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SUPPLEMENTARY MATERIAL

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Immuno- and glyco- histochemistry as a tool to evaluate the oregano supplemented feed effects in pig gut

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Materials and Methods

Animal recruiting and experimental design

Thirty-two Duroc x Large White pigs, previously randomly divided into 2 homogenous groups of 16 pigs each and housed in 3 × 3 m pens containing 4 pigs each, were used. The numerousness of animals for each group was calculated and considered optimal for a significance level of 0.05, a test power of 0.8 and an effect size of 1. In order to meet the nutrient requirements of Italian heavy pigs, the animals were fed according to a three-phase feeding program. During the finisher stage (87 days) the two dietary treatments were: degermed corn-barley-soybean-based diet (crude protein 14.08%; crude fat 2.73%; ash 4.89%; digestible energy 15.40 Mcal/kg) (CTR group); CTR diet supplemented (2 g/kg) with oregano aqueous extract (OAE; O group). Water was provided ad libitum and the diets were wet (water to feed ratio of 3:1). The animals were intended for human consumption and were slaughtered (10 months old) in a slaughterhouse in accordance with the European Union regulation on the protection of animals at the time of killing (Council Regulation (EC) No 1099/2009). Animal growth performances were summarized in Table S1.

Extraction and analysis of oregano aqueous extract (OAE)

The OAE was obtained by a process of bio-liquefaction based on enzyme bio-catalysis[1] as previously described by Franciosini *et al*.[2] Briefly, the OAE was obtained by a process of bio-liquefaction based on enzyme bio-catalysis placing the plant material in boiling water, and adding a specific enzymatic preparation. After four hours of hydrolysis, the plant material was filtered. The OAE obtained was analysed to quantify:

- antioxidant capacity, measured in terms of radical scavenging ability using the stable radical DPPH[3],

-t otal polyphenols, evaluated using the Folin-Ciocalteu reagent,[4]

- reducing sugars was evaluated using the ADNS method[5].

Results are summarized in Table S2. In order to enable the GC-MS analysis of the organic volatile compounds an organic phase extraction was performed on the OAE utilizing Petroleum Ether in 1:1 volume with respect to H_2O phase. Analysis of volatile compounds contained in the oregano aqueous extract was performed using a HP 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a MS (mass detector HP 5973, Agilent Technologies) mass selective detector in the electron impact mode (70 eV). Injector and MS transfer line temperatures were set at 200 and 300°C, respectively. One µL of diluted sample (1 mg in 10 mL petroleum ether) was injected in the split mode (ratio 1/60). The temperature

program was set at 50°C for 2 min, 50°C to 170°C at 5°C/min, 170°C to 200°Cat 2°C/min, 200°C to 290°C at 8°C/min, and 290°C for 7 min. Helium (purity 99.99999%) was the carrier gas, at a flow rate of 1 mL/min. The quadrupole MS operated in an electron impact ionization mode m/z at 70 eV; mass scanning was from 40 to 650 atomic mass unit. The components were identified based on the comparison of their retention indices (RI) and mass spectra with those of standards of the GC-MS system and literature data. The percentage composition of present volatile compounds in the OEA evaluated by means of GC-MS is reported in Table S3; components present in % under 1 were gathered as an only group.

References

1. Setti L, Zanichelli D. Bioliquefaction as a bio-refinery's approach for the production of natural bioactive compounds for functional cosmetics. In: Morselli L, Passarini F, Vassura I, editors. Waste recovery: strategies, techniques and applications in Europe. Milan; Franco Angeli; 2009; p. 122-8.
2. Franciosini MP, Casagrande-Proietti P, Forte C, Beghelli D, Acuti G, Zanichelli D, et al. Effects of oregano (Origanum vulgare L.) and rosemary (Rosmarinus officinalis L.) aqueous extracts on broiler performance, immune function and intestinal microbial population. J Appl Anim Res 2016;44:474-9.
3. Donglin Z, Yasunori H. Phenolics, ascorbic acid, carotenoids and its antioxidant activity of broccoli and their changes during conventional and microwave cooking. Food Chem 2004;88:503-9.
4. Ainsworth EA, Gillespie KM. Estimation of total phenolics content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. Nat Protoc 2007;2:875-7.
5. Bailey MJ, Biely P, Poutanen K. Interlaboratory testing of methods for assay of xilanase activity. J Biotechnol 1992;23:257-70.

