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In-feed supplementation of clinoptilolite favourably modulates intestinal and systemic immunity and some production parameters in weaned pigs

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ABSTRACT: The ban on dietary antibiotic growth promoters (AGP) in swine production has focused increasing research efforts on the development of alternative feed supplements. One such alternative to AGP is the dietary zeolite clinoptilolite (CPL). The aims of this study were to evaluate, in weaned pigs, the effects of CPL on: (a) growth performance, (b) gut health (reduction of harmful bacteria and, incidence/severity of diarrhoea) and (c) circulating or ileal mucosal subsets of lymphoid immune cells, over the course of five weeks post weaning. The non-treated pigs received standard Phase 1 diet from Day 0 to 21 and Phase 2 diet from Day 22 to 35, whereas both diets of experimental pigs were supplemented with 0.5% CPL. The pigs receiving diet supplemented with CPL had significantly higher average daily gain at Day 28 but significantly lower daily gain at Day 35 of the experiment ($P < 0.05$). The CPL group exhibited non-significantly improved feed conversion ratio (1.83 *vs.* 2.17) for the total duration of the experiment (Day 0 to Day 35). Although shedding of haemolytic/enterotoxigenic *E. coli* was more frequent in the CPL group, the sum of their diarrhoea severity score was 12.96% lower (47 *vs* 54) than that of the non-treated controls. The proportions of circulating lymphoid cell subsets tested ($CD45^+$, $CD4^+$, $CD21^+$), were significantly ($P < 0.05$ to $P < 0.01$) higher in CPL-treated pigs between Day 21 and Day 35 of the experiment. Immunohistology/morphometry of ileal segments revealed an increased recruitment of $CD45RA^+$ cells in interfollicular ($P < 0.05$), but not in follicular areas of ileal PP of CPL-treated pigs at Day 35. In conclusion, CPL did not improve growth in weaned pigs, and generally it failed to improve their feed conversion efficiency. Further, it did not suppress faecal shedding of enterotoxigenic *E. coli*; however, it was shown to be effective as an immunomodulatory agent by promoting the recruitment of circulating and intestinal immune cell subsets.

Keywords: clinoptilolite; immunomodulation; performance; bacterial counts; piglet

Porcine post-weaning diarrhoeal disease caused by enterotoxigenic *Escherichia coli* (ETEC) is a common enteric infection in weaned pigs that causes considerable economic losses due to high morbidity and moderate mortality rates, growth retardation and increased cost of medication (Fairbrother et al. 2005). For more than 50 years the problems of ETEC and other enteric infections in young pigs have been overcome by adding sub-therapeutic doses of antibiotic growth promoters (AGP) in-feed to enhance production efficiency in the swine industry (Cromwell 2002) by increasing growth rate, improving feed utilisation and reducing mortality from clinical disease (Thacker 2013).

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However, concerns about the potential risks for human health due to use and misuse of AGP in animal feeds (Dewey et al. 1997), and their residues in meat products (Vondruskova et al. 2010), have led to their ban throughout the EU since 2006 (Regulation EC No. 1831/2003). The withdrawal of AGP from use in the swine industry means that it now becomes urgent to provide relevant gut health criteria for the large-scale production of pigs and scientifically founded recommendations for alternatives to AGP. These criteria which are commonly accepted within the EU must be usable for objective assessment of alternatives to AGP (Gallois et al. 2009) and must be acceptable for swine producers, feed manufacturers and consumers. In the past two decades intensive efforts have been focused on the development of alternatives to AGP to maintain swine health and performance. Many research papers and excellent reviews have been published on this subject, describing immunomodulators of natural (Gallois and Oswald 2008; Gallois et al. 2009) and synthetic origin (Valpotic et al. 2013; Valpotic et al. 2014), probiotics (Cho et al. 2011), prebiotics (Halas and Nochta 2012) as well as less traditional alternatives such as selected organic acids, amino acids, protein sources, plant extracts (Lalles et al. 2009), peptides, clay minerals, egg yolk antibodies, essential oils, fatty acids, rare earth elements (Thacker 2013) and naturally extracted clay smectite or artificially composed zeolite with antibacterial activities in the gut (Almeida et al. 2013; Islam et al. 2014). However, only a small number of these have been shown to be effective. Amongst the vast variety of alternatives to dietary AGP tested, clinoptilolite (CPL), which is the most common member of the naturally occurring zeolite family of crystalline hydrated aluminosilicate minerals (Mumpton 1999), has shown promise as a growth-promoting (Defang and Nikishov 2009; Prvulovic et al. 2012), immune enhancing/antiviral (Jung et al. 2010) and gut health restoring (Vondruskova et al. 2010; Hrenovic et al. 2012) antibacterial dietary supplement in pigs. However, studies reported thus far have shown inconsistent results, suggesting that the reported potential of dietary CPL may have certain limitations due to the lack of standardisation of its physicochemical properties (especially particle size), and the heterogeneity of rearing conditions, e.g. exposure of pigs to pathogens.

