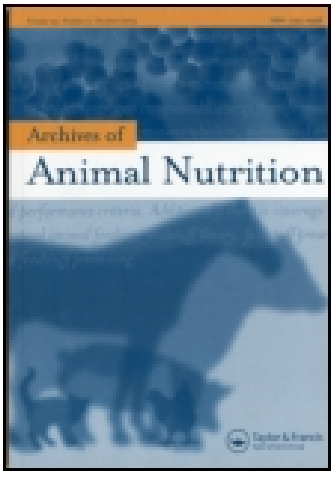

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THE INFLUENCE OF *LENTINUS EDODES* (SHIITAKE MUSHROOM) PREPARATIONS ON BACTERIOLOGICAL AND MORPHOLOGICAL ASPECTS OF THE SMALL INTESTINE IN PIGLETS[1]

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Among substances intended to replace growth promoting antibiotics in pig nutrition, non-digestible oligosaccharides or polysaccharides could be potential alternative compounds. Therefore, the influence of β-1,3-1,6 glucans on bacteriological, biochemical and morphological aspects of the small intestine in weaned piglets was investigated. As sources of β-glucans, Lentinan (extract of *Lentinus edodes* mycelium) or dried *L. edodes* mycelium were added to the diet. Four homogenous groups of 5 newly weaned piglets (4 weeks of age) received one of four diets: control diet (C), C supplemented with Avilamycin (50 mg/kg, positive control), C supplemented with 0.1% of Lentinan and C supplemented with 5% of dried *L. edodes* mycelium powder. A first group of 10 piglets was euthanized after 11 days and the remaining 10 on day 12 of the experiment. The gastrointestinal tract was divided in segments and samples taken from digesta (stomach, proximal and distal jejunum, caecum), mucosal scrapings (jejunum) and ring shaped tissue samples (1 cm) of proximal and distal jejunum. Bacterial counts were made with digesta and mucosal samples, and short-chain fatty acids (SCFA), lactic acid and ammonia concentrations were determined. Tissue samples of both jejunal sites were embedded in paraffin wax for morphometrical (villus length, crypt depth) and histological observations (numbers of intraepithelial lymphocytes (IEL), goblet cells, apoptotic enterocytes on villi, mitotic cells in crypts). Only the diet containing 5% of dried *L. edodes* consistently resulted in lower viable counts (ca. $1-2$ $\log_{10}$ CFU) of total bacteria, *E. coli*, streptococci and lactic acid bacteria, and luminal and mucosal effects agreed very well. With this diet, acetate and butyrate concentrations in the distal jejunum were doubled, which is favourable in view of the trophic effect on enterocytes and colonocytes. Villus length (V) was increased with both diets containing β-glucans while crypt depth (C) was not altered, but V/C was higher. IEL counts were decreased by both diets although bacterial numbers, which is only one parameter of bacterial load, were only diminished with the *L. edodes* feed. The three supplemented feeds lowered the number of apoptotic enterocytes on the villi, but these numbers were very low (control diet : 44 cells per 100 villi), making clear interpretation difficult. The mitotic index was slightly lower with the *L. edodes* feed, although not statistically significant. Decreased viable counts observed with the latter diet is a favourable effect as it is accepted that a lower bacterial load causes lower turnover rates of the intestinal epithelial cells, while there is also less competition for specific substrates. A higher V/C ratio, a smaller number of IEL in the epithelium and a lower apoptotic index also indicate slower turnover rate of the mucosa when Lentinan and *L. edodes* diets were fed. The inconsistent effects observed with Lentinan were probably due to the low amount added to the diet. It should be taken into account that the influence of *L. edodes* mycelium powder was more likely due to the presence of antibacterial compounds (eg.

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lenthionine, lentinamycin, terpenoids, polyphenols), rather than to an immunostimulating action of β-glucans with increased release of IgA onto the mucosa surface.

Keywords: Piglets; Oligosaccharides; Beta-glucans; *Lentinus edodes*; Intestinal microorganisms; Intestine; Morphology

