of 2.4%. However, both results were not significant. This points towards a high variation in the response of the individual animals to this type of feed additives [23].

Parameters	Probiotic Dose (mg/kg feed)				
	N ¹	Control	1000	1500	2000
Digestibility ² (in %)	5				
Dry matter		76.4 ± 6.90	73.2 ± 10.39	67.2 ± 2.22	75.7 ± 9.52
Crude fat		75.1 ± 5.48	71.2 ± 2.60	69.0 ± 9.11	70.0 ± 3.77
Crude fibre		51.1 ± 7.82	54.5 ± 7.48	52.3 ± 5.79	56.4 ± 2.31
Digesta pH	3				
Duodenum		5.54 ± 0.96	5.74 ± 0.68	5.87 ± 0.83	6.51 ± 0.77
Jejunum		6.24 ± 0.38	6.17 ± 0.66	6.29 ± 0.51	6.56 ± 0.85
Ileum ³		7.05 ± 0.43 ^a	6.43 ± 0.77 ^b	6.41 ± 0.16 ^b	5.25 ± 0.12 ^c
Caecum		5.62 ± 0.13	5.65 ± 0.20	5.79 ± 0.39	5.55 ± 0.09
Colon		5.87 ± 0.27	6.19 ± 0.38	6.27 ± 0.37	6.18 ± 0.43

Table 11. Effects of probiotic preparation on apparent nutrient digestibility and digesta pH of defined intestinal segments (Mean ± SD).

¹ Number of animals.

² Crude nutrients were determined by Weende scheme.

³ Significant differences, indicated with different superscripts.

Feeding probiotic preparation slightly increased the crude fiber digestibility compared to the control group in the range of 3.4%, 1.2% and 5.4% at supplementations with 1000, 1500

and 2000 mg/kg feed, respectively. With graded levels of the probiotic preparation pH of the chyme of ileum and caecum was slightly decreased, in contrast the pH of duodenum and jejunum was slightly increased [63]. The low effect of pH was agreement with digestibility results. The pH results in the duodenum and jejunum is in contrast to former results reported by [35]. This is possibly caused by the combination of different strains used in this study.

Two authors [19] supplemented the diets of growing pigs with LFP preparation (*Lactobacillus Fermentation Produced*) and observed that a supplementation of 0.72 mg LFP/kg feed increased the crude fiber digestibility with 14.2% compared to the control group ($P < 0.05$).

These authors assumed that the rate of passage of feed through the digestive tract was decreased by feeding LFP, which allowed more time for digestion of crude fiber. Also the urinary nitrogen excretion was greater than faecal excretion but both combined were less than intake, thus resulting in a positive nitrogen balance. In total, the digestibility of dry matter was decreased 0.4% and the digestibility of crude protein did not change, compared to the control. Another author [58] showed the influence of *Lactobacillus acidophilus* in broiler chicks on growth, feed conversion and crude fat digestibility. The addition of *Lactobacillus acidophilus* in broiler chicks diet decreased the digestibility of crude fat.

		Probiotic Dose (mg/kg feed)			
		Control	1000	1500	2000
1 st week of trial	<i>Lactobacilli</i> spp.	95	120	150	170
	<i>Enterococci</i> spp.	0.01	0.94	1.12	1.23
	<i>Escherichia coli</i> .	10	10	32	2
6 th weeks of trial	<i>Lactobacilli</i> spp.	683 ± 584	223 ± 191	345 ± 403	767 ± 306
	<i>Enterococci</i> spp.	0.018 ± 0.031	0.1 ± 0.131	0.011 ± 0.01	0.028 ± 0.02
	<i>Escherichia coli</i> .	2.35 ± 3.60	15 ± 21.8	0.05 ± 0	0.083 ± 0.057

Table 12. The effect of probiotic preparation on the microbial composition of faeces (CFU*10⁶/g wet weight) (Mean ± SD).