Since the 1980's intriguing reports have suggested that a variety of natural or synthetic zeolites, mostly CPL, when applied as feed supplement may improve feed efficiency, growth and carcass quality of growing and finishing swine (Cool and Willard 1982; Pond et al. 1988; Coffey and Pilkington 1989; Yannakopoulos et al. 2000; Alexopoulos et al. 2007). Conversely, some authors have found that growth rates of either fattening or weaned pigs were unaffected by dietary CPL (Shurson et al. 1984; Pearson et al. 1985; Paulsen and Oksbjerg 1995). More recent studies have shown that dietary CPL had positive effects on growth performance of pigs during the growing phase, whereas in the finishing phase body weight gain was found to be decreased and feed efficiency was unaffected (Prvulovic et al. 2007). The same authors reported that CPL supplementation positively affected daily weight gain and feed conversion ratio in young growing pigs (Prvulovic et al. 2012). Such contradictory effects seem to be related to the type and geographical source of the zeolite (i.e. CPL) tested, its purity, chemical and structural properties as well as the concentration used in the diets, the health status of the treated pigs and the environmental conditions (Pearson et al. 1985; Pond et al. 1988; Paulsen and Oksbjerg 1995). In accordance with such an idea was the finding of significantly higher daily weight gain in pigs fed a diet containing 4% natural CPL (Zikeevski origin) from Russia during the grower and finisher phases (Defang and Nikishov 2009). It is well known that zeolites exert ion exchange capacity, nonspecific adsorption and related molecular sieve properties. The affinity of CPL for ammonium ions (Leung et al. 2007), bacterial enterotoxins (Ramu et al. 1997) and mycotoxins (Dakovic et al. 2005) may prevent adverse effects on health/welfare, could be beneficial to the physiological status of the gut epithelium and, thus, may influence the digestive and absorption processes leading to improved pig performance. In particular, these biological activities of CPL resulted in a reduction in the incidence/severity of diarrhoeal disease in pigs (Benatti et al. 1994; Rodriguez-Fuentes et al. 1997; Papaioannou et al. 2004; Vondruskova et al. 2010). These findings may be ascribed to the antibacterial properties of either natural (Hu et al. 2013) or artificial zeolites (Islam et al. 2014) that could decrease intraluminal bacterial counts, adsorb/partially inactivate thermo-labile *E. coli* enterotoxins, and thus, reducing their attachment to enterocyte receptors and the incidence/severity of postweaning scours as suggested by Ramu et al. (1997). However, Flohr et al.

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(2014) reported that dietary CPL failed to ameliorate the increased osmotic diarrhoea observed in response to high sodium sulphate water in nursery pigs. The aforementioned effects of CPL did not adversely affect overall health status of growing and fattening pigs in terms of changes in their serum biochemical and haematological parameters (Alexopoulos et al. 2007).

Data on the effects of in-feed zeolites on the development of immune responses within porcine gut-associated lymphoid tissues (GALT) are scarce and should be studied in more detail in the future, as their effects are mainly expected in this compartment. Indeed, the available literature highlights the effects of dietary zeolites on systemic immunity related to either general immune-enhancing activity which reinforces the clearance of porcine circovirus type 2 in experimentally infected weaned pigs (Jung et al. 2010), or increased serum IgG levels in growing/finishing swine (Islam et al. 2014). Local intestinal immune responses to zeolite supplements have received only little attention, and until now the data are restricted to controversial effects of zinc oxide supported by zeolite on cytokine expression in jejunal mucosa of weaned pigs (Hu et al. 2013). Therefore, of particular relevance to the current study is the question of whether or not dietary CPL may have modulatory effects on porcine cellular immune responses, both systemic and local, at the intestinal mucosal surfaces of weaned pigs. It is well known that mucosal responses can occur independently of systemic immunity, and thus, the study of systemic immune responses may not reflect immune functions and dysfunctions occurring in the GALT (Hannant 2002).

Thus, the aims of this study were to evaluate the effects of dietary CPL as a potential alternative to AGP in weaned pigs on: (1) growth performance/feed efficiency, (2) gut health/reduction of harmful bacteria numbers and, hence, incidence/severity of diarrhoea, and (3) proportion/number of either circulating immune cell subsets or $CD45RA^+$ lymphoid cells residing in GALT compartments of the ileum, respectively, over the course of the five weeks of the experiment.

