

Evaluation of oral supplementation of free and nanoencapsulated *Minthostachys verticillata* essential oil on immunological, biochemical and antioxidants parameters and gut microbiota in weaned piglets

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Abstract

Early weaning is an important stressor that impairs the piglet's health, and essential oils appear as promising candidates to improve it. The aim of this study was to evaluate the effect of oral supplementation of free and nanoencapsulated *Minthostachys verticillata* essential oil (EO and NEO, respectively) on immunological, biochemical and antioxidants parameters as well as on gut microbiota in weaned piglets. EO was extracted by hydrodistillation and nanoencapsulation was performed by high-energy method using Tween 80 and Span 60 as surfactants. EO and NEO were chemically analyzed by gas chromatography-mass spectrometry (GC-MS). The cytotoxic effects of both EO and NEO was evaluated on Caco-2 cell line. For *in vivo* assay, piglets were randomly distributed in six groups of six animals each ($n = 6$) and received orally EO (10 mg/kg/day) or NEO (2.5, 5 and 10 mg/kg/day) for 30 consecutive days. Animals not treated or treated with surfactants mixture were evaluated as control and vehicle control. Subsequently, histological, hematological and biochemical parameters, cytokines production, oxidative markers, CD4⁺/CD8⁺ T cells and gut microbiota were evaluated. GC-MS analysis was similar in both EO and NEO. The NEO was more toxic on Caco-2 cells than EO. Oral supplementation of EO or NEO (10 mg/kg/day) increased growth performance compared to control group or NEO (2.5 or 5 mg/kg/day) ($p < 0.05$) groups. NEO (2.5, 5 and 10 mg/kg/day) did not alter the morpho-physiology of digestive organs and decreased MDA levels in liver ($p < 0.05$), resulting safer than EO. In addition, NEO (10 mg/kg/day) showed an increase in CD4⁺/CD8⁺ T cells ratio ($p < 0.001$), and induced the highest serum levels of IL-10 ($p < 0.01$). Serum triglycerides levels were significantly lower in animals treated with EO or NEO (2.5, 5 and 10 mg/kg/day) compared to control group ($p < 0.001$). Gut microbiota analysis showed that NEO (10 mg/kg/day) favor the development of beneficial intestinal microorganisms to maintain an anti-inflammatory microenvironment. In conclusion, EO and NEO improved parameters altered by early weaning in piglets however, NEO was safer and powerful. Therefore, NEO should be further studied to be applied in swine health.

Introduction

Pigs are exposed to various stressors, with early being weaning one of the most important, which alters the oxidative balance of the intestine and lead to the pro-inflammatory mediator's release that can result in reduced pig health, growth, and feed intake, particularly during the first week after weaning (Campbell et al. 2013). This affects not only animal health with immune compromise and muscle degeneration, but also performance and quality of its products (Mota Rojas et al. 2014; Yin et al. 2014; Omonijo et al. 2018). In concordance with the global trend to reduce the use of antibiotics in animal production within the "One Health" concept promoted by the World Organization for Animal Health (WOAH) and the World Health Organization (WHO), there has been an increase in research aimed at boosting the animal's immunity through prophylactic measures and beneficial supplements without compromising animal health, livestock production, the environment and human health (Windisch et al. 2008; Trevisi et al. 2014). In this context, the essential oils from medicinal plants have received a lot of attention as a safe and eco-friendly alternative to improve the health of animals under stress (Lakhani et al. 2019). *Minthostachys*

verticillata (Griseb.) Epling (Lamiaceae), known as *peperina*, is an aromatic medicinal plant that grows in South American countries, principally in the northwest and central regions of Argentina (Cariddi 2013). The native communities use their infusions to treat digestive and respiratory disorders as well as topical for wounds (Cariddi 2013; Ojeda and Karlin 2015). Due to its aroma, the traditional Andes cuisine uses leaves and flowers of *peperina* as a condiment or food flavoring. In addition, the leaves are used to flavor beverages and in the preparation of “yerba mate compuesta” (Ojeda et al. 2001; Schmidt-Lebuhn 2008). In recent years, multiple beneficial properties of *M. verticillata* essential oil (EO) as antimicrobial or insecticidal have been reported (Palacios et al. 2009; González and Marioli 2010; Rossi et al. 2012; Montironi et al. 2016; Cerioli et al. 2018; Cecchini et al. 2021). Furthermore, our research group has demonstrated the ability of EO to modulate the immune response in experimental mouse models administered intraperitoneally, subcutaneously, intramammary and orally. These discoveries contributed to the knowledge of a biological potential for EO not known until then (Cariddi et al. 2006, 2007, 2011, 2013; Montironi et al. 2019, 2022; Campa et al. 2021, 2022). In a recent study, we have reported the impact of EO oral administration on gastrointestinal and immune health in mice. EO improved mice growth performance, modulated systemic adaptive immunity with increase in the percentage of CD4⁺ T cells, improved total antioxidant capacity by decreasing malondialdehyde (MDA) concentrations, and up-modulated the anti-inflammatory cytokines’ (IL-4 and IL-10) levels (Montironi et al. 2022). These results served as a basis and stimulated us to carry out studies in weaned pigs. However, the limitations in the use and application of oils or their compounds are mainly attributed to their photosensitive, volatile and hydrophobic nature, which has stimulated the search for new conservation and stability systems to take advantage of their great potential. In previous studies we have demonstrated that micro or nanoencapsulated *M. verticillata* EO showed adjuvant properties of the immune response (Campa et al. 2022) or antimicrobial activity (Cecchini et al. 2021), higher than free EO.

