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Fenugreek seed affects intestinal microbiota and immunological variables in piglets after weaning

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Abstract

Fenugreek seed has been shown to affect the intestinal microbiota and immunological responses in animals. A feeding trial with male castrated piglets was performed over 28 d without or with the addition of 1·5 g fenugreek seeds/kg complete diet in ten and eleven piglets, weaned at 21 d. In the intestinal tract, pH, lactate and SCFA were measured as major bacterial metabolites. Immune cell phenotypes, phagocytic activity and lymphocyte proliferation after stimulation with pokeweed mitogen, concanavalin A and phytohaemagglutinin M were measured by flow cytometry. Health status and performance of the piglets were not affected by fenugreek. The pH in the caecum and colon were reduced compared with the control ($P<0·05$). Higher concentrations of L-lactic acid were recorded in the small-intestinal digesta (average concentrations from the duodenum, jejunum and ileum; $P<0·05$), while the concentrations of SCFA remained unchanged except an increase in *n*-butyric acid in colon contents ($P<0·05$). The piglets fed the fenugreek diet had higher *Lactobacillus* and clostridium cluster I concentrations and lower *Escherichia*, *Hafnia* and *Shigella* concentrations in the small intestine. The addition of fenugreek increased the relative concentration of the γδ T-cell population ($TCR1^+CD8\alpha^-$) in the blood with a simultaneous reduction of antigen-presenting cells ($MHCII^+CD5^-$) ($P<0·05$). Proliferation rate and phagocytosis activity of monocytes were not affected by the additive. In conclusion, fenugreek seeds might be interesting as a feed ingredient for young piglets due to their effects on the intestinal microbiota and immunological variables. The impact on performance and animal health has to be further evaluated.

Key words: **Piglets: Fenugreek: Lactate: SCFA: Lymphocytes**

Fenugreek (*Trigonella foenum graecum* L.) is a legume mainly cultured in the Middle East and in Asia. Fractions of fenugreek have different therapeutic properties, for instance antidiabetic, cholesterol-lowering, antioxidant and anti-inflammatory effects[1]. The seed is interesting as a dietary ingredient in human and animal nutrition due to some remarkable bioactive properties. Fenugreek seeds contain a variety of constituents. Those which are functionally interesting are mainly polysaccharides and flavonoids. They contain 32 % insoluble and 13·3 % soluble fibre, and have high concentrations of galactose and mannose[2]. The galactomannan content of fenugreek varies depending on growth conditions and genotype and can be 12·8–21·5 %[3]. The hypoglycaemic and hypocholesterolaemic effects seem to be mainly linked with the specific composition and biological activity of carbohydrates and the high saponin content[1,4]. There is increasing evidence that fenugreek can affect immunological variables in humans and animals. Isolated galactomannans from fenugreek increased the phagocytic rate of peritoneal macrophages in rats[5]. The galactomannans used in the same study resulted in an activation of human lymphoma cells, increasing the secretion of IgM. The alkaline-extracted polysaccharide fraction was more effective to stimulate the phagocytic activity than the aqueous extract. In mice, the use of an aqueous fenugreek seed extract at 50, 100 and 250 mg/kg body weight had a dose-dependent effect on different immune parameters[6]. In obese rats, fenugreek-supplemented diets reduced liver weight and circulating levels of TNF-α[7]. In a study with cyclophosphamide-treated rats, fenugreek alleviated leucopaenia, and increased weights and cellularity of lymphoid organs. Serum γ-globulin levels and delayed type of hypersensitivity response were higher in treated rats compared with the control group[8].

Recently, it has been shown that fenugreek addition to bacterial cultures from porcine colonic digesta resulted in a significant amount of acetate, propionate and butyrate formation[9]. Besides their potential as a microbial substrate, galactomannans from fenugreek can effectively interfere with ganglioside GM1-binding sites of cholera toxin and of human and porcine *Escherichia coli* heat-labile enterotoxin[10].

Abbreviation: MHCII, major histocompatibility complex class II molecules.

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This would offer perspectives for interventional nutrition with the potential of preventing bacterial enterotoxin-induced diarrhoea, especially in the young piglet after weaning.

Up to now, no feeding studies have been published on fenugreek in pigs to our knowledge. The seed might be interesting due to its potential impact on the intestinal microbiota and its immune-modulating effects. The present study hypothesised that these effects might affect the digestive physiology of piglets after weaning. We have demonstrated an impact of a diet supplemented with 1·5 g fenugreek seed/kg on the intestinal microbiota in piglets and on selected traits of the immune system.