MATERIAL AND METHODS

Pigs. Forty-six crossbred piglets (Topigs®) of both sexes (females and castrates) and with body weights of approximately 7.1 kg, which were the progeny of four litters (from 3rd parity sows) from a commercial swine farm in eastern Croatia were used. The pigs were weaned at 26 days of age, housed, managed and fed with a standard weaner diet (without antimicrobials or growth promoters) according to the rearing technology of the farm. Experimental and animal management procedures were conducted in accordance with the "Directive for the Protection of Vertebrate Animals used for Experimental and other Purposes" (86/609/EEC).

Experimental design and treatment. The weaned pigs were randomly divided into two groups comprising 23 animals each, ear-tagged with numbers 1–23 and kept in the same rearing facilities of the commercial farm in separate pens as detailed previously (Valpotic et al. 2014). All experimental diets were corn- and soybean meal-based and formulated to meet the NRC (1998) nutrient requirements for pigs (Table 1). At 28 days

Table 1. Ingredients and chemical composition of experimental diets

Item	Diet	
	Phase 1 (SO-0)	Phase 2 (SO-1)
Ingredients (% air-dry diet)		
Maize	44.88	45.85
Barley	11	14
Soybean meal (44% CP)	11	11
Fish meal	4	3.5
Dried skim milk	12	4
Soybean, full fat	14.5	15.95
Monocalcium phosphate	0.8	0.9
Limestone	0.8	0.9
Salt	0.3	0.3
Vitamin and mineral mix*	0.5	0.5
Lysine	0.12	0.1
Methionine + cysteine	0.1	0
Calculated analysis (% air-dry diet)	100	100
Crude protein	21.61	19.78
Metabolic energy (MJ/kg)	13.84	13.74
Lysine (g/kg)	13.7	11.6
Methionine + cysteine (g/kg)	8.3	6.7

*Provided the following amounts of minerals and vitamins per kilogram of diet: 18 mg copper, 110 mg zinc, 0.2 mg iodine, 110 mg iron, 50 mg manganese, 0.3 mg selenium, 20 000 IU of vitamin A; 3200 IU of vitamin D; 120 000 IU of vitamin E; 7000 mg of vitamin K; 70 mg of vitamin B_{12}; 200 mg of folic acid; 120 000 mg of pantothenic acid; 4000 mg of pyridoxine; 16 000 mg of riboflavin; 3000 mg of thiamine

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of age the pigs were treated as follows: control pigs received a standard weaner Phase 1 (from Day 0 to Day 21) or Phase 2 (from Day 22 to Day 35) diet (SO-0 or SO-1; Zito, Osijek, Croatia), whereas both diets for the experimental pigs were supplemented with 0.5% of CPL (Vetamin®, Panaceo, Austria). The experiment was conducted throughout a period of 35 days; the pigs were monitored daily (for diarrhoea and/or other clinical signs of gut health disorders, such as anorexia and weight loss) and weighed/sampled (for samples of peripheral blood and rectal swabs) at seven day intervals starting at Day 0 before the treatment.

Growth performance and feed efficiency evaluation. The pigs were weighed at weekly intervals during the experiment and changes in their body mass were recorded. The changes in body mass within the experimental group of pigs were calculated based on differences between either body weight at the beginning of the experiment (Day 0 = 100% of body mass) or average group body weight at Days 7, 14, 21, 28 and 35 of the experiment in comparison to average body weight of the pigs from the non-treated group. Feed intake was recorded on a weekly basis, and at the end of the experiment a total group feed intake, feed conversion ratio and total group body weight gain were calculated in relation to Day 0. In order to determine the kinetics of growth in the experimental pigs, as well as feed intake and feed conversion, we analysed average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) over the course of five weeks following weaning. These parameters were corrected according to pig losses during the experiment.

Diarrhoea evaluation. The pigs were monitored daily for diarrhoea and/or other clinical signs of gut disorders (such as anorexia and weight loss) and the incidence/severity of diarrhoea were recorded. Severity of diarrhoea was scored as follows: 0 = normal faeces, 1 = soft faeces, 2 = fluid faeces and 3 = projectile diarrhoea. Pigs with scores of either 2 or 3 were defined as diarrhoeic. After collection we summarised our data and calculated a diarrhoea severity score (DSS), which represented the sum of diarrhoea severity over the course of 35 days.