1. INTRODUCTION

The phasing out of the use of growth promoting antibiotics in animal nutrition will be completed by the end of 2005 in the European Union. This has resulted in intensive research efforts searching for alternatives with similar effects on growth, such as organic acids, probiotics and non-digestible oligosaccharides (NDO). Some of these NDO showed prebiotic properties (Gibson and Roberfroid, 1995). A common characteristic of these chemically different substances is their ability to modulate the bacterial population in the gastrointestinal tract. It seems that NDO are not digested by endogenous enzymes in the small intestine, thus reaching the hindgut and available for fermentation by bacteria, *e.g*. lactobacilli and bifidobacteria in small and large intestine (Gibson and Roberfroid, 1995; Crittenden, 1999). Growth of less favourable species or pathogens is lowered by mechanisms which are not completely understood, but may be related to the formation of short-chain fatty acids (SCFA) (Djouzi and Andrieux, 1997). However, analogous to the mechanism of action of antibiotic growth promoters (Vervaeke *et al*., 1979; Dierick *et al*., 1986a,b; Decuypere *et al*., 1991), it is evident that mechanisms underlying any growth promoting effect of NDO must be sought in processes at the intestinal level. In the case of antibiotic growth promoters, these mechanisms have been related to the following phenomena: increased availability of nutrients for productive processes and decreased formation of potentially toxic substances (e.g. ammonia, amines) when bacterial numbers are decreased and/or a slower turnover rate of the small intestinal mucosa, resulting in lower endogenous losses (protein, mucins) (Visek, 1978; Corring *et al*., 1981).

β-1,3-1,6 glucans (e.g. Lentinan, MacroGard) are polysaccharides and like NDO they cannot be digested in the small intestine, thus reaching the hindgut, available for fermentation. However, another interesting aspect is the fact that they are also known as direct immunostimulators after uptake by the M cells associated with Peyer's patches (GALT: gut associated lymphoid tissue), which is not the case for classical NDO. They activate macrophages through specific receptors, stimulate production of several cytokines with activation of helper T-cells, cytotoxic T-cells and natural killer cells. They also stimulate differentiation of B-lymphocytes and increase production of immunoglobulins (IgA) (Quinn, 1990; Engstad and Robertsen, 1993; Bohn and BeMiller, 1995). On the other hand, they can also act as modulators of the gut bacterial flora by influencing its composition in a qualitative (ratio G + /G- species, proteolytic/saccharolytic species) and quantitative manner through fermentation of substrates (Gibson and Roberfroid, 1995). Such changes can affect the bacterial (antigen) load of the small intestinal mucosa with ultimate influences on its morphology, immunological properties and functionality (Moreau and Coste, 1993; Sharma *et al*., 1995; Gaskins, 1997; Guy-Grand *et al*., 1998; Hamann *et al*., 1998; Ichikawa *et al*., 1999).

In the current study, β-1,3-1,6 glucans present in *Lentinus edodes* (Shiitake mushroom) preparations (Lentinan and dry *L. edodes* mycelium powder) were added to the diet of weaned piglets. Their effect on bacteriological, biochemical and

morphological aspects in the digestive tract was investigated. Newly weaned piglets were taken as experimental animals because it is well-known that the first 2–3 weeks after weaning is the most critical phase of the growth process. Indeed, weaning induces villous atrophy in the small intestine, partly caused by separating the piglets from the sow (stress), while inadequate feed intake may also contribute to the changes in intestinal morphology (Kelly *et al.*, 1991; Cera *et al.*, 1988; Nabuurs *et al.*, 1993; van Beers-Schreurs *et al.*, 1998). So, it is reasonable to expect that during this phase, any beneficial effects of additives would be at their most obvious and consequently, easier to detect and demonstrate.

2. MATERIAL AND METHODS

2.1. Animals

A group of twenty cross-bred piglets (Seghers hybrid × Piétrain) weaned at 4 weeks of age was divided into four homogenous (sex, weight) subgroups of 5 animals. They were housed per subgroup under standard conditions in a clean temperature (28°C) controlled facility, and each subgroup received one of four experimental diets. Mean live weight of the piglets at the start of the experiment was 6.72 ± 1.45 kg (± SD). The animals had free access to feed and water. Ten piglets were euthanized (overdose of Nembutal®, natrii pentobarbitalum; Ceva Santé Animale, Brussels, Belgium) after 11 days and the remaining 10 on day 12. Immediately after euthanasia, the gastrointestinal tract was removed and divided into the following sections: stomach, proximal jejunum (J1: 0–3 m distal to pylorus), distal jejunum (J2: 3–0 m proximal to caecum) and caecum. The different segments were emptied and their contents weighed. The jejunum segments were then flushed with saline (0.85 g of NaCl l^{-1} of water) until the effluent was clear. The segments were cut longitudinally and the mucosa was carefully and completely scraped off the underlying muscular layers with a microscopy slide. After the pH of the contents was determined, they were homogenized (Ultra-Turrax T25, Janke & Kunkel GmbH & CoKG, Staufen, Germany; 30 s, 13 500 rpm) and samples for bacterial counts and biochemical parameters were taken. Mucosal scrapings were also homogenized and sampled for bacterial counts. From proximal and distal jejunum, 3–4 segments (1 cm of length) were transversally cut and fixed in neutral buffered formalin for 24 h.