* Four faeces samples/every group were collected/every week, during the experimental period.

The effect of probiotic preparation on the microbial composition of faeces was examined early, one week after supplementation, because the first week after weaning is critical period for tends to shift the balance of the gut microflora away from beneficial bacteria towards pathogenic bacteria. One week after weaning piglets fed with the probiotic preparation showed increased the concentration of *Lactobacilli* spp. and *Enterococci* spp. compared to the control treatment. Feeding 2000 mg probiotic preparation/kg feed induced a reduction of *Escherichia coli*. At the end of the experiment piglets fed with 1500 and 2000 mg probiotic preparation/kg feed had reduced *Escherichia coli* compared to the control. These results indicate that the probiotic preparation may be less suppressive to the *Escherichia coli*. [40]

observed the similar microbial changes in the faeces of weaned piglets, fed with the same combined probiotic preparation.

		Control	Probiotic Dose (mg/kg feed)		
			1000	1500	2000
Jejunum	<i>Anaerobe bacteria.</i>	13.92 ± 14.15	12.22 ± 12.45	8.75 ± 8.60	12.98 ± 13.07
	<i>Lactobacilli spp.</i>	10.24 ± 10.44	12.58 ± 12.78	8.36 ± 8.38	11.60 ± 11.55
	<i>Enterococci spp.</i>	7.02 ± 6.98	8.03 ± 8.22	7.00 ± 7.19	7.01 ± 6.97
	<i>Escherichia coli.</i>	7.57 ± 7.74	8.60 ± 8.72	6.00 ± 0.00	7.90 ± 8.02
Ileum	<i>Anaerobe bacteria.</i>	13.17 ± 13.36	13.21 ± 13.20	13.21 ± 13.20	12.60 ± 12.72
	<i>Lactobacilli spp.</i>	12.87 ± 13.11	12.69 ± 12.73	12.72 ± 12.95	13.68 ± 13.89
	<i>Enterococci spp.</i>	6.00 ± 0.00	8.82 ± 9.06	7.33 ± 7.55	7.02 ± 7.22
	<i>Escherichia coli.</i>	8.17 ± 8.17	11.00 ± 11.23	12.01 ± 12.25	12.05 ± 12.23
Caecum	<i>Anaerobe bacteria.</i>	13.90 ± 13.85	12.69 ± 12.84	13.75 ± 13.87	13.98 ± 14.12
	<i>Lactobacilli spp.</i>	13.28 ± 13.48	12.60 ± 12.84	13.43 ± 13.65	13.83 ± 14.05
	<i>Enterococci spp.</i>	6.86 ± 7.04	10.00 ± 10.23	7.80 ± 8.03	6.84 ± 6.70
	<i>Escherichia coli.</i>	12.69 ± 12.93	10.00 ± 10.23	10.82 ± 11.06	10.86 ± 11.04
Colon	<i>Anaerobe bacteria.</i>	14.72 ± 14.92	13.04 ± 13.06	13.95 ± 14.18	13.93 ± 14.15
	<i>Lactobacilli spp.</i>	12.55 ± 12.49	13.01 ± 13.23	13.84 ± 14.08	13.92 ± 14.10
	<i>Enterococci spp.</i>	8.82 ± 9.06	9.00 ± 9.23	12.01 ± 12.25	9.12 ± 9.36
	<i>Escherichia coli.</i>	13.44 ± 13.68	11.30 ± 11.53	12.69 ± 12.93	12.39 ± 12.59

Table 13. The effect of probiotic preparation on the microbial composition of digesta, one week after probiotic supplementation. (log CFU/g wet weight), (Mean ± SD; n = 3).

The effects of the probiotic preparation on the microbial composition of the chyme showed no dose–dependent effects. However there was a tendency for increasing of the concentration of *Lactobacilli spp.* and *Enterococci spp.* in the colon compared to the control.