Experimental methods

Animals and housing

The experiment was approved according to the rules of the German Animal Welfare Law by the Landesamt für Gesundheit und Soziales, Berlin (registration no. 0267/08). A total of twenty-one male castrated piglets (Duroc × Piétrain), weaned at day 21 with an average body weight of 8·26 (SD 0·83) kg, were used in the study. The piglets were randomly allocated into the control group with eleven animals and the fenugreek group with ten animals. The animals were kept pairwise over a period of 4 weeks in flat-deck pens on a plastic slatted floor. The room temperature was 28°C initially and was decreased to 25°C within 10 d. Humidity was kept constant at approximately 65 %. A light programme ensured a 16 h light and 8 h dark phase. Daily feed intake was recorded for each pen and the individual body weights of the animals were recorded weekly. Health status was examined daily for clinical signs of disease, in particular the presence of diarrhoea.

Feed and feed analysis

The animals had free access to water. The daily feed allowance was adjusted according to the calculated energy requirements. The diets were offered as mash and met the requirements for piglets at this age[11,12] (Table 1). The proximate nutrient concentrations of the feed were determined by Weende analysis[13]. Ca, P and Na were determined after ashing using atomic absorption spectrometry. P was measured by the ammonium vanadate/molybdate method. Chromium oxide was included as an indigestible marker for the measurement of nutrient digestibility (data not reported). The galactomannan concentration of the fenugreek seed was 28 % and the concentration of apigenin-7-glycoside was 0·672 % (analysis by Belan Laboranalytik).

Table 1. Ingredients and nutritional composition of the basal piglet diet (as-fed basis)

Ingredients (%)	
Wheat	36·00
Barley	30·00
Soyabean meal	18·00
Maize	10·00
Limestone	1·40
Monocalcium phosphate	1·19
Premix*	1·18
L-Lys	0·20
DL-Met	0·04
Salt	0·29
Soyabean oil	1·50
Chromium(III) oxide	0·20
Fenugreek seed†	–
Analysed chemical composition (%)	
DM	89·3
Crude protein	17·0
Crude ash	5·15
Crude fibre	48·2
N-free extracts	59·5
Crude fat	2·75
Starch	28·7
Ca	0·97
P	0·69
K	0·68
Na	0·31
Calculated amino acid (%) and energy content	
Lys	0·85
Met	0·44
Thr	0·58
Trp	0·28
Metabolisable energy (MJ/kg)	12·75

* Vitamin and mineral premix (Spezialfutter Neuruppin) containing per kg: 600 000 IU vitamin A (36 000 μg retinol), 120 000 IU vitamin D_3 (3000 μg cholecalciferol), 8000 mg vitamin E, 300 mg vitamin K, 250 mg thiamin, 250 mg riboflavin, 2000 μg cobalamine, 2500 mg nicotinic acid, 25 000 μg biotin, 100 mg folic acid, 1000 mg pantothenic acid, 133 g Na (NaCl), 55 g Mg (magnesium oxide), 5000 mg Fe (Fe(II) carbonate), 1000 mg Cu (copper(II) sulphate, pentahydrate), 5000 mg Zn (zinc oxide), 6000 mg Mn (manganese(II) oxide), 45 mg I (calcium iodate), 35 mg Se (sodium selenite).

† Fenugreek seed was added at 1·5 g/kg diet only for the fenugreek group.

Sampling

At days 28 and 29 of the experiment, blood samples were taken from the anaesthetised animal by cardiac puncture by a blood collecting system (S-Monovette®; Sarstedt). The piglets were killed 2 h after their last meal. They were anaesthetised with ketamine hydrochloride (Ursotamin®, 10 %; Serumwerk Bernburg AG) and azaperone (Stresnil®; Jansen-Cilag) (0·2 ml/0·1 ml per kg body mass), and killed with tetracaine hydrochloride, mebezonium iodide and embutramide (T61®; Intervet). Subsequently, the stomach, distal jejunum, ileum and the caecum and colon contents were removed.