2.2. Diets

Four diets were formulated and their composition is shown in Table I. Diets were balanced for total protein content, net energy value (9.66 MJ kg^{-1}), Ca, P and some essential amino acids (based on small intestinal digestibility). All ingredients were ground and diets fed in the dry form. Lentinan (Shiitake extract; min. 25% of polysaccharides) and *L. edodes* mycelium (dried Shiitake) were bought from Galaxy Rear Mushroom Farm (Qingyuan, Zheijang, China). The feed supplemented with Avilamycin (Maxus®, Elanco, Brussels, Belgium; 50 mg kg^{-1}) was used as a positive control diet. Avilamycin has been shown to act as a growth promoter for pigs and is active against gram-positive organisms (Jones *et al.*, 1987; Anderson *et al.*, 1999). Feeds were analysed for Weende components (dry matter, ash, crude protein, crude fat)

TABLE I Composition of the diets [g kg^{-1}]

Ingredients	*Control diet*	*Control and Avilamycin*	*Control and Lentinan*	*Control and* L. edodes
Barley	350.00	350.00	350.00	350.00
Maize (press. cooked)	100.00	100.00	100.00	100.00
Soya beans Danex [1]	150.00	150.00	150.00	150.00
Wheat feed flour	150.00	150.00	150.00	150.00
Soya bean meal [2]	109.93	110.04	110.16	54.60
AA Protamyl SF [3]	10.00	10.00	10.00	10.00
Soya bean oil	2.36	2.47	2.57	2.06
Methionine	1.95	1.95	1.95	2.51
Lysine	4.25	4.25	4.25	3.94
Threonine	1.92	1.92	1.92	1.71
Tryptophane	0.23	0.23	0.23	0.29
NaCl	4.63	4.63	4.63	4.42
Mono Ca – phosphate	9.41	9.41	9.41	8.14
Limestone	11.03	11.03	11.03	11.05
Whey permeate	57.69	57.69	57.19	57.69
Wheat flour	33.30	32.83	32.36	40.39
Vit E 50%	0.10	0.10	0.10	0.10
Choline 60% (dry)	0.30	0.30	0.30	0.23
Copper sulphate 25%	0.56	0.56	0.56	0.56
Premix vit. pigs 3/2.5 [4]	1.12	1.12	1.12	1.12
Premix minerals pigs 3/4 [4]	1.20	1.20	1.20	1.20
Maxus 20 (Avilamycin) [5]		0.25		
Lentinan [6]			1.00	
Lentinus edodes [7]				50.00

[1]Expanded full fat soya bean (DANIS N.V., Belgium).
[2]Solvent extracted
[3]Potato protein
[4]VITAMEX N.V., Belgium
[5]50 mg/kg (Elanco, Belgium)
[6]Shiitake extract (min. 25% polysaccharides).
[7]Shiitake mushroom mycelium (dried and ground).

according to EU standard methods (Anonymous, 1971, 1972). Total dietary fibre (TDF) analysis was carried out as described by Prosky *et al*. (1985).

2.3. Enumeration of viable bacteria and biochemical parameters

Bacterial counts on intestinal contents and mucosal scrapings were done using the ring-plate technique described by Van Der Heyde and Henderickx (1963). The following selective media (Oxoid, Basingstoke, England) were used : Reinforced Clostridial Medium, supplemented with 0.001% of hemin for total bacterial counts, Rogosa agar for lactobacilli, Slanetz & Bartley medium for streptococci and Eosin Methylene Blue agar (only greenish to brown colonies) for *E. coli*. Incubation conditions were as follows: RCM agar : anaerobic, 48 h; Rogosa agar : anaerobic, 48 h; Slanetz & Bartley agar : aerobic, 48 h; EMB agar : aerobic, 24 h. Samples taken from gastrointestinal contents and mucosa were serially diluted (10-fold), using a sterile peptone solution (per l of distilled water: 1 g of peptone, 0.4 g of agar, 8.5 g of NaCl). As colonies growing on Rogosa agar were not checked for microscopic appearance, use of the term lactic acid bacteria instead of lactobacilli seems to be more appropriate.

Contents sampled in J1, J2 and caecum were acidified (2% w/v of H_2SO_4 18N final concentration) after measuring pH and stored at −18°C until further analysis. Lactic acid, SCFA and ammonia were determined as described by Van Nevel and Demeyer (1977).

