Effect of dietary natural supplements on immune response and mineral bioavailability in piglets after weaning

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ABSTRACT: Plants and plant extracts (PPEs) have gained increasing interest as feed additives and possible replacing antibiotics for pig productions. The effects of dietary *Chlorella vulgaris* (1%), sodium alginate (0.1%), inulin (1.5%), and a mixture of essential oils (0.04%) supplements on immune response, and bioavailability of some micronutrients (iron – Fe, copper – Cu, manganese – Mn, and zinc – Zn) were investigated in weaned piglets in this study. The results showed that the concentration of IgG was increased in the plasma of pigs fed the PPEs supplemented diets being significantly higher ($P < 0.05$) in the case of dietary sodium alginate supplementation in comparison to the control (6.00 vs. 4.03 mg/ml). In liver, PPEs, especially inulin and essential oils, were able to modulate the level of cytokine production and mineral retention, resulting in higher liver concentration of IL-1β (125.4 and 88.9%), IL-8 (136.9 and 61.3%), TNF-α (296.6 and 121.6%), and IFN-γ (51.2 and 107.28%), Cu (71.31 ppm), and Fe (192.56 ppm) in comparison to the control. The results of this experiment indicate that natural supplements investigated herein, especially inulin, essential oils, and sodium alginate had the ability to potentiate both the immune function and mineral retention during the initial post weaning period.

Keywords: pig; natural supplements; minerals; immune response

Since the ban of the in-feed antibiotics in the European Union, plants and plant extracts (PPEs) have gained increasing interest as possible alternatives to the in-feed antibiotics, and also as feed additives for pig production (Van Nevel et al., 2005; Windisch et al., 2008). In many studies health benefits obtained via their immunostimulatory properties were observed after the use of different PPEs in animal diets. For example, thymol and carvacrol, active components of plant essential oils are able to increase the percentage of CD4+, CD8+, MHC class II, and non-T/non-B cells in peripheral blood,

and CD4+ CD8+ double-positive T lymphocytes in peripheral blood and mesenteric lymph nodes in growth-retarded, low-weight growing-finishing pigs (Walter and Bilkei, 2004). Thymol used alone enhances total IgA and IgM serum levels, and exhibits some local anti-inflammatory properties, as indicated by reduction of TNF-α mRNA in the stomach of post-weaned pigs (Trevisi et al., 2007). Plants from the *Echinacea* family are also known to modulate immune functions. Pharmacological data inferred that *Echinacea* preparations stimulate the innate immune system and increase the resistance

to infection (Bauer et al., 1999). The response to a vaccine against *Erysipelothrix rhusiopathiae* was enhanced by the inclusion of *E. purpurea* into the diet of finishing pigs (Maass et al., 2005).

The PPEs used in the present study, namely *Chlorella vulgaris* powder, Na-alginate, inulin, and a mixture of essential oils are known for their potential to modify microbial gut population increasing the beneficial lactic acid bacteria and to have strong antibacterial effects (Castillo et al., 2006; Janczyk et al., 2009a, b; Lallès et al., 2009). Dietary administration of inulin resulted in increase of faecal lactobacilli and bifidobacteria and in decrease of clostridia, gram-positive cocci, and *Bacteroides* (Mitsouka et al., 1987; Kleessen et al., 1997; Harmsen et al., 2002). Alginate is considered to influence digestibility and availability of nutrients from the diet (Terada et al., 1995; Bach Knudsen, 2001; Drochner et al., 2004) and for the unicellular microalga *Chlorella vulgaris* immune-modulating and anti-cancer properties have been reported (Tanaka et al., 1984, 1986; Noda et al., 1996; Yasukawa et al., 1996; Justo et al., 2001). Active components of essential oils are known to have antioxidative (Grassmann et al., 2001) and anti-inflammatory (Santos and Rao, 2000; Peana et al., 2002; Kim et al., 2003) effects.

It was observed that, to induce any response, the composition, source, and the rate of PPEs inclusion in the diet are very important. For example, Lee and Kim (2009) showed that dietary *Chlorella vulgaris* intake may prevent insulin resistance in Wistar rats fed high fat diet, but 10% dietary Chlorella was more effective for blood glucose regulation than the diet with 5% Chlorella. Also, saponin from different sources (*Quillaja saponaria* and yucca plant extracts) improved (Cromwell et al., 1985) or not (Turner et al., 2002) growth rate and feed efficiency of weanling pig at the dose of 125 ppm.