Bacteriological analysis of intestinal microbiota. In order to monitor gut health we also determined bacterial species/serovars isolated from the rectal swabs and jejunal content. The rectal swabs were taken from five pigs per group on Days 0, 14, 21 and 35 of the experiment for isolation and serotyping of the enteric bacteria. On either Day 0 or Day 35 of the experiment five pigs per group were euthanized by intracardial injection of 0.3 ml/kg of T61 preparation (Hoechst®, München, Germany) and sampled for bacteriology and immunohistology. Immediately following euthanasia a 10 cm segment of mid jejunum with digestive content was first ligated and taken for counting of intraluminal bacteria. For determination of the total number of *E. coli* cells in 1 ml of the jejunal content (CFU/ml) the samples were diluted in serial dilutions up to 10^{10} in saline and each dilution was plated onto selected culturing plates (Winn et al. 2006). In order to isolate and count *E. coli* bacteria, 1 ml of each dilution was added into two Petri dishes into which Tryptone-bile glucuronic medium (TBX; contains bile salts No. 3 and 5-bromo-4-chloro-3-indolil β-D-glucuronic acid (BCIG)) was poured. Each serial dilution was plated in duplicate, and after 24 h of incubation at 37 °C the grown colonies were counted on an automatic computer-assisted counter and the number of CFU per ml were calculated as detailed earlier (Valpotic et al. 2014). For identification and serotyping one loop (0.1 ml) of the jejunal content was plated onto a blood agar base with 5% of defibrinated sheep blood (Blood Agar Base No. 2 OXOID CM 271) and XDL agar. The most common fimbrial antigens of *E. coli* F4, F5, F6 and F18 were serotyped using Minca medium by rapid slide agglutination with specific commercial antisera (Denka Seiken, Japan). The haemolytic isolates of *E. coli* were identified by plating of the jejunal content onto TSB with 5% of defibrinated sheep blood with esculine. The identification of haemolytic isolates of *E. coli* bacteria from rectal swabs and their further serotyping was performed using the same procedure as for those isolated from the jejunal content (Valpotic et al. 2014).

Analysis of peripheral blood immune cell subsets using flow cytometry (FCM). At the same weekly intervals blood samples (1 ml) from seven out of 23 pigs from each group (ear-tagged with numbers 1–7) were taken from *vv. cava cranialis* into glass tubes (Beckton Dickinson Plymouth, UK) with ethylenediaminetetraacetic acid (EDTA) (Sigma, St. Louis, USA) as an anticoagulant for FCM analysis. The monoclonal antibodies (mAbs) reactive with swine leukocyte surface molecules, i.e. cluster of differentiation (CD) antigens and fluorescent dye conjugates that we used for identifica-

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tion/quantification of CD45 lymphoid cells as well as for CD4/CD8 T and CD21 B cell subsets have been described previously (Valpotic et al. 2014). Single cell suspensions (100 µl) were prepared in triplicates (comprising 10 000 cells each) and incubated with mAbs (50 µl) and processed as described previously (Bozic et al. 2002). The fluorescence of the mAb-labelled porcine lymphoid cells was quantified using a Coulter EPICS-XL flow cytometer (Beckman Coulter Miami FL, USA) as detailed earlier (Valpotic et al. 1994).

Immunohistological and histomorphometric analyses of intestinal immune cells. Immediately following euthanasia on either Day 0 or Day 35 of the experiment, five specimens of mid ileum from each of five pigs per group, respectively (either 5–6 cm or 7–8 cm proximal to the ileocaecal junction of 4-week-old pigs and 9-week-old pigs, respectively), were fixed in 10% neutral-buffered formalin (pH 7.0–7.6) for 24 h until used for immunohistology analyses. The paraplast-embedded sections were processed for an indirect immunoperoxidase (IP) method as detailed earlier (Lackovic et al. 1997; Valpotic et al. 2014). The primary mAbs reactive with CD45RA$^+$ lymphoid cells and secondary polyclonal Abs conjugated with horseradish peroxidase that were used to study *in situ* identification, distribution and quantification patterns of these cells residing in the ileal mucosa of weaned pigs have been described previously (Valpotic et al. 2014). Histomorphometric analyses of CD45RA$^+$ lymphoid cells within compartments of ileal mucosa such as interfollicular areas (IFA) and follicular areas (FA) of Peyer's patches (PP) were performed using the Lucia G commercial software imaging program (version 4.11) for digital image analysis (DIA) as described previously (Kovsca-Janjatovic et al. 2009; Valpotic et al. 2014).