Bacterial cell concentrations in the stomach and gastrointestinal segments

Quantification of bifidobacteria, enterobacteria, lactobacilli (*L. amylovorus*, *L. reuteri* and *L. johnsonii)*, the *Escherichia/Hafnia/Shigella* group, clostridium clusters I and XIVa and streptococci in the jejunal and caecal digesta was accomplished by quantitative PCR. Total nucleic acids were extracted by shearing 1 g sample with a 4 M-guanidinisothiocyanate solution and 3 g of glass beads in a bead beater. After phenol–chloroform extraction, the nucleic acids were collected by isopropanol precipitation and purified with commercial spin columns (Macherey-Nagel). DNA content was determined by fluorometric quantification (NanoDrop ND

3300; Fisher Scientific) using the Hoechst 33 258 dye and calf thymus DNA as a reference. Cell numbers for the *Escherichia* group (*Escherichia* spp., *Hafnia* spp. and *Shigella* spp.) were detected with a Taqman assay[14]. Lactobacilli[15], bifidobacteria[16], clostridium clusters I and IV[17] as well as *L. reuteri*, *L. johnsonii* and *L. amylovorus*[18] were detected using the stated published primer sequences. Streptococci were detected using the following primer combination, which was validated in the institute: Str1-f 5′-CCG CAT AAC AGC TTT TGA CA-3′ and Str1-r 5′-GGT AGG CCG TTA CCC TAC CT-3′. All primers were purchased from MWG Biotech. A Stratagene MX3000p (Stratagene) was used for PCR amplification and fluorescent data collection. The mastermix consisted of 12·5 μl Brilliant SYBR Green QPCR Mastermix (Stratagene) or 12·5 μl HotStartTaq Mastermix (Qiagen) for Taqman assays, 0·5 μl of each primer (10 μM), 0·75 μl 5-carboxy-X-rhodamine (ROX) reference dye (1:500 diluted) and 10·75 μl water. Before PCR amplification, 1 μl sample was added. All amplification programmes included an initial denaturation step at 95°C for 15 min to activate the polymerase. All PCR programmes featured an annealing time of 30 s, and a 30 s extension at 72°C. The annealing temperature was 50°C for *Escherichia* cell numbers, 55°C for lactobacilli, streptococci, both clostridium clusters as well as *L. reuteri*, and 58°C for bifidobacteria, *L. johnsonii* and *L. amylovorus*. As a quantification procedure[19], a series of autoclaved pig faecal samples were spiked with different bacterial species and known cell numbers (10^9–10^3 cells/g wet weight). After extraction and purification, these extracts were used as PCR calibration samples, and the results were expressed as cell numbers/g sample wet weight.

pH and bacterial metabolites in the digesta

pH was measured in the digesta by a pH electrode connected to a pH meter (seven Multi; Mettler Toledo). D- and L-lactate concentrations in the digesta of intestinal contents were determined by a HPLC system (Agilent 1100; Agilent Technologies). For the analysis of lactate, 0·5 g digesta were mixed at a ratio of 1:1 with ice-cold 0·5 M-copper sulphate solution. After centrifugation, the supernatant was mixed with 50 μl of 85 mM-$K_4[Fe(CN)_6].3H_2O$ (Carrez I), and incubated on ice for 5 min. Then, 50 μl of 250 mM-$ZnSO_4.7H_2O$ (Carrez II) were added and the samples were again incubated on ice for 5 min. The supernatant was filtered through cellulose acetate syringe filters and separated on a Phenomenex Chirex 3126 (D)-penicillamine (150 × 4·6 mm; Phenomenex Company) fitted with a Phenomenex pre-column (C18 4·0 mm × 2·0 mm inner diameter; Phenomenex Company). For the analysis of SCFA, 300 mg digesta were diluted in distilled water, homogenised and centrifuged (Heraeus Instruments) at 13 000 rpm for 15 min. Hexanic acid was used as an internal standard (0·5 mmol/l). The sample (1·0 μl) was analysed by a gas chromatograph (Model 19095N-123; Agilent Technologies), fitted with a HP-INNOWax column A (length 30 m, internal diameter 530 μm, with a film thickness of 1·0 μm). The initial temperatures of the oven, injector and FID detector were 70, 230 and 250°C. Hydrogen gas was used as a carrier gas (gas generator: Parker ChromGas; Parker Hannifin Corporation) at a flow rate of 30 ml/min.

Blood count

Blood count was carried out with EDTA blood (Laboklin).