However some PPEs have no unambiguous ability in immune modulation. β-glucans from *Astragalus membranaceus* do not have an effect on immune response, the concentration of specific ovalbumin antibody, IgG, IL-4, and IL-10 being not affected by different dietary levels of the plant (Mao et al., 2005; Yuan et al., 2006). Nevertheless, the potential of plant extracts and their components to enhance pig health and immunity has been scarcely evaluated *in vivo*, and further studies would be required to confirm their effects.

The aim of the present study was to determine whether some selected dietary plants and plant extracts can influence the bioavailability of several micronutrients important for the immune response (Fe, Cu, Mn, and Zn) and to assess if the dietary PPEs supplementation could potentiate an immune response which would improve pig health status after weaning.

MATERIAL AND METHODS

Animals and sampling

The experiment was performed on a total of 20 male starter piglets of German Landrace sows in the 2nd to 6th parity (Janczyk et al., 2009a). To reduce the genetic difference between litters, all piglets had one father boar. Piglets were weaned at 28 days of age and the whole litter from one dam (8–11 piglets of both sexes) was allocated to a pen of 4.20 m^2 as a treatment group. After weaning, piglets were offered a barley-wheat based starter diet (reference diet, RD) (Janczyk et al., 2009a) or the RD supplemented with PPEs: *Chlorella vulgaris* powder (RD + CV), Na-alginate (RD + A), inulin (RD + I), and a mixture of essential oils (RD + EO) for 11days. The PPEs were added to the starter diet according to supplier's recommendations in the following concentrations: 1.0% of *Chlorella vulgaris* as a bullet-milled micro algae powder (IGV GmbH, Nuthetal/Bergholz-Rehbrücke, Germany)(Janczyk et al., 2009b), 0.1% of Na-alginate (Bioalgeen®, Schulze & Hermsen GmbH, Dahlenburg, Germany), 0.04% of essential oil mixture containing 25 g limonene, 5 g eugenol and 12 g pinene per kg product, closed in micro-capsules (provided by Delacon Biotechnik GmbH, Steyregg, Austria), and 1.5% inulin (Raftiline® HP, Orafti S.A., Oreye, Belgium). The RD was formulated to meet all nutritional requirements of starter pigs (Table 1); diets and water were available *ad libitum* and pigs were weighed individually in the beginning and at the end of the trial; no feed intake was recorded in this study. After 11 days of feeding the PPEs (39 days of age), four piglets per treatment group were sacrificed by an intracardial injection of T61 (Intervet, Unterschleißheim, Germany), after collecting of blood samples. Liver and spleen were dissected, weighed and an aliquot (30–50g) was immediately frozen in liquid nitrogen and stored at −80°C. The remaining organ samples were homogenized, frozen at −20°C, and finally lyophilised in vacuum at low temperature.

Table 1. Formulation of the control and experimental diets

Ingredients (%)	
Barley meal	30.0
Wheat meal	29.7
PEF (44% starch)	5.00
Whey powder	8.00
Wheat bran	2.50
Soycomil (soy concentrate)	4.00
Maize starch[1]	4.00
Potato protein, purified	5.00
Maize gluten meal	2.20
Sunflower meal	2.50
Limestone	1.02
Mono calcium phosphate	0.78
Trace min.-vit. premix[2]	0.40
Methionine (99%)	0.11
L-lysine-HCl (79%)	0.34
Tryptophan (99%)	0.031
Threonine (98%)	0.03
Palm oil + soybean oil	3.10
Molasses	1.009
NaCl	0.28
Calculated nutrient content (g/kg)	
Dry mater	888
Crude protein	191
Ash	55
Crude fibre	34
Crude fat	50
Starch + sugar	455
Lysine	12.5
Ca	7.2
Total P	6.1

[1]Maize starch was reduced to 3% in RD + CV, 3.9% in RD + A, 2.5% in RD + I, and 3.96% in EO; 1, 0.1, 1.5, and 0.04% of *Chlorella vulgaris*, Na-alginate, inulin, and essential oil mixture (limonene 0.01, eugenol 0.002, pinene 0.0048 g/ kg) was added instead, respectively

[2]Trace mineral-vitamin premix (0.4%) supplies per kg diet as follows: vit. A 1750 IU, vit. D3 200 IU, vit. E 11 IU, vit. K1 0.5 mg, vit. B_1 1.0 mg, vit. B_2 4 mg, d-pantothenic acid 9 mg, niacin 12.5 mg (available), biotin 50 μg, vit. B_{12} 15 μg, folic acid 0.3 mg, vit. B_6 1.5 mg, choline 400 mg, Fe 80 mg, Zn 54 mg, Mn 30 mg, Co 0.15 mg, I 0.14 mg, Se 0.25 mg, antioxidants (E310, 320, 321) 50 mg, and maize starch as carrier

The study protocol was approved by the Animal Care and Use Committee of the Ministry of Nutrition, Agriculture, Forestry, and Fishery of the State Mecklenburg Vorpommern, Schwerin, State Mecklenburg-Vorpommern, Germany (permission No. VI 522a-7221.31-1-018/99).