Statistical analyses. Numerical data were analysed using Student's *t*-test for dependent samples using the StatisticaSixSigma software (StatSoft, Inc.). The generalised linear mixed model (SAS 9.3) was used to analyse the body weight. The statistical model included the fixed effects of group, day and their interactions. Animal effects on repeated measures over time were included in the model by

Table 2. Effect of clinoptilolite supplementation on the performance of weaned pigs during 35 days of the experiment

Items	Day	Non-treated*	CPL**
ADG g/pig per day	0–7	214 ± 25	204 ± 22
	8–14	172 ± 25	193 ± 24
	15–21	273 ± 40	303 ± 29
	22–28	261 ± 54	414 ± 31[a]
	29–35	562 ± 36[a]	356 ± 56
	0–35	296 ± 17	294 ± 22
ADFI g/pig per day	0–7	419	318
	8–14	464	450
	15–21	780	619
	22–28	565	626
	29–35	975	674
	0–35	641	537
FCR	0–7	1.96	1.56
	8–14	2.70	2.33
	15–21	2.86	2.04
	22–28	2.16	1.51
	29–35	1.74	1.90
	0–35	2.17	1.83

Groups comprised 23 pigs each at Day 0 of experiment

*standard weaner diet only

**the diet supplemented with 0.5% CPL

[a]values differ significantly at $P < 0.05$

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Table 3. Incidence and severity of diarrhoea in weaned pigs fed diet supplemented with CPL

Items	Day	Non-treated*	CPL**	*P*-value
Number of diarrhoeic pigs	0–35	12/23 (52.17%)	7/23 (30.43%)	0.1486
DSS				
Sum of DSS	0–35	54	47	0.3540
% difference			–12.96	

DSS (diarrhoea severity score): 0 = normal faeces, 1 = soft faeces; 2 = fluid faeces or 3 = severe diarrhoea

*standard weaner diet only or diet supplemented with 0.5% of CPL

**during 35 days of experiment

RANDOM statement with RESIDUAL option and compound-symmetry structure. Multiple comparison test of the least-square means with Bonferroni correction was performed using the SLICE option to compare each level of the group within each level of time. The binary variable DSS was analysed using the GENMODE procedure with log link function and binomial distribution. Differences between treated and non-treated groups of pigs were considered as significant at $P < 0.05$.

Table 4. Difference in proportion (%) of lymphoid cell subsets (mean ± SEM) in the peripheral blood of weaned pigs fed diet supplemented with CPL

Item	Day	Non-treated*	CPL**
$CD45^+$	0	55.31 ± 1.41	49.79 ± 0.64[a]
	7	56.28 ± 1.31	53.74 ± 0.75
	14	58.30 ± 0.75	58.84 ± 0.24
	21	60.51 ± 0.46	63.67 ± 0.43
	28	61.27 ± 0.35	66.16 ± 0.41[a]
	35	61.46 ± 0.27	66.35 ± 0.37[a]
$CD4^+$	0	19.34 ± 0.50	17.37 ± 0.22[a]
	7	19.70 ± 0.47	18.90 ± 0.31
	14	20.46 ± 0.23	20.55 ± 0.09
	21	21.18 ± 0.16	25.45 ± 0.18[a]
	28	21.53 ± 0.10	26.45 ± 0.16[a]
	35	21.60 ± 0.13	26.56 ± 0.15[a]
$CD8^+$	0	11.06 ± 0.28	9.94 ± 0.12[a]
	7	11.14 ± 0.23	10.73 ± 0.15
	14	11.73 ± 0.12	11.77 ± 0.05
	21	12.10 ± 0.09	12.73 ± 0.09
	28	12.27 ± 0.07	13.23 ± 0.08
	35	12.34 ± 0.10	13.50 ± 0.09
$CD21^+$	0	22.07 ± 0.60	19.89 ± 0.26[a]
	7	22.11 ± 0.53	21.44 ± 0.31
	14	23.16 ± 0.35	23.53 ± 0.34
	21	24.35 ± 0.24	22.28 ± 0.15
	28	24.51 ± 0.14	26.68 ± 0.11[a]
	35	24.52 ± 0.13	26.59 ± 0.24[a]

*standard weaner diet only or the diet supplemented with 0.5% of CPL

**groups comprised seven pigs each at Day 0 of the experiment

[a]values differ significantly at $P < 0.05$

RESULTS

Growth performance and feed efficiency

Non-treated pigs had much lower ADG (261 *vs.* 414) between Day 22 and Day 28 ($P < 0.05$), but the pigs supplemented with CPL had significantly lower ADG (356 *vs.* 562) between Day 29 and 35 ($P < 0.05$). Over the course of the entire experimental period (Day 0 to Day 35) the ADG was observed to be similar in both groups of pigs (296 g *vs.* 294 g). Feed efficiency was lower in pigs supplemented with CPL (1.83) as compared to non-treated pigs (2.17) during the trial although the results were not statistically significant (Table 2).

Diarrhoea and intestinal microbiota

The sum of DSS recorded in the pigs that received dietary CPL was lower by 12.96% (47 *vs.* 54) than in the non-treated pigs, but this difference was not significant (Table 3). The control pigs had a much higher incidence of diarrheal disease (52.17% *vs.* 30.43%). Numbers of haemolytic (Hy) *E. coli* isolates were slightly, but not significantly higher ($P > 0.05$) in the experimental compared to the non-treated pigs (8 *vs.* 6) over the five weeks of the experiment. The average bacterial load in the jejunum was much lower in the CPL-treated pigs (19×10^7 *vs.* 19×10^8 CFU/ml) at the end of the experiment but these differences were not statistically analysed.