Flow cytometry for immune cell phenotyping

Peripheral blood mononuclear cells were isolated by density gradient centrifugation using Ficoll. Flow cytometry was carried out as described previously[20]. Staining of purified immune cell preparations for CD4 and CD8α was performed with labelled primary antibodies in a one-step incubation. All other immune cell antigens were detected using unlabelled primary antibodies followed by washing and incubation with a fluorescence-labelled secondary antibody (see below). Cells were assayed by flow cytometry using a FACSCalibur flow cytometer equipped with a 488 nm argon laser (FACS Calibur; Becton Dickinson).

Labelled primary antibodies used for flow cytometry are as follows: mouse anti-porcine CD4 clone 74-12-4, conjugated to fluorescein isothiocyanate; mouse anti-porcine CD8α clone PT8, conjugated to R-phycoerythrin. Unlabelled primary antibodies used for flow cytometry are as follows: mouse anti-porcine CD2, clone MSA4 (kind gift from Professor A. Saalmüller, University of Veterinary Medicine Vienna, Vienna, Austria); mouse anti-porcine CD5, clone b53b7 (gift from Professor A. Saalmüller); mouse anti-porcine CD8β clone PG164A (VMRD); mouse anti-porcine CD14, clone MIL2 (Serotec); mouse anti-porcine CD21, clone BB6-11C9.6 (Southern Biotech); mouse anti-porcine CD25, clone K231.3B2 (Acris); mouse anti-porcine TcR1-N4 (δ-chain), clone PGBL22A (VMRD); mouse anti-porcine MHCII, clone MSA3 (VMRD); mouse anti-porcine CD45 RC, clone 3a56 (gift from Professor A. Saalmüller); mouse anti-porcine CD172a, clone 74-22-15A (BD Pharmingen). Secondary antibodies are as follows: goat anti-mouse IgG, conjugated to fluorescein isothiocyanate or to phycoerythrin (PE) (Southern Biotech).

Phagocytosis assay

Phagocytosis of opsonised fluorescein isothiocyanate-labelled *E. coli* bacteria was measured using the commercial test kit Phagotest™ following the instructions of the manufacturer (PHAGO-Test®; ORPEGEN Pharma GmbH).

Proliferation assay

The assay was carried as follows: leucocytes (4×10^5 cells/well) were cultured in ninety-six-well flat-bottom tissue culture plates. To stimulate peripheral blood mononuclear cells, pokeweed mitogen (2·5 μg/ml), concanavalin A (5 μg/ml) and phytohaemagglutinin (10 μg/ml) were used. Incubation was performed at 37°C and 100 % relative humidity in a 5 % CO_2 atmosphere. After 48 h of incubation, bromodeoxyuridine (60 μM) was added and cells were incubated under the same conditions for another 24 h. To measure bromodeoxyuridine

incorporation, cells were fixed with ethanol (10 min on ice). Cells were washed and stained with a fluorescein isothiocyanate-conjugated antibody (mouse anti-bromodeoxyuridine; BD Pharmingen™) according to the manufacturer's instructions. The incorporation was detected using a FACSCalibur flow cytometer (Becton Dickinson).

Statistical analysis

Statistical analysis was conducted using the statistical program PASW 18.0 (IBM). Data distribution was tested using the Kolmogorov–Smirnov test with Lilliefors correction. For normally distributed data, a two-sided *t* test was performed. For non-normally distributed data, the Mann–Whitney test was applied. A *P* value of $\leq 0{\cdot}05$ was considered significant for all data. The experimental unit for the performance data (body weight, weight gain, feed intake and feed conversion) was the pen, and for the post-mortem data the individual.

Results

All animals showed an unimpaired general condition; diarrhoea did not occur. Initial body weight (8·23 (SD 0·78) *v.* 8·30 (SD 0·86) kg), final body weight (16·71 (SD 1·51) *v.* 17·43 (SD 1·30) kg), daily weight gain (0·30 (SD 0·04) *v.* 0·33 (SD 0·03) kg), daily feed intake (0·59 (SD 0·10) *v.* 0·64 (SD 0·10) kg) and feed conversion (1·96 (SD 0·25) *v.* 1·95 (SD 0·24)) were comparable in the control group and the fenugreek group.

pH and bacterial metabolites in the digesta

Compared with the control group, the pH was reduced in the fenugreek group in the caecum and colon (Table 2). A significant difference between the control animals and the fenugreek group was observed in the L-lactic acid concentrations in the small intestine ($P<0{\cdot}05$). The lactic acid concentration declined in the large-bowel digesta and was not different between the two groups. The total concentration of SCFA in the small intestine was very low and showed no feed-related differences. In the caecum, the levels of SCFA increased. However, absolute levels and the distribution of the acids were similar. In the colon digesta, butyric acid was higher in piglets fed the fenugreek diet compared with the control group ($P<0{\cdot}05$).