Plasma biochemical parameters measurement

Plasma concentrations of Ca, P, Mn, total protein, urea, glucose, bilirubin, and the activity of alkaline phosphatase (ALKP), glutamate pyruvate transaminase (TGP), and glutamate oxaloacetate transaminase (TGO) were determined on a BS-130 Chemistry analyzer (Shenzhen Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, P.R. China).

Measurement of minerals (Fe, Cu, Mn, Zn) in liver and spleen

The lyophilised samples of liver and spleen were prepared for microelement analysis (Cu, Fe, Mn, and Zn) via microwave digestion (model BERHOF, Speedwave, MWS-2 Comfort, Eningen, Germany), including mineralization with $HNO_3 : H_2O_2$ (5 : 2), and the following digestion conditions: 8 min at 130°C, 80% energy; 5 min at 155°C, 80% energy; 12 min at 170°C, 80% energy. Maximal microwave oven power was 1000 W. After full cooling at room temperature, the obtained solutions were filtered and mineral analyses were conducted by flame atomic absorption spectrophotometry (Pye Unicam, Thermo Electron, Solaar M). All analyses were performed in duplicate, and mineral concentrations were reported on a wet basis (ppm), respectively.

Plasma antioxidant capacity detection

Antioxidant level in plasma of pigs from different groups was measured by Trolox equivalent antioxidant capacity (TEAC) assay (Sigma, Saint Louis, USA). Briefly, 10 μl of plasma or Trolox solution plus 20 μl of myoglobin solution, and 150 μl of 3mM ABTS solution (final concentration 300μM) were added to 96-well microplate, mixed by vibration, and incubated at room temperature for 5 min according to the manufacturer's instructions.

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Reaction was stopped with 100 µl of stop solution. The end point absorbance was read at 405 nm using a microplate reader (TECAN, Sunrise, Austria).

Measurement of plasma total immunoglobulin subsets (IgG, IgA, IgM)

Blood was collected from the heart on EDTA (1.6 mg EDTA/ml blood). After centrifugation in the Multifuge 3L-R (Heraeus, Hanau, Germany) at 2500 rpm at 4°C for 10 min, the plasma was transferred to a fresh tube, frozen, and further stored at –20°C until immunoglobulin analysis was performed. Total concentration of immunoglobulin subsets was measured by ELISA (Bethyl, Medist, Montgomery, USA) after plasma dilution: 1/4000 (IgA), 1/60 000 (IgG), and 1/6000 (IgM) as previously reported (Marin et al., 2006), and according to the manufacturer's instructions. Absorbance was read at 450 nm using a microplate reader (TECAN, Sunrise, Austria), and results were expressed as mg/ml of plasma.

Measurement of cytokine production

Samples of fresh liver were weighed and homogenized in phosphate buffer containing igepal 1%, sodium deoxycholate 0.5%, SDS 0.1%, and complete (EDTA-free) protease inhibitor cocktail tablets. The homogenates were kept 30 min on ice, and then centrifuged at 10 000 *g* at 4°C for 10 min. IL-1β, IL-8, TNF-α, and IFN-γ concentration in the supernatants were determined by ELISA, using the commercially available kits (R&D Systems, Minneapolis, USA), according to the manufacturer's instructions (Marin et al., 2010). Optical densities were measured on an ELISA reader (Tecan, Sunrise, Austria) at a wavelength of 450 nm. Dilution of recombinant swine IL-1β, IL-8, TNF-α, and IFN-γ were used as standards, and data were analyzed against the linear portion of the generated standard curve. Results were expressed as picograms of cytokine/ml supernatant.

Statistical analyses

All data are expressed as mean ± standard error of the mean (SEM). Each pig was considered an experimental unit. ANOVA & *t*-test analyses were performed to investigate the statistical differences between groups for all parameters analysed. Further differences between means were determined by Fisher's procedure of the least square difference. Values of $P < 0.05$ were considered significant.