Table S1. Animal performances.

Growth performance	CTR diet	O diet
Mean body weight at beginning of finisher stage (kg)	120.2	119.8
Mean body weight at ending of finisher stage (kg)	183.9	184.2
Average daily gain (g)	732	740
Feed conversion efficiency	4.05	3.85

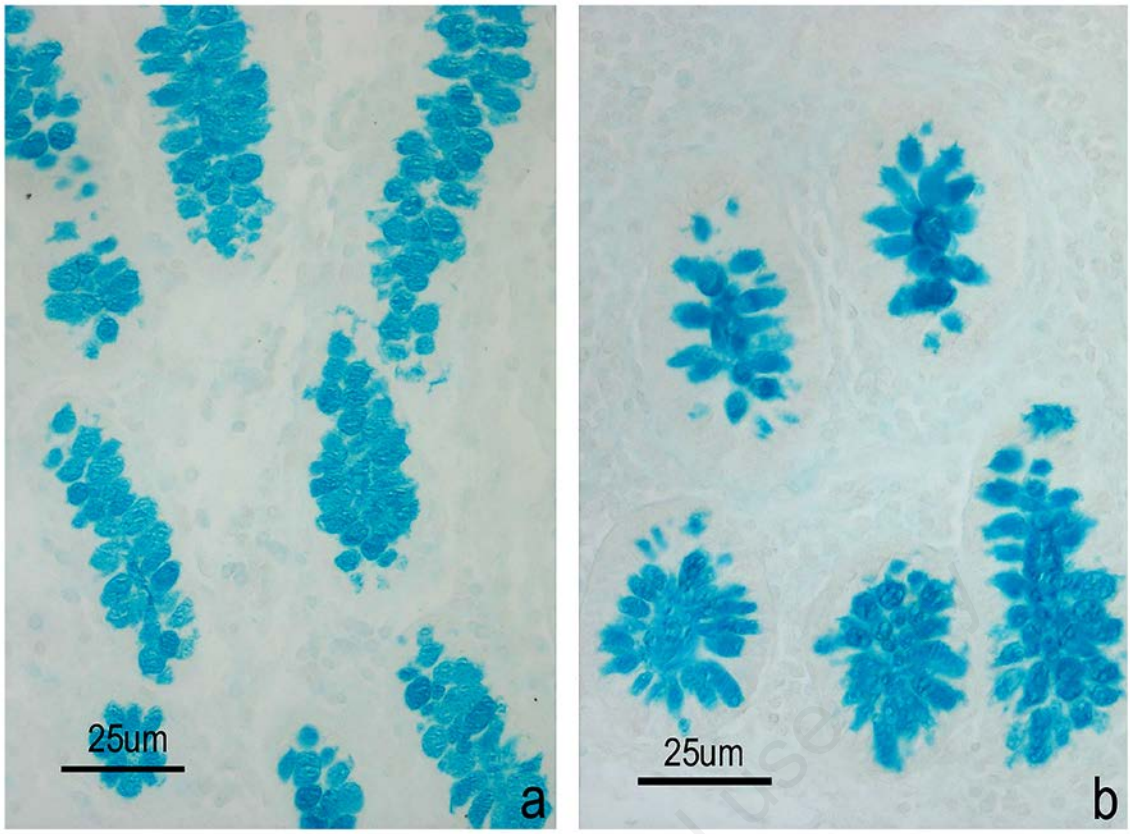
CTR, degermed corn-barley-soybean-based diet; O, CTR diet supplemented (2 g/kg) with an oregano aqueous extract.