A group of authors [1] supplemented the pig diets with a combination of *Lactobacillus fermentum* 14 and *Streptococcus salivarius* 312 for 4 days and observed a significant reduction in the *Escherichia coli* count in both the stomach and duodenum. A significant reduction of *Escherichia coli* number in the stomach was also found, when *Lactobacillus fermentum* was supplemented separate. In cases of diarrhoea caused by *Escherichia coli* the treatment as described here was not effective because the count of *Escherichia coli* in the duodenum of culture-fed pigs was still greater than 10⁶/g. However, if the antibacterial effect of strain 14 could be increased some effect on scouring due to *Escherichia coli* should follow. This might be accomplished by the feeding of large numbers of organisms or by the administration in a concentrated form of the inhibitory factors produced by *Lactobacillus fermentum* strain 14. [15] showed that the application of 10⁸ colony forming units (CFU) of a *Bacillus cereus* preparation/kg feed to piglets reduced counts for *Lactobacilli spp.*, *Bifidobacteria*, *Eubacteria* and *Escherichia coli* in the duodenum and jejunum, but increased respective CFU in the ileum, caecum and colon.

Two authors [35] showed a significant reduction of *Escherichia coli* CFU in the small intestine of piglets was also noted when an *Enterococcus faecium* preparation was applied. However, at the same time *Lactobacilli spp.* and *Enterococci spp.* counts increased as a trend and statistically significant, respectively [24].

The results of studies on the ability of probiotic bacteria to reduce the colonization of pathogenic bacteria are ambiguous. Challenge studies with piglets and *Escherichia coli* O141:K85 showed no influence on clinical symptoms, mortality or excretion of hemolytic *Escherichia coli* [8]. A group of authors [24] showed that the colonization with mucosa associated *Enterobacteria spp.* was reduced when a probiotic *Bacillus cereus* preparation was supplemented.

The probiotic had no influence on the occurrence of pathogenic *Escherichia coli* as measured with a PCR assay [16]. These results point to the fact that hygienic conditions in scientific institutes may sometimes be too favorable to investigate effects of pathogenic bacteria without challenge trials [54].

These and the other studies imply that probiotics are able to reduce/enhance specific bacterial groups, but the reduction of total bacterial cell numbers as recorded for antibiotics is probably not a probiotic mode of action. In order to understand the casual relationships which lead to the observed improvements in weight gain and feed conversion or general health of animals, possible interactions between bacteria in the intestine and host animal must be studied. Of special significance are interactions between the metabolism of the host and metabolic activity of intestinal bacterial populations [54].

20. Conclusions

The supplementation of the combined probiotic preparation induced slightly the performance data. In extensive farm condition, a significant difference of daily weight gain (DWG) was documented four weeks after probiotic supplementation. A positive effect of the probiotic on feed conversion ratio (FCR), kg feed/kg weight gain and vitality was observed, also. We recommend the level of 1000mg/kg feed combined probiotic as the optimal dose.

Combined probiotic preparation induced slightly the performance data in intensive farm condition, also. However the differences were not significant. Feeding probiotic preparation slightly increased the crude fibre digestibility in all treated groups. With graded levels of the probiotic preparation pH of the chyme of ileum and caecum was slightly decreased, in contrast the pH of duodenum and jejunum was slightly increased. The probiotic preparation showed increased the concentration of *Lactobacilli spp.* and *Enterococci spp.* compared to the control. The results indicate that the probiotic preparation may be less suppressive to the *Escherichia coli*. The effects of the probiotic preparation on the microbial composition of the chyme showed no dose-dependent effects. However there was a tendency for increasing of the concentration of *Lactobacilli spp.* and *Enterococci spp.* in the colon compared to the control. Possibly this was due to the combined probiotic preparation. At the end, we recommend the level of 1500 mg/kg feed combined probiotic as the optimal dose.

* Approved by competent authority according to Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States, regarding the protection of animals used for experimental and other scientific purposes.

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