2.4. Gut morphology and histology

2.4.1. Morphometry

After fixation in neutral-buffered formalin, intestinal tissue samples were processed under standard conditions in an automatic tissue processor (Shandon, Pittsburgh, PA, USA). Processing consisted of serial dehydration with ethanol, clearing with xylene and impregnation with paraffin wax. Four slides (silane-prep^{TM} slides, Sigma, St. Louis, MO 63178, USA) were prepared for each piglet and each slide contained one section cut at 5 μm. One slide was used for hematoxylin-eosin (HE) staining. Another one was stained with periodic acid and Schiff reagent (PAS) for counting goblet cells. The remaining slides were used for determination of the apoptotic and mitotic index. For sections stained with HE, villus length (V : from tip to base) and crypt depth (C : from base to opening) of all well-oriented villi and adjacent crypts were measured using a microscope equipped with a camera and computer with appropriate software (Image Archiving Plus 4.51; Lucia, Laboratory Imaging s.r.o., Praha 4 – Hája, Czechia). The ratio V/C was calculated and per section (piglet), mean values for V,C and V/C were calculated, resulting in 5 observations per diet ($n = 5$). These mean values per diet were further used for statistical evaluation.

2.4.2. Enumeration of intra-epithelial lymphocytes (IEL)

For HE stained sections, 10 villi were selected for counting IEL, based on morphological characteristics. Series of 40 – 110 enterocytes were defined in the optical field and IEL present in the area were counted. Finally, the sum was made of all counted enterocytes and IEL per section, and the latter expressed as number of IEL per 100 enterocytes. Following this procedure, one value per piglet was obtained or 5 observations per diet. Then, mean values per diet were calculated and used for statistical treatment.

2.4.3. Enumeration of goblet cells

For the sections stained with PAS reagent, goblet cells (positive cytoplasm) on the villi and in the crypts were counted. Therefore, per section, ten villi and crypts were selected and positive cells counted. For villi, numbers of goblet cells were expressed per 100 μm of circumference while for the crypts, cells were expressed per 100 μm of crypt depth.

2.4.4. Determination of the apoptotic index

This parameter was determined only on samples taken from the distal jejunum. Apoptotic enterocytes on the villi were visualized using the TdT-FragEl DNA Fragmentation Detection Kit (Oncogene Research Products, Boston, MA 02118, USA). The protocol provided by the manufacturer was strictly applied. As apoptotic

(positive) cells were generally very scarce, all intact villi present on a section were counted as well as the apoptotic enterocytes thereupon and the latter expressed per 100 villi. Mean apoptotic index (n = 5) was calculated and used for statistical treatment.

2.4.5. Determination of mitotic index

Proliferating cells in the crypts were determined by immunohistochemical labelling utilizing the Ki67 Antigen Kit (Novocastro Lab. Ltd, Newcastle upon Tyne, England). The protocol provided by the supplier had to be slightly changed: incubation with the primary antibodies (clone MM1) was performed overnight instead of during 60 min. Cells in mitosis were then clearly stained and could be counted, on condition that the crypt was perfectly oriented (base to mouth). Positive and negative cells were counted in 7–13 crypts per section. Per crypt, mitotic cells (distal jejunum only) were expressed as% of total cells (mitotic index). This procedure resulted in 5 observations per diet and mean values were calculated and used for statistics.

2.5. Statistical analysis

Statistical evaluation was performed using the GLM ANOVA procedure and mean values were compared by the LSD test. All calculations were carried out using the SPSS 7.5 program for Windows (SPSS Inc., Chicago IL, USA).

3. RESULTS

3.1. Animals and diets

As the experiment only lasted 11–12 days, data concerning performance (daily feed intake and weight gain, feed conversion ratio) are considered as being not very relevant. However, feed intake (200–240 g d^{-1}) and growth values (150–210 g d^{-1}) were normal. Only one animal suffered from chronic diarrhoea and showed no growth at all (Avilamycin diet). It was decided to discard all results obtained with this piglet, thus decreasing the number of animals (observations) on this diet to four.

Diet 3 contained 0.1% of Lentinan powder (min. 25% of polysaccharides), which should correspond to approximately 0.025% of β-glucans. The amount of β-glucans in diet 4 (5% of dried and ground *L. edodes* mycelium) was not known.

The results of the Weende analysis are shown in Table II. The slightly lower protein content of the diet containing *L. edodes* was due to the lower crude protein content of *L. edodes* mycelium. A somewhat higher TDF content of this diet was the result of the high fiber content of the dried mycelium. These small differences were not important as comparison of effects on growth parameters was not the aim of the experiment.