Table 2. Selected plasma biochemical parameters in piglets fed a reference diet (RD) or supplemented with *Chlorella vulgaris* (RD + CV), Na-alginate (RD + A), inulin (RD + I), and a mixture of essential oils (RD + EO) for 11 days after weaning*

Biochemical parameters	Normal** value	Supplemental plant extract, diet (%)					SEM	*P*-value
		RD 0	RD + CV 1.0	RD + A 0.1	RD + I 1.5	RD + EO 0.04		
Phosphorus (mg/dl)	5.5–9.3	8.36	8.25	8.43	8.66	9.80	0.32	0.372
Magnesium (mg/dl)	1.3–2.5	1.37	1.35	1.37	1.38	1.39	0.03	0.986
Total protein (g/dl)	5.8–8.3	4.94	5.00	4.97	5.05	4.75	0.091	0.409
Urea (mg/dl)	8.2–24.6	14.27	13.47	13.38	14.60	13.75	0.56	0.964
Creatinine (mg/dl)	0.8–2.3	1.08[c]	1.27[ab]	1.17[bc]	1.24[ab]	1.32[a]	0.03	0.009
Bilirubin (mg/dl)	0.0–0.5	0.03[b]	0.03[b]	0.03[b]	0.03[b]	0.04[a]	0.02	0.010
Alkaline phosphatase (ALKP, IU/l)	41–176.1	146.43	135.70	149.97	132.30	138.28	3.85	0.529
Glutamate oxaloacetate transaminase (TGO, IU/l)	21.7–46.5	30.70	28.85	32.43	33.03	30.83	1.12	0,826
Glutamate pyruvat transaminase (TGP, IU/l)	15.3–55.3	24.70[b]	31.30[a]	32.55[a]	24.70[b]	24.03[b]	1.29	0.009

*At the end of the experiment plasma from 4 piglets per group was used to measure the blood biochemical parameters. Data are means ± SEM. Comparison between control and treated animals

**Reference Guides (1998)

[abc]mean values within a row with unlike superscript letters were significantly different ($P < 0·05$)

RESULTS

Performance

All pigs fed either RD or PPEs supplemented diets appeared clinically normal during the whole experiment period (11 days). There were no differences among the groups in the body weight ($P > 0.05$). The initial weight of the piglets was 8.6 ± 1.7 kg, and final body weight was 10.2 ± 1.6, 9.3 ± 1.5, 10.8 ± 0.7, 10.2 ± 1.6, 10.0 ± 1.7 kg in the RD, RD + CV, RD + A, RD + I, and RD + EO groups, respectively.

Plasma biochemical parameters

To determine whether the diets supplemented with PPEs had an effect on general health status of weaning piglets, concentrations of plasma biochemical parameters mentioned above were determined. Pigs fed diets supplemented with PPEs had significantly increased creatinine concentration ($P < 0.05$) (Table 2). The supplementation of 0.1% Na-alginate and 0.04% essential oils to the RD also increased the activity of glutamate pyruvate transaminase and the bilirubin concentration in the plasma of treated animals, respectively ($P < 0.05$) (Table 2). Other plasma biochemical parameters were not affected by the dietary treatments (Table 2).

Mineral (Fe, Cu, Mn, Zn) concentration in liver and spleen

To determine whether PPEs interfere with micronutrients, the concentration of trace elements, Cu, Fe, Mn, and Zn in liver and spleen was measured. The results expressed in Figure 1 showed an increase in the liver concentration of Cu and Fe in response to the diets supplemented with PPEs. Compared to the control, the increase of Cu was significantly higher ($P < 0.05$) in liver for all the four treatments: 47.07 ppm (RD + CV), 48.98 ppm (RD + A), 71.31 ppm (RD + I), and 49.22 ppm (RD + EO) vs. 28.29 ppm (RD). Level of Fe was also higher ($P < 0.05$) in RD + I (192.56 ppm), RD + CV (188.77 ppm), and RD + EO (176.18 ppm) groups. No effect on Zn and Mn concentration was registered in the liver, except for a slight numerical increase produced by RD + EO, and a decrease caused by RD + A in Zn. In the spleen, the concentration
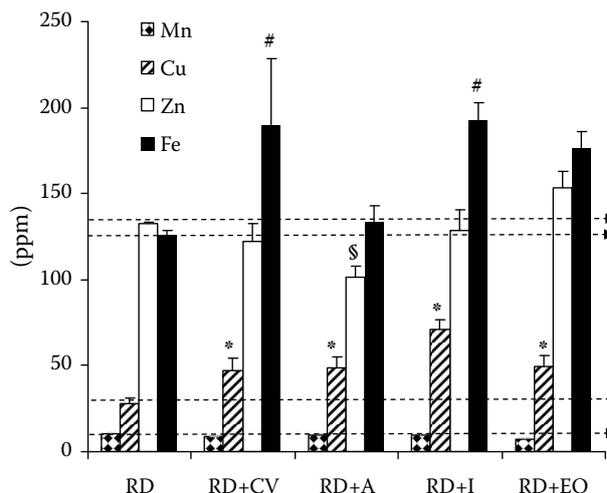