Bacterial cell numbers in the jejunal and caecal digesta

The bacterial cell numbers in the jejunum are shown in Table 3. Compared with the control diet, the counts of the *Lactobacillus* group were higher when feeding fenugreek ($P=0{\cdot}018$). A cell count increase was evident for *L. johnsonii* ($P=0{\cdot}016$). The jejunal concentration of the *Escherichia* group was significantly reduced by the addition of fenugreek ($P=0{\cdot}048$). Ingestion of fenugreek led also to increased cell numbers of clostridium cluster I ($P=0{\cdot}048$), while clostridium cluster XIV remained stable. In the caecum, *L. reuteri* and *L. johnsonii* were increased ($P=0{\cdot}034$ and 0·016) by the addition of fenugreek. The differences were only marginal for the other groups and species.

Blood counts, immune cell phenotyping and proliferation assays

Among the haematological parameters and the differential blood counts, no differences between the feeding groups were identified (data not shown). The piglets fed with fenugreek seed showed somehow altered composition of peripheral blood mononuclear cells (Table 4). The phenotypic analysis of circulating T cells showed a relative increase in $CD8^-$ γδ T cells, while no changes were observed concerning the portions of circulating $CD8^+$γδ T cells. The percentages of

Table 2. pH and concentrations of D- and L-lactic acid and SCFA in the small intestine, caecum and colon digesta in piglets fed the control or fenugreek diet

(Mean values and standard deviations)

Diet...	Gut segment											
	Small intestine†				Caecum				Colon			
	Control		Fenugreek		Control		Fenugreek		Control		Fenugreek	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
pH	6·1	0·61	5·4	0·52	5·7	0·37	5·3*	0·24	6·0	0·23	5·5*	0·36
Lactic acid (mmol/l)												
D	15·8	21·5	17·4	7·80	4·81	14·6	7·31	12·6	ND		ND	
L	24·5	13·5	40·3*	17·9	4·91	4·42	7·73	12·9	ND		ND	
SCFA												
Total (mmol/l)	2·40	2·87	4·31	3·26	101·2	22·6	106·5	21·0	110·0	15·1	111·1	21·7
Acetic acid (mol%)	82·5	6·48	77·2	6·17	57·0	4·04	55·8	3·12	58·4	3·75	55·4	4·65
Propionic acid (mol%)	10·1	5·82	10·7	5·02	29·7	3·25	30·5	3·98	26·5	4·72	26·6	3·55
i-Butyric acid (mol%)	0·77	2·05	0·73	0·91	0·90	1·97	0·36	0·24	0·68	3·57	0·68	0·55
n-Butyric acid (mol%)	2·14	1·76	3·68	2·17	9·91	2·38	11·1	2·99	11·1	1·72	13·5*	1·32
i-Valeric acid (mol%)	3·63	3·41	6·66	6·97	0·30	0·07	0·31	0·24	0·76	0·37	0·80	0·72
n-Valeric acid (mol%)	0·87	0·53	1·02	0·86	2·18	1·86	2·07	1·13	2·57	1·02	2·99	1·12

ND, not determined.
*Mean value was significantly different from that of the control group ($P<0{\cdot}05$).
† Data represent the average concentrations from three sampling sites (duodenum, jejunum and ileum), except the pH, that was measured in the proximal jejunum.