3.2. Bacterial counts

Table III shows the results of viable counts of total bacteria, *E. coli*, streptococci and lactic acid bacteria. Compared with the control, only with the *L. edodes* diet were significant decreases in numbers of all bacterial groups observed in digesta (stomach, jejunum) and mucosal scrapings of the small intestine at both sites (J1 and J2). Effects of the diet supplemented with Avilamycin and Lentinan were negligible. The number of

TABLE II Chemical components (Weende analysis) of the diets and *L. edodes* mycelium [g kg^{-1}]

Diet	*Dry matter*	*Org. matter*	*Crude ash*	*Crude protein*	*Crude fat*[1]	*TDF*[2]
Control	886.1	827.2	58.9	184.0	63.4	173.9
Avilamycin	894.1	831.0	63.1	190.2	64.8	186.1
Lentinan	894.4	828.8	65.6	177.1	64.7	170.7
L. edodes	891.2	824.7	66.5	169.0	61.6	190.7
L. edodes mycelium	893.1	850.4	42.7	155.6	6.9	467.5

[1]After acid hydrolysis.
[2]Total dietary fibre (corrected for ash and protein in TDF).

TABLE III Effect of various diets on viable counts [$\log_{10}$ CFU g^{-1}] in samples of digesta and mucosa[1]

Bacteria	*Diet*	*Stomach*	*Jejunum 1*	*Jejunum 2*	*Caecum*	*Mucosa 1*	*Mucosa 2*
Total count	Control	6.9^{A}	6.9^{A}	7.5^{A}	7.9^{A}	5.6^{a}	5.7^{ab}
	Avilamycin	6.9^{A}	6.4^{B}	7.0^{AB}	8.1^{A}	5.3^{ab}	5.5^{ab}
(RCM)	Lentinan	6.6^{A}	6.4^{B}	7.3^{AB}	8.0^{A}	5.2^{ab}	6.0^{a}
	L. edodes	6.5^{A}	5.4^{C}	6.8^{B}	7.7^{A}	4.8^{b}	5.2^{b}
	RSD[2]	0.6	0.4	0.5	0.5	0.4	0.5
	Effect diet $P =$	0.58	0.00	0.22	0.72	0.08	0.11
E. coli	Control	2.0^{a}	3.7^{a}	4.3^{a}	n.d.[3]	4.0^{A}	4.2^{A}
	Avilamycin	2.4^{a}	3.5^{a}	4.5^{a}		3.6^{BC}	3.9^{AB}
(EMB)	Lentinan	1.4^{ab}	3.4^{a}	4.8^{a}		4.0^{AC}	4.3^{A}
	L. edodes	0^{b}	1.8^{b}	2.3^{b}		3.5^{B}	3.4^{B}
	RSD	1.5	1.1	1.2		0.4	0.7
	Effect diet $P =$	0.14	0.07	0.02		0.13	0.23
Streptococci	Control	5.6^{a}	5.6^{a}	5.3^{A}	n.d.	3.9^{A}	4.1^{A}
	Avilamycin	5.1^{a}	4.7^{a}	3.8^{A}		2.8^{A}	3.3^{A}
(Slan.& Bartley)	Lentinan	5.6^{a}	5.2^{a}	5.4^{A}		4.0^{A}	4.6^{A}
	L. edodes	3.3^{b}	2.8^{b}	4.6^{A}		3.0^{A}	3.7^{A}
	RSD	0.9	1.2	1.9		1.1	1.3
	Effect diet $P =$	0.02	0.01	0.59		0.29	0.51
Lactic acid bacteria	Control	7.3^{A}	7.3^{a}	8.0^{a}	n.d.	5.5^{a}	5.9^{A}
	Avilamycin	7.8^{A}	6.9^{a}	7.4^{ab}		5.2^{a}	5.7^{A}
(Rogosa)	Lentinan	7.3^{A}	6.9^{a}	7.7^{a}		5.3^{a}	6.0^{A}
	L. edodes	6.0^{B}	5.4^{b}	6.8^{b}		4.6^{b}	5.2^{B}
	RSD	1.1	0.7	0.6		0.4	0.4
	Effect diet $P =$	0.14	0.005	0.04		0.01	0.03

Mean values bearing different superscripts are significantly different at $P < 0.05$ (a,b,c) or $P < 0.01$ (A,B,C).
[1]Mucosa 1 and 2 resp. jejunum 1 and 2.
[2]RSD: Residual standard deviation.
[3]n.d.: not determined.

bacteria per g of digesta was much higher than in mucosal samples, except in the case of *E. coli*. It is also interesting to note that decreases in bacterial counts in jejunal contents, caused by *L. edodes* supplementation, could also be observed in mucosal samples indicating that effects induced in the intestinal lumen were reflected on the mucosa or *vice versa*.