Figure 1. Liver micronutrients (Cu, Fe, Mn, Zn) concentration in pigs fed diets supplemented with PPEs for 11 days after weaning

After weaning (28 day) pigs were fed with a starter reference diet (RD) or RD supplemented with *Chlorella vulgaris* powder (RD + CV), Na-alginate (RD + A), inulin (RD + I), and essential oil mixture (RF + EO) for 11 days. Liver lyophilisates samples were collected and prepared for microelement analysis (Cu, Fe, Mn, and Zn) via microwave digestion including mineralisation with $HNO_3 : H_2O_2$ (5 : 2). Mineral analyses were conducted by flame atomic absorption spectrophotometry and mineral concentrations were reported on a wet basis, respectively. Data are means ± SEM ($n = 4$) #, §, * mean value significantly different from that of the control group ($P < 0.05$)

of investigated minerals was lower than in the liver (3.72–4.02 for Cu, 51.52–56.84 for Fe, 1.15–1.47 for Mn, and 91.88–95.33 for Zn, respectively), and PPEs supplementation did not affect any of the investigated trace elements (data not shown).

Plasma antioxidant capacity

To assess the antioxidant activities of the investigated PPEs, the antioxidant level in plasma of pigs from different groups was measured by Trolox equivalent antioxidant capacity (TEAC) assay. No difference between the four PPEs and the RD in the plasma levels of TEAC was observed (Table 3).

Plasma immunoglobulin profile

Blood samples were collected from the piglets fed the treatment diets for 11 days, and the effect of dietary PPEs on the humoral immune response was assessed by the measurement of total immu-

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Table 3. Plasma TEAC* in piglets fed a reference diet (RD) or supplemented with *Chlorella vulgaris* (RD + CV), Na-alginate (RD + A), inulin (RD + I), and a mixture of essential oils (RD + EO) for 11 days after weaning

Diets	TEAC
RD	0.236
RD + CV	0.204
RD + A	0.220
RD + I	0.245
RD + EO	0.262
SEM	0.008
P-value	0.11

*Antioxidant level in plasma of pigs from different groups was measured by Trolox equivalent antioxidant capacity (TEAC) assay according to the manufacturer's instructions. Data are means ± SEM (*n* = 4). ANOVA test was performed to analyze the effect of different treatments on TEAC level. No differences were obtained between the groups

noglobulin subsets, IgA, IgM, and IgG in plasma. As shown in Figure 2, the presence of PPEs in the diet of pigs did not have any influence on IgA and IgM levels with the exception of a slight increase of these Ig produced by RD-EO diet. In contrast, all dietary supplements increased the plasma IgG and pigs fed RD + A diet had significantly (*P* < 0.05) higher plasma IgG concentration than pigs from RD (6.00 vs. 4.03 mg/ml).
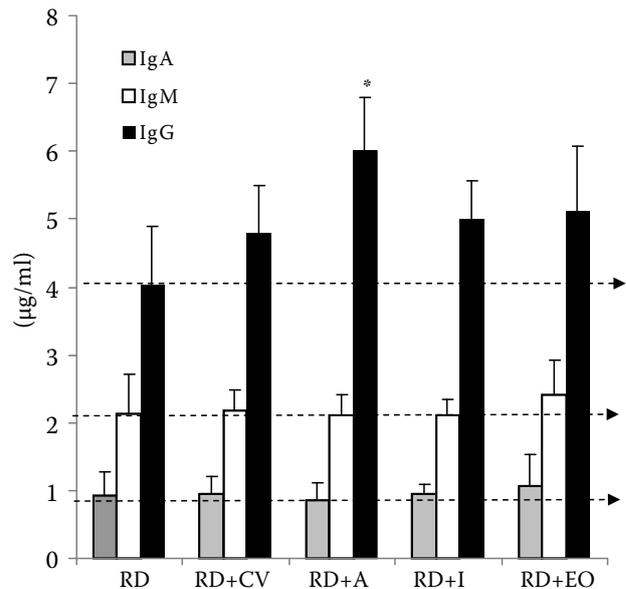