Table S2. OEA antioxidant activity.

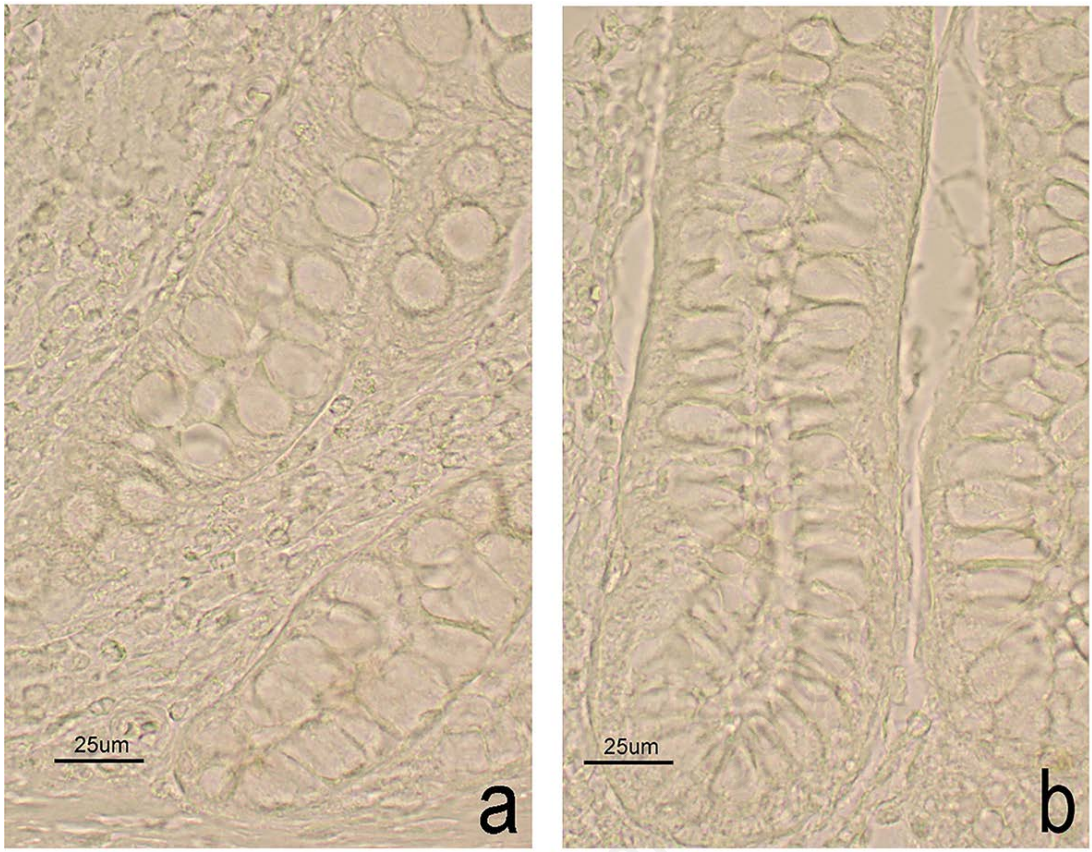
Antioxidant capacity ORAC/L	17,780 ± 260
Total polyphenols	2.5 ± 0.2
Reducing sugars	3.3 ± 0.2

Table S3. Percentage composition of volatile compounds in OEA.

Volatile component	%
Carvacrol	27
Thymol	27
γ-Terpinene	13
2-Hexen-1-ol	7
p-Cymene	7
Limonene	4
α-Terpinene	3
Germacrene	2
Methyl carvacrol	2
1.8-Cineole	1.5
β-Myrcene	1.5
Others compounds (%<1)	5



Supplementary Figure 1.
AB reactivity of ileum (a) and caecum (b) pig sections in Sialidase-free buffer solution.



Supplementary Figure 2.
BAX negative controls of ileum (a) and caecum (b) pig segments.