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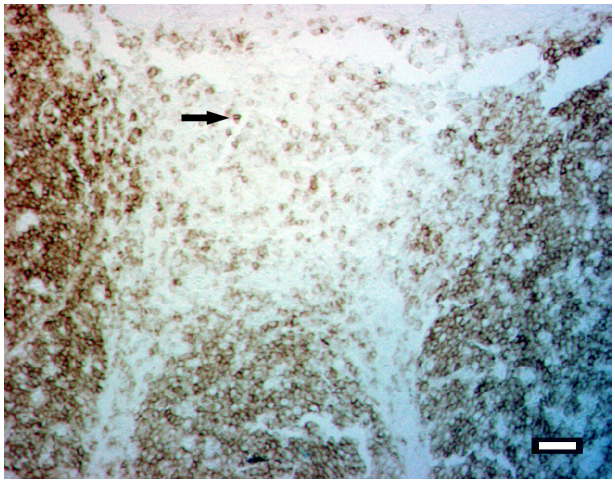

Figure 1. CD45RA$^+$ lymphoid cells within interfollicular areas (IFA) of ileal mucosa of a pig from the non-treated group after five weeks of the experiment; immunoperoxidase (IP) method, scale bar = 50 μm

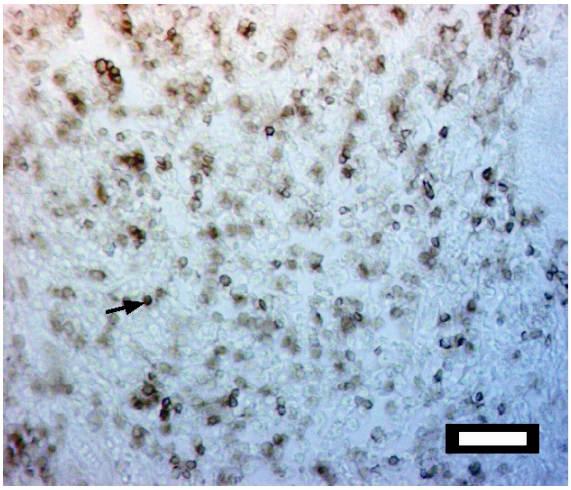

Figure 2. CD45RA$^+$ lymphoid cells within interfollicular areas (IFA) of ileal mucosa of a pig from the clinoptilolite (CPL)-treated group after five weeks of the experiment; immunoperoxidase (IP) method, scale bar = 50 μm

Proliferation rates of circulating and intestinal immune cell subsets

Flow cytometry based analysis of the kinetics of the differential expression of immune cell subsets with respect to the proportions of CD45$^+$ lymphoid cells, CD4$^+$ and CD8$^+$ T cells as well as of CD21$^+$ B cells in the peripheral blood of weaned pigs over the 5 weeks of the in-feed treatment with CPL are shown in Table 4.

With the exception of CD8$^+$ T cells, the proportions of lymphoid cell subsets tested were significantly higher in the CPL-treated pigs than in the non-treated pigs between Day 28 and Day 35 of the experiment (Table 4). Moreover, on Day 21 of the experiment we recorded a significantly increased proportion of CD4$^+$ T cells ($P < 0.05$). Interestingly, the proportions of either CD45$^+$ lymphoid cells, CD4$^+$ and CD8$^+$ T cells or CD21$^+$ B cells were significantly lower in the experimental pigs before the treatment with CPL at Day 0 ($P < 0.05$, respectively) than in the non-treated pigs.

Distribution patterns and frequency of CD45RA$^+$ cells within IFA and FA of the ileal PP from the CPL-

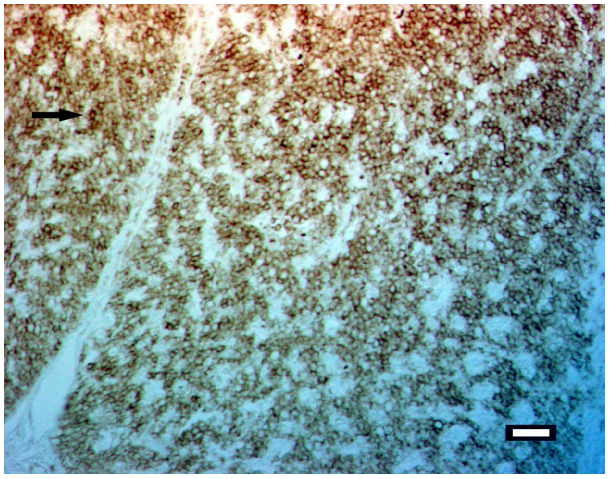

Figure 3. CD45RA$^+$ lymphoid cells within follicular areas (FA) of ileal Peyer's patches (PP) of a pig from the non-treated group after five weeks of the experiment; immunoperoxidase (IP) method, scale bar = 50 μm