Figure 2. Plasma immunoglobulins (IgA, IgM, IgG) concentration in pigs fed diets supplemented with PPE for 11 days after weaning

Total concentration of immunoglobulin (Ig) subsets from blood plasma derived from weaned pigs fed for 11 days with a starter reference diet (RD), or RD supplemented with *Chlorella vulgaris* powder (RD + CV), Na-alginate (RD + A), inulin (RD + I), and essential oil mixture (RF + EO) was measured by ELISA. Results are expressed as IgA, IgM, or IgG content in the plasma of piglets, mean ± SEM, (*n* = 4/group)

*mean value significantly different from that of the control group (*P* < 0.05)

Cytokines production in liver and spleen

To investigate the effect of PPEs on cellular immune response, liver and spleen samples were collected and the synthesis of cytokines (IL-1β, IL-8, TNF-α, and IFN-γ) was measured by ELISA. In liver, dietary PPEs treatments, especially RD + EO and RD + I were able to modulate the level of IL-1β,

Table 4. Cytokine concentrations* in liver of pigs fed a reference diet (RD) or supplemented with *Chlorella vulgaris* (RD + CV), Na-alginate (RD + A), inulin (RD + I), and mixture of essential oils (RD + EO) for 11 days after weaning

Cytokine (pg/ml)	Supplemental plant extract, diet (%)					SEM	*P*-value
	RD 0	RD+CV 1.0	RD+A 0.1	RD+I 1.5	RD+EO 0.04		
Il-1β	1372.01[b]	1360.61[b]	1439.08[b]	3092.25[a]	2591.30[a]	183.40	0.0001
IL-8	1784.96[c]	1688.85[c]	1986.05[bc]	4229.45[a]	2878.50[b]	258.69	0.0005
TNF-α	1025.23[c]	927.40[c]	876.34[c]	4066.39[a]	2272.25[b]	331.42	0.0004
IFN-γ	51798.75[c]	57856.58[bc]	44562.75[c]	78301.67[b]	107367.29[a]	6048.57	0.0003

*Concentration of cytokines, IL-1β, IL-8, TNF-α, and IFN-γ was measured by ELISA using R&D Systems kits, according to the manufacturer's instructions. Data are means ± SEM (*n* = 4). ANOVA test was performed to analyze the effect of different treatments on cytokine production
[abc]means with different superscripts within a row differ significantly from each other (*P* < 0.05)

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Table 5. Cytokine concentrations* in spleen of pigs fed a reference diet (RD) or supplemented with *Chlorella vulgaris* (RD + CV), Na-alginate (RD + A), inulin (RD + I), and a mixture of essential oils (RD + EO) for 11 days after weaning

Cytokine (pg/ml)	Supplemental plant extract, diet (%)					SEM	*P*-value
	RD 0	RD + CV 1.0	RD + A 0.1	RD + I 1.5	RD + EO 0.04		
Il-1β	202.47	215.33	84.34	129.46	238.80	21.33	0.099
IL-8	2754.05	2304.80	2857.77	2790.33	nd	261.45	0.001
TNF-α	nd	nd	nd	nd	nd	nd	–
IFN-γ	6720.90[b]	7703.20[a]	6772.18[b]	6448.63[b]	7703.20[a]	200.37	0.007

*Concentration of cytokines, IL-1β, IL-8, TNF-α, and IFN-γ was measured by ELISA in samples of spleen collected at the end of the experiment, using R&D Systems kits (according to the manufacturer's instructions). Data are means ± SEM (n = 4). ANOVA test was performed to analyze the effect of different treatments on cytokine production
[abc]means with different superscripts within a row significantly differ from each other ($P < 0.05$)
nd = not detected

IL-8, TNF-α, and IFN-γ production (Table 4). Indeed, in comparison with the control, in liver of animals fed RD + I and RD + EO a higher concentration of IL-1β (125.4 and 88.9%), IL-8 (136.9 and 61.3%), TNF-α (296.6 and 121.6%), and IFN-γ (51.2 and 107.28%) was observed ($P < 0.05$). A slight numerical increase of IFN-γ level was also produced by consumption of RD + CV (Table 4).

In spleen, the influence of PPEs on cytokine response was less consistent. Thus, *Chlorella vulgaris* and essential oil supplements triggered an increase of IFN-γ, RD + A, and RD + I resulted in a non significant decrease in IL-1β production while no difference between control and PPEs diets for IL-8 production was registered. TNF-α was detected neither in control nor in the pigs fed the diets supplemented with PPEs as well as IL-8 in the spleen from pigs fed RD + EO (Table 5).