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Table 3. Bacterial groups and species in the jejunal and caecal digesta of piglets fed the control or fenugreek diet
(Mean values and standard deviations)

Diet...	Jejunum				Caecum			
	Control		Fenugreek		Control		Fenugreek	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Bifidobacteria (log/g)	6·05	1·02	6·59	0·49	7·07	0·56	7·28	0·71
Lactobacilli (log/g)	7·67	0·56	8·41*	0·74	9·40	0·57	9·67	0·53
L. amylovorus	6·92	0·46	7·34	0·73	9·72	0·57	9·74	0·87
L. reuteri	6·55	0·95	7·67	1·54	7·59	0·67	8·27*	0·70
L. johnsonii	7·73	0·60	8·34*	0·41	8·45	0·40	9·15*	0·45
Escherichia/Hafnia/Shigella (log/g)	5·90	0·56	5·40*	0·48	7·23	0·99	7·25	0·71
Clostridium cluster I (log/g)†	6·09	0·70	6·99*	1·22	9·05	0·79	9·08	1·24
Clostridium cluster XIVa (log/g)‡	6·53	1·53	6·12	0·96	10·37	0·35	10·31	0·36
Streptococci (log/g)	7·65	0·25	7·61	0·28	8·71	0·71	8·82	0·49

*Mean value was significantly different from that of the control group ($P \leq 0{\cdot}05$).
† *C. acetobutylicum*, *C. botulinum*, *C. butyrium*, *C. carnis*, *C. celatum*, *C. cellulovorans*, *C. chauvoei*, *C. cochlearium*, *C. fallax*, *C. haemolyticum*, *C. intestinale*, *C. peptidivorans*, *C. perfringens*, *C. putrefaciens*, *C. saccharobutylicum*, *C. septicum*, *C. sardiniensis*, *C. subterminale*, *C. tetani*.
‡ *C. aminovalericum*, *C. coccoides*, *C. herbivorans*, *C. indolis*, *C. nexile*, *C. oroticum*, *C. polysaccharolytium*, *C. scindens*, *C. xylanolyticum*, *C. xylanovora*.

the other T-cell populations measured during the animal study ($CD4^+$ and $CD8^+$) showed no clear alterations. NK cells ($CD2^+CD5^-$) also seemed to be unaffected. In the case of the antigen-presenting cells, a significant effect was observed in the outcome in that the percentage of major histocompatibility complex class II molecules (MHCII)-bearing CD5 cells was significantly reduced in the blood of the fenugreek group. Likewise, the median values of $CD14^+$ cells (monocytes) and $SWC3^+$ cells (all myeloid cells) were numerically lower in the group of fenugreek-treated piglets, while no changes were observed between the groups concerning the relative numbers of $CD21^+$ cells (B cells) in the blood. The proliferation assays carried out with peripheral blood mononuclear cells from the different treated groups did not show any alterations. Phagocytosis also was not affected by the feeding of the piglets (data not shown).

Table 4. Phenotypes of immune cells in the peripheral blood of piglets fed the control or fenugreek diet
(Medians and minimal (Min) and maximal (Max) measurements)

	Diet			
	Control		Fenugreek	
Lymphocytes (%)	Median	Min-Max	Median	Min-Max
$CD2^+CD5^+$	39·9	33·6–49·6	41·5	27·1–47·2
$CD2^+CD5^-$	22·6	10·2–31·3	18·0	9·42–35·7
$CD4^+CD8\beta^-$	16·3	8·60–28·4	13·4	5·45–23·9
$CD4^+CD25^+$	3·10	0·83–4·88	2·20	1·40–4·64
$CD8\beta^+CD4^-$	11·3	10·0–24·3	13·9	9·17–24·0
$TCR1^+CD8\alpha^+$	3·40	1·93–7·60	4·10	1·84–7·13
$TCR1^+CD8\alpha^-$	9·40	3·92–20·7	17·2*	7·28–35·0
$CD14^+$	11·3	4·16–23·4	6·30	3·13–17·2
$CD8\alpha^+TCR1^-$	24·8	20·7–39·9	26·2	18·3–43·2
$CD21^+$	16·8	8·78–22·9	17·3	8·51–26·6
$MHCII^+CD5^-$	30·5	11·2–43·4	20·9*	16·4–36·0
$CD45RC^+$	77·9	64·6–90·5	83·5	74·1–88·7
$CD45RC^-$	21·7	9·44–35·4	16·2	11·1–25·7

*Median value was significantly different from that of the control group ($P \leq 0{\cdot}05$). Mann–Whitney test was used for non-normally distributed data.

Discussion

Fenugreek seed has been previously described to have interesting properties affecting digestive function and the immune system of animals. We have demonstrated that fenugreek affected the intestinal microbiota as well as selected aspects of the immune function of piglets in the post-weaning period.