3.3. Biochemical parameters in the intestinal tract

pH values were not altered by the various diets, except in the stomach of piglets receiving the *L. edodes* diet where lower values were observed (2.3 *vs.* 3.6 for control). In the proximal jejunum, the only effect that could be demonstrated was a decrease in acetic acid concentration with the *L. edodes* diet (control diet 1.4 mmol kg^{-1} *vs.* 0.8 mmol kg^{-1}). In the distal part however, this diet caused higher acetate (control diet 3.5 mmol kg^{-1} *vs.* 6.1 mmol kg^{-1}) and butyrate (0.5 mmol kg^{-1} *vs.* 1.0 mmol kg^{-1}) concentrations. No differences in lactic acid and ammonia N were observed for the various diets. In caecal contents only the concentration of valeric acid was altered. Compared with the control feed, higher amounts were found with the three experimental diets (control diet: 0.7%; Avilamycin: 2.3%; Lentinan: 2.6%; *L. edodes*: 1.4%). For the four diets, there was no difference in the concentration of total SCFA (approx. 100 mmol kg^{-1}).

3.4. Morphometry and histological analysis of the small intestine

The influence of the various diets on villus length (V), crypt depth (C) and V/C is shown in Table IV. Feeding diets supplemented with Lentinan and *L. edodes* resulted in higher villi, although only statistically significant with the latter diet. Crypt depth was not changed, but V/C was higher with both diets. Enumeration of goblet cells on villi and in crypts revealed no differences between the four diets (results not shown), except with the *L. edodes* feed where in the distal part of the jejunum, small increases were noted (villi: control 1.4 cells per 100 μm of circumference vs. 1.9 cells for *L. edodes* feed; crypts: 9 cells per 100 μm depth vs. 10.7 cells). IEL are one of the components of the mucosal immune system and are involved in the elimination of damaged or infected cells (Cerf-Bensussan and Guy-Grand, 1991; Gaskins, 1997). Therefore, it was decided to determine the number of IEL (per 100 enterocytes) on HE stained sections (Table V). Diets supplemented with β-glucans resulted in lower counts of IEL in the villous epithelium of proximal and distal jejunum.

Turnover rate of the intestinal mucosa is the resultant of cell proliferation in the crypts and loss of cells at the villus tips. As it was thought that apoptotic- and mitotic index could provide information concerning turnover rate of the intestinal mucosa, both parameters were determined and results given in Table VI. Only the distal site of

TABLE IV Influence of the various diets on villus length [μm] and crypt depth [μm] at both sites of jejunum

	Jejunum 1			*Jejunum 2*		
Diet	*Villi [V]*	*Crypts [C]*	*V/C*	*Villi [V]*	*Crypts [C]*	*V/C*
Control ($n = 5$)	424^{BD}	321^{A}	1.33^{c}	413^{AB}	300^{A}	1.38^{BC}
Avilamycin ($n = 4$)	391^{CD}	294^{A}	1.34^{bc}	361^{B}	298^{A}	1.21^{C}
Lentinan ($n = 5$)	443^{AB}	292^{A}	1.54^{a}	431^{AB}	276^{A}	1.56^{AB}
L. edodes ($n = 5$)	470^{A}	302^{A}	1.58^{a}	473^{A}	302^{A}	1.59^{A}
RSD[1]	43	28	0.08	77	32	0.17
Effect diet $P =$	0.09	0.38	0.00	0.22	0.54	0.02

[1]RSD: Residual standard deviation.
Mean values per column bearing different superscripts are significantly different at $P < 0.05$ (a,b,c) or $P < 0.1$ (A,B,C).

TABLE V Number of intra–epithelial lymphocytes (IEL per 100 enterocytes) on villi in proximal and distal jejunum

Diet	*Jejunum 1*	*Jejunum 2*
Control ($n = 5$)	35.1 ^{AC}	36.4 ^{a}
Avilamycin ($n = 4$)	41.4 ^{A}	33.1 ^{a}
Lentinan ($n = 5$)	26.5 ^{B}	23.8 ^{b}
L. edodes ($n = 5$)	29.2 ^{BC}	22.8 ^{b}
RSD[1]	7.4	5.2
Effect diet $P =$	0.04	0.002

[1]RSD: Residual standard deviation.
Mean values per column bearing different superscripts are significantly different at $P < 0.05$ (a,b,c) or $P < 0.1$ (A,B,C).