DISCUSSION

In the present study, we determined whether selected dietary plant and plant extracts (*Chlorella vulgaris*, sodium alginate, inulin, and a mixture of essential oils) investigated *in vivo* as alternatives to antibiotics could influence the humoral and cellular immune function (immunoglobulin and cytokine production), as well as the status/bioavailability of several trace elements supporting the immune response (Fe, Cu, Mn, and Zn), in pigs after weaning. The results of this trail indicate that PPEs supplements investigated herein had the ability to boost the IgG immune response during the initial post weaning period. Plasma of pigs fed dietary PPEs had higher concentration of IgG than that from animals fed no supplements, significantly higher for Na-alginate group. A similar increase in IgG concentration was also observed after feeding other polysaccharides like pectin (Khramova et al., 2009), ginseng (Wang et al., 2001) or FOS/inulin (Vidal et al., 2008) in mice or different plant extracts like cinnamon, thyme, oregano (Namkung et al., 2004), and saponin (Ilsley et al., 2005) in pigs. By contrast, no effect on IgG was found with dietary curcumin supplement (Ilsley et al., 2005) or with oregano essential oil supplementation (Ariza-Nieto et al., 2011) in pig. Immunoglobulin G plays a crucial role in the clearance of invading microbes and the generation of long-lasting immunity. Furthermore, immunoglobulin G feedbacks on the generation of novel IgG antibodies by activated B cells, and participates actively in maintaining and returning to the steady state (Nimmerjahn and Ravetch, 2010). The mechanism by which PPEs lead to an increase of IgG is largely unknown, and needs to be demonstrated. It is possible that the active molecules from PPEs (polyphenols, vitamins, minerals, etc.) interact as additional ligands with Fc-receptors for IgG (FcgammaRs) and then influence the immune response (Nimmerjahn and Ravetch, 2010).

For some of the investigated PPEs the influence on humoral immune function was accompanied by changes in cellular immune response as measured by cytokine production. After 11days of treatment, liver from animals fed RD + I and RD + EO diets contained higher ($P < 0.05$) level of IL-1β, IL-8, TNF-α, and IFN-γ than liver from animals fed no supplements. A higher IFN-γ production was also found in the spleen of animals fed RD + EO

and RD + CV. Our findings support the hypothesis that PPEs potentiate the immune reaction through increase of IFN-γ production reported also by Hasegawa et al. (1997) and Quieroz et al. (2002) for *Chlorella vulgaris* which might involve a Th1 rather than Th2 type of cellular immunity. Indeed, an increase in the synthesis of pro-inflammatory cytokines IL-1β, IL-6, TNF-α, IL-12, IFN-γ was noticed in mice and pigs after treatments with inulin, alginate, and essential oils (Iwamoto et al., 2005; Pié et al., 2007; Benyacoub et al., 2008; Nam et al., 2008; Yang and Jones 2009) or other PPEs, propolis (Moriyasu et al., 1994), β-glucans from Astragalus (Lamm and Riggs, 2001) etc. The mechanism by which polysaccharides like inulin or alginate impact immunity still needs further investigation. It is possible that they directly interact with the macrophages and dendritic cells underlying the gut mucosa (Benyacoub et al., 2008) and activate the NF-kappaB pathway through the macrophage receptors (toll-like receptors). Treatment with alginate solution caused responses that closely paralleled stimulation by lipopolysaccharide in timing and magnitude (Iwamoto et al., 2005; Yang and Jones, 2009).

On the other hand, many studies have shown an anti-inflammatory action of PPEs. For example, Cherng et al. (2010) observed that peptides (Val-Glu-Cys-Tyr-Gly-Pro-Asn-Arg-Pro-Gln-Phe) derived from Chlorella (Chlorella-11 peptide) is able to diminish the expression of iNOS mRNA, iNOS, NF-kappaB proteins and the levels of TNF-alpha and PGE2 after LPS activation. Rodrigues et al. (2009) reported that treatment of mice and mice macrophages with water extract and essential oils of clove (both forms containing eugenol as major component) inhibited the production of IL-1β and IL-6. The use of a mixture of long-chain inulin and oligosaccharide in HLA-B27 transgenic rats exhibited some local anti-inflammatory properties, through the reduction of gross cecal and inflammatory histological scores in the cecum and colon, the decreased levels of the pro-inflammatory cytokine IL-1β, and the increased of the anti-inflammatory TGF-β (Hoentjen et al., 2005). The results of Hirota et al. (2010) suggest that low concentrations of limonene from Yuzu, a traditional medicine used in Japan have anti-inflammatory effect by inhibiting cytokines, ROS production, and inactivating eosinophil migration. Limonene and beta-myrcene from essential oils of two species of Asteraceae produced also significantly less IFN-γ and IL-4