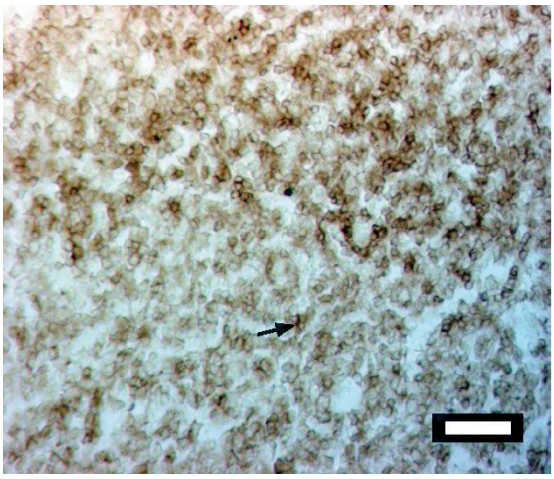

Figure 4. CD45RA$^+$ lymphoid cells within follicular areas (FA) of ileal Peyer's patches (PP) of a pig from the clinoptilolite (CPL)-treated group after five weeks of the experiment; immunoperoxidase (IP) method, scale bar = 50 μm

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Table 5. Numbers of CD45RA+ naive lymphoid cells in interfollicular areas (IFA) and follicular areas (FA) of the ileal Payer's patches (PP) of weaned pigs supplemented with CPL

Items		Day	Non-treated*	CPL**	% difference
$CD45RA^+$ cells in IFA***		35	15.60 ± 1.70	28.00[a] ± 3.01	12.40[b]
$CD45RA^+$ cells in FA***		35	504.80 ± 25.60	502.60 ± 31.70	–2.20
Change of the mean no. ileal $CD45RA^+$ cells[a] (%)	IFA	35	100	+1.79	95
	FA	35	100	1.00	–0.4

*standard weaner diet only or the diet supplemented with 0.5% of CPL

**five pigs per group were euthanized and sampled on either Day 0 (data not shown) or Day 35, respectively

***number of the cells per μm^2 of randomly selected 12 microscopic fields (average area of digital image field was 71 168.87 μm^2) in each of five serial sections of mid ileum per pig

[a]increase/decrease (%) of the mean values in CPL pigs *vs.* the mean values in non-treated pigs (100%)

[b]values differ significantly at $P < 0.05$

treated pigs on Day 35 of the experiment are shown in Figures 1 and 2 and Figures 3 and 4, respectively.

Densely distributed $CD45RA^+$ naive lymphoid cells were observed in the lamina propria of intestinal villi, within Lieberkühn's crypts and in the ileal submucosa. These cells were rarely visible adjacent to the basal membrane of enterocytes, but were frequently present in the middle of villous lamina propria and in the IFA (Figures 1 and 2). $CD45RA^+$ cells were noticed to be more numerous in the FA of the ileal PP (Figures 3 and 4).

Microscopic examination revealed an increased recruitment of $CD45RA^+$ cells in the IFA, but not in the FA of the ileal PP of CPL-treated weaned pigs (Table 5).

This observation was confirmed quantitatively using DIA within the IFA (28.00 *vs.* 15.60) ($P < 0.05$). The values obtained within the FA (502.60 *vs.* 504.80) were not significantly different ($P < 0.05$). The pigs supplemented with CPL had a significantly increased number of $CD45RA^+$ cells in IFA ($P < 0.05$) as compared to non-treated pigs on Day 35 of the experiment. When these differences (CPL *vs.* non-treated) were expressed as the percentage of increase in the number of naive lymphoid cells tested, it was even more evident that their recruitment was strongly stimulated in the IFA (by 79%) of the ileal PP by dietary CPL (Table 5).

DISCUSSION

Considerable efforts have been devoted to understanding porcine post-weaning diarrhoea, including attempts directed toward understanding the aetiology and the biology of pathogens, host resistance as well as therapy, in which antibiotics had a major role in the development and growth of the swine industry. By contrast, less is known regarding the prevention of this disease through dietary and non-dietary strategies because these problems have been overcome thus far by adding AGP and zinc and copper in feed (Thacker 2013). Before the EU ban on AGP (in 2006), this prophylaxis was highly effective in early-weaned pigs, which are particularly prone to digestive disorders. Concerns about potential risks to human health include the possibility of AGP residues in meat (Vondruskova et al. 2010), unapparent carriage of anti-microbial drug-resistant bacteria, exchange of plasmids from antibiotic-resistant porcine bacteria to human pathogens making them resistant to antibiotics, and further transfer of these naturally cloned new pathotypes to both animals and humans (Van der Fels-Klerx et al. 2011).