Therefore, the aim of this study was to evaluate the effect of oral supplementation of free and nanoencapsulated *Minthostachys verticillata* essential oil (EO and NEO, respectively) on immunological, biochemical and antioxidants parameters as well as on gut microbiota in weaned piglets.

Materials and methods

Plant material

The aerial parts (leaves, thin stems and flowers) of *Minthostachys verticillata* (Griseb.) Epling (Lamiaceae) (WFO 2023) were collected from cultivated plants grown in a field located in Alta Gracia, Department of Santa María, Córdoba, Argentina, Coordinates Latitude: 31°38'12", longitude: 64°26'16". The samples were obtained on February 2021. The morphological characterization of the plant was performed macro and microscopically to confirm the identity of this specimen in the Botany Area (Natural Sciences Department, Universidad Nacional de Río Cuarto, Argentina). A specimen was deposited in the Herbarium under number 7801.

Essential oil extraction and nanoencapsulation

Essential oil (EO) extraction was performed from 100 g of aerial parts of the plant by hydrodistillation using a semi-laboratory scale steam entrainment essential oil extractor (Figmay, Córdoba, Argentina) according to the manufacturer's instructions. EO nanoencapsulation was performed by the high-energy method according to the protocol described by Cecchini et al. (2021), with some modifications. Briefly, the EO (20% v/v) was mixed with Tween 80 (0.75% v/v) (Anedra, Argentina) and Span 60 (0.25% w/v) (SIGMA, St. Louis, USA), used as surfactants, in a magnetic stirrer at 800 rpm for 30 min. Then, deionized water (79% v/v) was added dropwise at a flow rate of 2 mL/min. The mixture was stirred at 800 rpm for 30 min. Subsequently, an ultrahomogenization for 5 minutes (8000 rpm), using a Heidolph DIAx 900 homogenizer (Germany) equipped with a 10 G disperser, was performed. Final homogenization was achieved through a sonication process for 30 minutes in a Cole-Parmer ultrasonic bath (model 8848) at 60 Hz 19 W. The nanoemulsion obtained was transferred to a sterile container, hermetically covered and stored at room temperature protected from light until use. The nanoencapsulated EO (NEO) formulation used in this study was selected from a previous study in which different NEO formulations were evaluated (EO (20% v/v), water (79% v/v) with different proportions of Tween 80/Span 60 (1% v/v final). The optimal NEO formulation was chosen based on results obtained from physical-chemical characterization (pH (5.78), conductivity (72.2), absorbance (0.443) and transmittance percentage (36.7%), drop size (105.6 nm), polydispersity index (0.301) and Z potential (-22.5). Furthermore, transmission electron microscopy (TEM) revealed spherical morphology and low droplet size dispersion (Cecchini et al. 2022).

EO and NEO chemical characterization

To perform the gas chromatography-mass spectrometry (GC-MS) analysis of EO and NEO, a gas chromatograph *Clarus 600*, PerkinElmer (Shelton, Connecticut, USA) serial number 664N9100105 fitted with a DB5 capillary column (60 m, 0.25 mm ID, 0.25 µm particle) was used, following the methodology described by Montironi et al. (2016) and Cecchini et al. (2021).