and inhibited cell migration when administered orally in a mouse model of pleurisy induced by zymosan and lipopolysaccharide (LPS) (Souza et al., 2003). The anti-inflammatory effect of PPEs is in many cases carried out by the activation of the NF-kappaB pathway. Despite different effects on the same immune parameters, all above observations revealed that PPEs are able to modulate the immune response. Conflicting results (pro- or anti-inflammatory) may arise probably from variations in the origin, structure, period of supplementation, and level of added compound, or might be attributed to their different mechanism of action; it would be of interest to test these mechanisms in the future.

Mineral bioavailability is mainly influenced by diet composition (Rodrigues Lobo et al., 2009). The present study indicates that pigs fed a diet supplemented with PPEs for 11 days showed an increase in plasma Fe concentration and in Fe and Cu liver concetration evaluated as a measurement of mineral bioavailability. *Chlorella vulgaris* fed to pregnant mice also resulted in increase of serum Fe concentration (Janczyk, 2005), as it has also been reported for feeding rats with *Spirulina* (Kapoor and Mehta, 1993). Vyas et al. (2009) demonstrated also in humans that the daily serving of a lucerne leaf concentrate powder was as effective as a daily tablet of Fe (60 mg as $FeSO_4$) in the treatment of anaemia in Indian adolescent girls, suggesting a better bioavailability of Fe and/or a synergistic effect of other components of leaf concentrate, even though it contains a lower Fe concentration (5 mg Fe) (Vyas et al., 2009). Similarly, dietary supplementation with 10% inulin type fructan for 15 days resulted in higher intestinal absorption of some macro- and microminerals (Ca, Mg, Cu, and Zn) in rats (Rodrigues Lobo et al., 2009). Among the plant extracts herein investigated, inulin and essential oils supplement produced the highest effect on liver Cu and Fe concentration. Inulin and other fructo-oligosaccharides are the most investigated dietary non-digestible oligosaccharides with respect to their positive effects on mineral bioavailability. Mechanisms on how inulin-type fructans mediate this effect in rats include, among others, modulation of the paracellular and transcellular absorption, permeability of intestinal tight junctions, and gene expression of mineral transporters such as calbindin-D9k (for Ca), DMT-1, ferroportin (for Fe), and ZnT1 (for Zn), which increase mineral bioavailability (Rémésy, 1993; Scholz-Ahrens and

Schrezenmeir, 2007; Tako et al., 2008). By contrast, in our study no influence of plant extracts on Mn and Zn was observed.

Trace elements are implicated in many physiological processes contributing to the body's natural defences on three levels: support for physical barriers (skin/mucosa), cellular immunity, and antibody production (Maggini et al., 2007), as well as their critical role in enzyme activity (catalase, alkaline phosphatase, superoxid dismutase) has been increasingly recognized (Chandra and Chandra, 1986; Bourre, 2006; Rodrigues Lobo et al., 2009; Yuan et al., 2010). Supplementation with these micronutrients support also a Th-1 cytokine mediated immune response with sufficient production of pro-inflammatory cytokine, and enhanced innate immunity (Wintergerst et al., 2007; Wan-an et al., 2010). Indeed, in the present study the diets that induced an increase in liver concentration of Fe and Cu determined an increase in pro-inflammatory cytokines (IL-1β, IL-8, TNF-α, IFN-γ) production, which suggest the hypothesized role of PPEs in supporting innate immunity. This should be further studied.

CONCLUSION

In summary, the above findings suggest that plants and plant extracts have a potential to influence the immune functions in weaned pigs. The immunopotentiating properties of the plant extracts investigated in this study, especially sodium alginate and essential oils, lead to an increase in the secretion of plasma IgG, and inulin and essential oils were able to increase the level of cytokine (IL-1β, IL-8, TNF-α, IFN-γ) production in liver. The effect on spleen cytokine response was less consistent for all the investigated PPEs. These findings also revealed an improvement in mineral (Cu and Fe) stores in liver, produced by inulin, chlorella, and essential oils. Further feeding experiments using different PPEs dietary rates are required in order to study in more detail the health promoting effects of these plants and plant extracts and elucidate the likely mechanisms involved in these results.

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