The administration of 1·5 g fenugreek seeds/kg diet led to a lower pH of the digesta with significant diet-dependent changes in the caecum and colon of piglets. The pH reduction in the digesta by fenugreek seeds can display either an increase in the number of lactate-producing bacteria or their relevant metabolic activity. Fenugreek seeds contain over 50 % carbohydrates in the DM[21]. Soluble galactomannans seem to be the most interesting fraction, while the amount of insoluble fibre is small[22]. The hydrophilic galactomannans have a high water-holding capacity and the ability to form viscous gels[1], and seem to be a readily metabolisable substrate for the intestinal microbes, resulting in a higher production of organic acids. The concentrations of D-lactic acid were not affected by the seed. L-Lactic acid increased in the small intestine ($P < 0{\cdot}05$), but was not influenced in the large intestine by fenugreek. This indicates that galactomannans are readily fermented in the upper gut by lactic acid bacteria and that lactic acid has been effectively metabolised in the large intestine by resident lactate-consuming bacteria. However, no increase in propionic acid and acetic acid was observed. These acids are the typical fermentation products of lactate fermentation[23]. Using a porcine large-intestinal simulation model, the fermentability of fenugreek gum, containing 26·2 % galactose and 31·4 % mannose, was also high and resulted in a high yield of acetate and butyrate. Lactic acid production was higher compared with flaxseed but considerably lower compared with the incubation of caecal microbes with

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β-glucans[9]. This difference might either indicate a lack of coincidence of data from *in vivo* and *in vitro* models or it can also be explained by different amounts of test substances and compositional varieties of fenugreek seeds. In particular, the content of galactomannans may vary depending on various factors[3]. In rats, high levels of gums from fenugreek seed increased the concentrations of caecal SCFA, particularly propionic and acetic acid, and increased the caecal weight and the amount of water in the faeces. The higher propionic acid formation was correlated with a significant decrease in caecal pH[24]. In piglets, fenugreek seed, included at a considerably lower dietary level, had no effect on the total concentration and the pattern of SCFA in the small intestine and in the caecal digesta. Compared with the control group, the SCFA profile was similar. However, higher *n*-butyrate was seen in the colon contents of the fenugreek group. Next, to a general change in carbohydrate fermentation patterns, this may also indicate that a higher lactate concentration was available for *n*-butyrate-producing bacteria, which belong to heterogeneous clostridium clusters[25]. In the small intestine, a significantly increased L-lactate concentration as well as the strong numerical increases of lactobacilli, bifidobacteria and clostridium cluster I, which contains many butyrate-producing species, were observed. Thus, the increased *n*-butyrate formation may have been a result of increased lactic acid fermentation, which originated from the fermentation of soluble galactomannans in the upper gut. This finding is interesting due to the anticipated effects of butyrate as an energy-yielding substrate for the colonic epithelium in pigs[26,27] and prospectively for humans.

Indigestible carbohydrates may alter the composition of the intestinal microbiota in pigs. In the present study, the modified bacterial fermentation metabolites were accompanied by a marked change in the microbiota in the jejunal and caecal digesta. With the addition of fenugreek seeds, an increase in lactobacilli and some major representatives of this genus, including *L. johnsonii* and, as a trend, *L. reuteri*, was observed. These changes show that fenugreek seeds had apparently substrate effects in the intestine, although the concentration in the diet was relatively low. Diets containing mannans and galactomannans from different sources have been shown to affect the intestinal microbes in pigs. Piglets fed a diet with mannan oligosaccharides from yeast had higher faecal lactobacilli counts compared with the control animals[28]. The increase in lactic acid-producing bacteria is interesting from the perspective of intestinal micro-ecology and due to the dietary impact on the occurrence of enterobacteria. A lower cell number of enterobacteria was observed in piglets after administration of fenugreek seeds. The change was not more than half a logarithm step, but was statistically significant. The addition of 0·2 % mannan oligosaccharides decreased enterobacteria concentrations in the jejunum digesta of piglets from 9·1 to 8·0 log/g[29]. The impact of galactomannans from guar gum, locust bean gum or carob tree seed on the intestinal microbiota in pigs appeared inconsistent[30]. Total counts of bacteria were decreased by half a log unit in the proximal jejunum, while the number of *E. coli* increased in the stomach and in the jejunum. Increased ileal concentrations of total anaerobes, total aerobes, lactobacilli, enterobacteria and clostridia were determined in the ileum of piglets fed diets with very high concentrations (7 %) of guar gum[31]. From those findings, it can be concluded that the chemical composition of the indigestible carbohydrate fraction and the dietary inclusion level can have variable effects on the intestinal microbiota. Carbohydrates are considered to be most important, but other fractions of fenugreek seed, such as polyphenols, can have effects on the microbiota in pigs[32]. Regarding clostridia, a strong increase was observed for bacteria of clostridium cluster I in the small intestine, while clostridium cluster XIVa was not changed. Both clusters contain peptidolytic as well as saccharolytic species and therefore one cannot identify the exact net changes that occurred within both clusters. Proteolytic activities of the gut microbiota were not determined in the present study. However, given the increase in carbohydrate-fermenting lactic acid bacteria, it is conceivable that either saccharolytic clostridium species or lactate-fermenting clostridia gained an advantage.