Biocompatibility assay on Caco-2 cell line

The cytotoxic effect of NEO on human colorectal adenocarcinoma (Caco-2) cell line (CC20 [Human colon adenocarcinoma] (RRID:CVCL_2H54) was evaluated before its oral administration in weaned pigs following the methodology described by Montironi et al. (2022). Caco-2 cells were exposed to different concentrations (250, 500, 750, 1000 and 2000 µg/mL) of NEO dissolved in complete Dulbecco's Modified Eagle Medium (DMEM; Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Natocor, Villa Carlos Paz, Córdoba, Argentina) and Penicillin-Streptomycin (1%) (Gibco, Life Technologies, Grand Island, NY, USA). EO was assayed at the same concentrations of NEO to compare the cytotoxic effects. Cells cultured with complete DMEM medium alone and cells exposed to the surfactant mixture [Tween 80 (42.7 µg/mL) and Span 60 (4.5 µg/mL)] in complete DMEM were added as negative control and vehicle control, respectively, in all assays. The plate was incubated at 37 °C with 5% CO₂ and humidity for 24 h. Cells' viability was evaluated by the colorimetric reduction method using 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (MTT) (Sigma, Burlington, USA). For each concentration, triplicate wells in two independent assays were performed.

Piglets

This study was conducted in the Instituto Nacional de Tecnología Agropecuaria (INTA), Estación Experimental Agropecuaria, Marcos Juárez, in Marcos Juárez City, Córdoba Province, Argentina. A total of 36 male capon piglets (Large White x Landrace; body weight average of 10.35 ± 1.67 kg) weaned at 28 days of age were used. The piglets were housed individually in pens, equipped with full slatted plastic floors (Zhengzhou Jinhui Agricultural S&T Co., Ltd.) and metal divisors between pens. Environmental conditions were regulated by ventilation, force air circulation and infrared lamps to give an appropriate light, temperature (28–30 °C) and humidity (around of 60%). The animals had *ad libitum* access to water and food (Correa et al. 2022) and received a commercial balanced feed with two-phase (in relation to feed granulometry, animals age and nutritional balance): phase I from 0 to 14 days post-weaning and phase II from 15 to 30 days post-weaning. The zone was maintained through daily cleaning and disinfection (2% sodium hypochlorite).

Experimental design and sample collection

Thirty-six piglets were randomly assigned in six groups of six animals each ($n = 6$) using an online random number generator (<https://www.graphpad.com/quickcalcs/randomize1/>). The animals received different treatments orally with a syringe, at 1 hour before feeding, for 30 consecutive days. Group 1: Control group (not treated), Group 2: Vehicle control (Tween 80 0.75% v/v, Span 60 0.25% w/v and deionized water 99% v/v). Group 3: EO (10 mg/kg/day). Groups 4–6: NEO (2.5, 5 or 10 mg/kg/day, respectively). Concentrations of free EO and EO into nanocapsules were chosen based in a previous assay (Montironi et al. 2022). The animals were carefully monitored throughout the assay period. Parameters such as body weight gain (the animals were weighted at the beginning and at the end of the assay); fecal consistency (daily) score [(0) Normal, (1) Pasty, (2) Semi-liquid (fluid feces), (3) Liquid (severe diarrhea)], and body temperature (via a digital thermometer inserted into the rectum) were recorded (Alustiza et al. 2016). On day 30, before sacrifice, blood samples (20 mL) were collected, by venipuncture, into both nonheparinized and heparinized tubes to obtain serum and whole blood or plasma, respectively. The animals were subsequently euthanized by a captive bolt gun. Abdominal cavity was exposed immediately after sacrifice; samples of 2 cm² of surface of cardia region of stomach, 2 cm of length of jejunum, 2 cm of length of colon, 1 cm³ of a central region of liver and 1 cm³ of a cranial zone of kidney, between cortical and medullar region, were extirpated and placed in 10% of neutral-buffered formaldehyde for histological analyses. In addition, in these organs and femoral biceps muscle from left hind leg, oxidative stress was evaluated by lipid peroxidation which was estimated by measuring the thiobarbituric acid-reactive substances (TBARS). The intestinal content from the middle colon of individual piglets randomly selected from the control, EO and NEO (10 mg/kg/day) groups (3 animals per each group), was collected and stored at -80°C for gut microbiota analysis.

Histological analysis

Samples from stomach, jejunum, colon, liver and kidney were quickly collected and fixed in neutral buffer with 10% formaldehyde. The tissues were then embedded in paraffin and sectioned for histological evaluation. Haematoxylin/eosin staining was performed on 4- μ m-thick sections developed following the protocol described by Montironi et al. (2022).

TBARS determination

Thiobarbituric acid-reactive substances concentration was measured in samples of 0.5 g of five different sections of stomach, jejunum, colon, liver, kidney and femoral biceps muscle from left hind leg according to the method described by Mañas et al. (2013) to estimate oxidative stress. TBARS concentration was expressed as nmol of malondialdehyde (MDA)/g of tissue. All determinations were performed by triplicate.

Hematological parameters

Total leukocyte count was determined in heparinized blood whole samples following conventional experimental procedures (Hernández Barreto et al. 2002). The results were expressed as number of cells/ μ L.