TABLE VI Mitotic (crypts) and apoptotic (villi) index in distal jejunum[1]

Diet	*Mitotic index*	*Diet*	*Apoptotic index*
Control ($n = 5$)	61.6 ^{AB}	Control ($n = 5$)	43.6 ^{A}
Avilamycin ($n = 3$)	60.8 ^{AB}	Avilamycin ($n = 3$)	9.4 ^{B}
Lentinan ($n = 4$)	65.1 ^{A}	Lentinan ($n = 5$)	19.9 ^{B}
L. edodes ($n = 5$)	57.8 ^{B}	*L. edodes* ($n = 4$)	9.6 ^{B}
RSD [2)]	5.5	RSD	19.2
Effect diet $P =$	0.31	Effect diet $P =$	0.09

[1]Mitotic index: Mitotic cells in crypts (perfect orientation) were counted and expressed as % of total cells in the crypt (between 5 and 13 crypts depending on piglet).
Apoptotic index: The number of apoptotic enterocytes were counted on all intact villi on a section (varied between 23 and 203 villi depending on piglet) and then expressed per 100 villi.
[2]RSD: residual standard deviation.
Mean values per column bearing different superscripts are significantly different at $P < 0.05$ (a,b,c) or $P < 0.1$ (A,B,C).

the jejunum was considered. A lower apoptotic index was found after feeding the three supplemented diets. Mitotic index was slightly lower in piglets receiving the *L. edodes* feed. The effect on both indexes suggested a lower intestinal mucosal turnover rate feeding the latter diet.

4. DISCUSSION

4.1. Bacteriological enumeration

Total counts in the small intestine were about the size of Rogosa counts indicating that lactic acid bacteria were by far the most important fraction of the flora. Total counts on caecal contents were rather low in comparison to published data (Jensen, 1999), which could be due to the fact that measures taken to maintain strict anaerobiosis during dilution and inoculating were insufficient. Only in the piglets fed the *L. edodes* diet clear and consistent effects on bacterial numbers in stomach contents, in luminal contents and on the mucosa of the jejunum were observed. The total bacterial count in caecal contents was not altered. The higher bacterial numbers in luminal contents compared with the mucosa are in agreement with earlier work (Muralidhara *et al.*, 1977). However, this was not valid for *E. coli* numbers (Table III), possibly explained by the fact that this species is able to express fimbrial adhesins enabling a more efficacious

adherence to receptor glycans in the intestinal mucosa, compared with other bacterial groups (Stewart *et al.*, 1993). However, some caution is indicated as certainly not all *E. coli* strains express fimbriae. In general, it can be suggested that decreasing bacterial counts by the *L. edodes* feed was a favourable effect. Despite the fact that lactobacilli are presumed to be beneficial, lowering their numbers in the lumen or on the mucosa of the small intestine is not necessarily a negative factor, as far as growth of the animal is concerned. Indeed, antibiotics known as growth promotors (*e.g.* Virginiamycin, Avoparcin, Avilamycin, Flavomycin, ionophores) are all active against gram-positive organisms including lactobacilli (Anderson *et al.*, 1999). The bacterial load on the mucosa was lowered, possibly resulting in a slower turnover rate of this tissue coupled with lower endogenous losses (Visek, 1978; Corring *et al.*, 1981; Parker, 1990). A further benefit for the host animal could be the decreased competition for specific substrates (growth factors, e.g. vitamins) by the intestinal flora. Finally, it is very unlikely that influences on the intestinal flora with the *L. edodes* diet could be due to the very small differences observed in nutrient composition of this diet compared to the control feed.

4.2. Biochemical parameters

The lower pH measured in the stomach of piglets receiving the *L. edodes* feed could be related to a slower gastric emptying, but relating this phenomenon to *L. edodes* feeding is merely speculative (Kidder and Manners, 1978; Low, 1990). Feeding this diet also decreased the concentration of acetic acid in the proximal jejunum, which may be related to the lower counts of streptococci. However, in the distal part, acetate and butyrate were increased with the same diet, although bacterial counts were lower. Other mechanisms must be responsible for this observation (e.g. rate of passage, absorption rate). SCFA and in particular butyrate are known to exert trophic effects on enterocytes and colonocytes (Sakata, 1987, 1997; Salminen *et al.*, 1998).

Compared with the control, none of the diets caused changes in the concentration and pattern of metabolites in caecal contents, except for an increase in valeric acid proportions when the Avilamycin, Lentinan and *L. edodes* diets were fed. However, valeric acid proportions in the caecum were very low and unlikely to have nutritional significance, although valerate can act as a growth factor for bacterial species.

4.3. Morphometry and histological determinations

The higher V/C in piglets fed the Lentinan and *L. edodes* diets was indicative of a lower turnover rate of the intestinal mucosa, although interpretation of these results should be done with some caution in view of the relatively small number of animals involved ($n = 5$). It is generally accepted that an increase in villus length, in combination with lower or unaltered crypt depths causes a slower migration rate of enterocytes along the villi and decreased enterocyte loss from the villi. This situation can result in improved digestive and absorptive capacities of the small intestine as fully mature enterocytes have a higher activity of brush border enzymes (Hampson, 1986; Hampson and Kidder, 1986; Li *et al.*, 1990; Pluske *et al.*, 1997).