The phenotyping of lymphocytes in the peripheral blood was characterised by a high variability of the data that is also known from other experiments with piglets[33–35]. This variability makes careful interpretation of the observed diet-related differences mandatory. Fenugreek has been shown to affect immune variables in different animal models. The number of studies is scarce, but indicates some interesting effects. Galactomannans were able to affect phagocytosis in rat macrophages, and proliferation and IgM secretion in hybridoma cells[5]. In mice, cellular/humoral response variables and phagocytic capacity of macrophages were affected in a dose-related manner[6]. In rats, fenugreek alleviated symptoms of experimental immunosuppression[8]. $CD8^-$ γδ T cells were increased in piglets fed fenugreek compared with the control animals. Porcine γδ T cells have varying phenotypes and numerous functions, including a role in early responses against infections at epithelial surfaces, regulatory effects on different T-cell subsets and antigen-presenting cells, and are presumed to be crucial for both innate and specific immune responses in the young[36–39]. In the group of piglets fed with fenugreek seed, a significantly higher proportion of these so-called 'non-classical T cells'[40,41] was observed. In this group of piglets, $CD8^-$ γδ T cells outnumbered the populations of helper T cells as well as cytotoxic T cells, which was not the case in the control group. 'Non-classical T cells' have been reported to constitute approximately 15–25 % of lymphocytes in the peripheral blood of pigs[42], which is in line with the present results. Unfortunately, until now, little is known about the physiology of $CD8^-$ γδ T cells. In contrast to $CD8^+$γδ T cells, they do not respond to stimulation by mitogens including concanavalin A, phytohaemagglutinin and pokeweed mitogen, neither to foreign antigens[43,44]. However, an extract from the plant *Acanthospermum hispidum* resulted *in vitro* in a proliferation of porcine $CD8^-$ γδ T cells based on an IL-2-dependent mechanism mediated by CD4 helper cells[45]. Both γδ T-cell subpopulations are high in the young piglet and decrease during the growth period. Therefore, they are considered to be of special importance for the development and maturation of

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the juvenile immune system. The expression of the CD8α molecule occurs after activation and is age-dependent, together with increasing MHCII expression[37]. The expansion of the $CD8^-$ γδ T subset can be explained by the direct effects of fenugreek on the gut-associated immune system, or it might be the consequence of the observed changes in the intestinal microbiota. This finding makes fenugreek interesting as an immune-modulating ingredient. However, the characterisation of functional significance requires further studies and the influence on the health status of the animals cannot yet be assessed. Moreover, the same group of piglets showed another deviation in the composition of blood leucocytes. The portion of $MHCII^+CD5^-$ cells was significantly lower in the fenugreek-fed group compared with the control group. As CD5 is expressed on all porcine T cells, this $CD5^-$ MHCII-expressing population should be mainly B cells and monocytes. From the observation that the $CD21^+$ population did not show any differences between the groups, we assume that the affected population is mainly composed of monocytes. This assumption is strengthened by the fact that the median values of $CD14^+$ cells and SWC3a (CD172a and $SIRP\alpha^+$) cells were also decreased in the fenugreek group.

In conclusion, fenugreek had interesting effects in piglets, lowering the pH, and increasing the concentrations of lactic acid and lactobacilli in the digesta. Furthermore, the lower concentrations of enterobacteria indicate a change in the gut microbiota. The effects on the lymphocyte phenotypes in the peripheral blood of piglets have been noted. However, whether these findings affect colonisation resistance against invading pathogens and animal health has to be further evaluated.

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