The effects of dietary CPL on growth performance parameters that we have determined, with the exception of either higher body weight gain or ADG in the CPL-treated pigs ($P < 0.05$) on Day 28 and between Day 21 and Day 28, respectively, of the experiment are predominantly in agreement with the findings of those authors who reported that the agent did not promote growth in weaned pigs (Shurson et al. 1984; Pearson et al. 1985; Paulsen and Oksbjerg 1995). Although not significant ($P < 0.05$), the slight increase in feed efficiency recorded during the entire experimental period could be indicative of improvement, but generally, our results are in not in agreement with authors who recorded improved performance parameters in pigs fed diets with CPL (Yannakopoulos et al. 2000; Alexopoulos et al. 2007; Defang and Nikishov 2009; Prvulovic et al. 2012).

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The development of both innate and adaptive immunity at the mucosal intestinal surfaces is critical in preventing the potential harmful effects of heavy exposure to the intestinal pathogenic microbiota which is associated with a high incidence of diarrhoea during the weaning transition when pigs are subjected to major stressful events, making them highly sensitive to digestive disorders (Gallois et al. 2009). Although not significant ($P > 0.05$), the reduction in the sum of DSS in the CPL-treated pigs by 12.96%, could be rather ascribed to the high adsorption capacity of clay minerals than to its bactericidal properties (Vondruskova et al. 2010; Hu et al. 2013). Although not significant, our finding on Day 35 of a lower average bacterial load in the jejunum (19×10^8 *vs.* 19×10^7 CFU/ml) in pigs supplemented with CPL is in agreement with studies suggesting that CPL can reduce the counts of pathogenic bacteria in the gut of weaned pigs exposed to intestinal colonisation (Hu et al. 2013; Islam et al. 2014). We propose that the immunostimulatory effect of CPL on gut immunity, i.e. recruitment of $CD45RA^+$ lymphoid cells in the ileal mucosa established a protective immune response as the diarrhoea score was more favourable in the CPL group at the end of the experiment. Much interest has been paid to the effects of CPL on systemic immune responses (Jung et al. 2010; Islam et al. 2014), whereas reports dealing with effects of CPL on local intestinal immune responses in pigs are very scarce (Hu et al. 2013).

Since we did not find any data on the influence of dietary CPL on cellular immune responses either within porcine peripheral blood or the GALT, we can only discuss our results in relation to our previous research obtained following a single *per os* administration of the synthetic immunomodulators polyoxyethylene and polyoxypropylene (POE-POP) and levamisole (LEVA) as potential alternatives to in-feed AGP in the same model of weaned pigs (Valpotic et al. 2013; Valpotic et al. 2014). Both agents strongly stimulated the recruitment of circulating $CD45^+$ lymphoid cells, $CD4^+$ and $CD8^+$ T cells, and $CD21^+$ B cells of weaned pigs between Day 14 and Day 35 following the treatments. Dietary CPL also stimulated a significant proliferation of $CD45^+$ lymphoid cells, $CD4^+$ T cells and $CD21^+$ B cells, but failed to affect the proportion of $CD8^+$ T cells. The proportion of the latter subset was slightly increased on Day 35 of the experiment, but we did not follow-up these parameters further. The immunomodulatory effects of dietary CPL are also controversial with respect to its influence on the cellular immune responses within the GALT compartments when compared to the effects of POE-POP or LEVA. These treatments were shown to elicit significant increases in the number of $CD45RA^+$ cells in both the IFA and FA of the ileal PP five weeks following the treatments. The former observation is only in partial agreement with our present finding that the pigs fed with CPL supplement had significantly increased numbers of $CD45RA^+$ cells only in the IFA on Day 35 of the experiment.

However, these abilities of dietary CPL to stimulate both systemic and intestinal cellular immunity may indicate the suitability of the agent as a natural in-feed additive with immunostimulatory properties. It may be particularly useful for improving gut health and targeting the GALT of weaned pigs, which is well known to promote a tolerogenic rather than a protective immune response (Bailey et al. 2005).

CONCLUSIONS

Taking into consideration all the obtained data, it can be concluded that CPL: (1) did not improve performance in weaned pigs, but (2) showed potential to be effective as an immunostimulator for recruitment of circulating and intestinal immune cell subsets, and thus, may probably improve resistance to enteric infections, resulting in a reduction in the incidence/severity of post-weaning diarrhoea.

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