The small increase in number of goblet cells on villi and crypts observed when the *L. edodes* diet was fed is not fully understood, nor its implication for digestion. The number and phenotype of IEL are influenced by microbial colonization (bacterial load)

and hence by diet composition (Gaskins, 1997). Furthermore, a positive relationship between the number of IEL in the small intestinal mucosa and its turnover rate had been reported earlier (Guy-Grand *et al*., 1998; Nishiyama *et al*., 2002). Feeding both diets supplemented with specific β-glucans resulted in lower numbers of IEL. This observation was in agreement with the lower bacterial counts, seen only in the case of the *L. edodes* feed but not with Lentinan. This suggested that besides bacterial load on the mucosa, other factors must be involved in the migration of lymphocytes from the *lamina propria* to the villus epithelium.

Cell proliferation in the crypts and loss of epithelial cells at the villus tips are responsible for maintaining homeostasis of the normal intestinal epithelium (Hall *et al*., 1994; Jin *et al*., 1994; Potten *et al*., 1997). Epithelial cell proliferation in rats was stimulated by the presence of gut microbes, luminal trophical factors and feeding of probiotic bacteria (Sakata, 1987; Ichikawa *et al*., 1999). Compared with the control diet, the apoptotic index was lowered with all three supplemented diets. However, the number of apoptotic enterocytes on the villi was small. In their experiments with mice, Hall *et al*. (1994) calculated that under normal conditions, on average only one apoptotic cell per villus could be found, most frequently at the tip of the villi. This did not mean that apoptosis was not an important factor in the turnover rate of the small intestinal epithelium but apoptotic cells are cleared very rapidly through phagocytotic action of macrophages or even adjacent enterocytes (Iwanaga *et al*., 1994). Taking this into account, it can be questioned whether it is meaningful to perform counts of apoptotic cells. Perhaps only when drastic (pathological) effects on the small intestine are expected, determination of the apoptotic index is warranted. As IEL play a role in the induction of apoptosis, it is tempting to relate the lower IEL counts observed with both specific β-glucans supplemented diets to the decreased apoptotic index (Iwanaga, 1995; Shiner *et al*., 1998). Although statistical significance was not reached, the lowest mitotic index was found in piglets receiving the *L. edodes* feed, which was in agreement with the lower bacterial numbers (Sakata, 1987; Ichikawa *et al*., 1999).

The effect of *L. edodes* mycelium on the bacterial flora in the small intestine was originally thought to be the result of an immunostimulating action at the mucosal level with increased release of IgA onto the mucosa surface. Further careful consideration of the results however pointed to another possible explanation. It has been reported that several antibacterial substances (lenthionine, lenthionine disulphide, lentinamycin and various terpenoids and phenols) are present or could be extracted from dried *L. edodes* mycelium (Borchers *et al*; 1999; Hirasawa *et al*., 1999; Ishikawa *et al*., 2001). Taking into account the rather high amount of the dried mushroom in diet 4 (5% by weight), it is not impossible that these antibacterial substances were at least partially responsible for the lowered bacterial counts in the gastrointestinal tract. The lack of a consistent effect with Lentinan supplementation can also be explained. Lentinan is the result of a water or water/ethanol extraction of the dried *L. edodes* mycelium and Hirasawa *et al*. (1999) showed that the fraction extracted using this procedure had the weakest antibacterial action, compared with chloroform or ethyl acetate extraction. Therefore, 0.1% of Lentinan in the feed was probably insufficient to cause consistent effects on bacterial numbers, although a concentration of 0.025% of β-glucans (MacroGard) has been used to induce immunomodulation in pigs (Dritz *et al*., 1995; Decuypere *et al*, 1998; Hiss and Sauerwein, 2003). Finally, the experimental design did not permit to exclude the immunological aspect with increased IgA release at the mucosal level.

5. CONCLUSIONS

Results obtained in this experiment have shown that in piglets receiving the diet supplemented with *L. edodes* mycelium, all parameters involved indicated a lower turnover rate of the small intestinal epithelium and it seems reasonable to relate this phenomenon to the lower bacterial load. The effect of Lentinan was much less evident and inconsistent, probably due to the small amount administered (0.1%). Finally, whether the effects of *L. edodes* mycelium on intestinal parameters are reflected in improved growth performances of pigs and piglets remains to be investigated in properly designed experiments. Other important factors that need to be considered are: further characterization of the product (*e.g.* β-glucans content, identification of antibacterial compounds and their effect on intestinal bacteria), optimal dose in the feed and administration period (before and/or after weaning), and the possibility of the flora to develop adaptation or resistance to the antibacterial compounds.

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