T cells quantification

CD4⁺ and CD8⁺ T cell quantification was performed following the protocol previously described by Montironi et al. (2019). Briefly, the leukocyte population was obtained from whole blood samples subjected to red blood cell lysis. Subsequently, cells were stained with monoclonal antibodies (anti-CD4*FITC, anti-CD8*PE) (Invitrogen, USA). Isotype controls were performed and the samples were analyzed in duplicate by using a BD FACS Canto II flow cytometer (Becton Dickinson, San Jose, CA, USA). Data were obtained with the FACS Diva v. Software. 6.1.2.

Cytokine's measurement

Plasma was obtained by centrifugation of heparinized blood to measure the levels of pro-inflammatory (TNF- α , IL-1 β) and anti-inflammatory (IL-10) cytokines using ELISA kits (Invitrogen, USA) following what was specified by the manufacturers. Optical densities were measured at 450nm using a spectrophotometer (Labsystem Multiskan MS, Thermo, Vantaa, Finland). Cytokine's concentration was expressed as pg/mL.

Biochemical parameters

Serum was obtained by centrifugation from nonheparinized blood samples. Concentration of serum albumin, glucose, total cholesterol, triglycerides, glutamic-pyruvic transaminase (GPT) and glutamic-oxalacetic transaminase (GOT) were measured by standard reagents in a spectrophotometer.

Gut microbiota analysis

For gut microbiota analysis, total microbial DNA of intestinal content from the middle colon was extracted using the Macherey-Nagel kit (Bioanalysis, USA). DNA quantity and quality was analysed by

NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, NC, USA). The V3-V4 regions of bacterial 16S rRNA gene were amplified by PCR. The purified amplicons were quantified and sequenced on an Illumina MiSeq platform (INTA, Castelar, Buenos Aires, Argentina). The sequencing data were analyzed with the Quantitative Insight Into Microbial Ecology 2 program (QIIME2, <https://view.qiime2.org/>) (Bolyen et al. 2019) to monitor changes in the gut microbiota of pigs supplemented with EO or NEO (10 mg/kg/day). Alpha-diversity (Faith's phylogenetic diversity and Shannon indices), beta-diversity (UniFrac, Bray-Curtis distance and Jaccard similarity index) and taxonomic classification were analyzed.

Statistical analysis

Results were expressed as mean $\pm$ standard deviation (SD). The GraphPad Prism version 5.00.288 (San Diego, USA, 2007) was used. Data were compared with one-way analysis of variance (ANOVA) and the Tukey multiple comparison test. A $p < 0.05$ difference was considered statistically significant. Comparison of relative abundance data of taxonomic composition between samples was performed using the t-test. Alpha-diversity (Faith's phylogenetic diversity and Shannon indices) were analyzed with Kruskal-Wallis test in QIIME2 program.

Results

M. verticillata EO and NEO chemical analysis

Ten compounds were identified in both *M. verticillata* EO and NEO by GC-MS. The main oxygenated monoterpenes identified were pulegone (41.34% and 40.08%) and menthone (27.99% and 27.53%). Other oxygenated monoterpenes identified were berbenone (5.89% and 4.78%) and isopulegone (2.43% and 2.00%). The only hydrocarbon monoterpene identified was limonene (2.29% and 1.98%). A relatively high sesquiterpenes level identified as β -cubebene (1.46% and 1.13%), γ -elemene (6.88% and 5.56%) and spathulenol (1.39% and 1.13%) was also found (Supplementary Information Tables S1 and S2 and Fig. S1).

Biocompatibility assay on Caco-2 cell line

Cytotoxic effect of both EO and NEO on Caco-2 cell line was evaluated before its oral supplementation in weaned piglets. The results obtained showed that NEO was more toxic than EO on Caco-2 cells with a $CC_{50} = 650 \mu\text{g/mL}$ (Fig. 1). On contrary, EO or vehicle control (mix of Tween 80, Span 60 and water) behaved similarly to cells cultured with complete DMEM medium alone.

Clinical response of piglets

Weaned piglets orally supplemented with EO (10 mg/kg/day) or NEO (10 mg/kg/day) showed a significant increase in body weight gain compared with control group ($p < 0.05$) (Table 1). In addition, body weight gain in piglets orally supplemented with NEO (10 mg/kg/day) was higher than in those

supplemented with NEO (2.5 or 5 mg/kg/day) ($p < 0.05$). No diarrhea episodes (score = 0) or increase in body temperature were observed in piglets of the different experimental groups (Table 1).

Table 1
Clinical response of weaned piglets

Groups (n = 6)	Mean Body Weight Gain ^a , kg	Body temperature, °C
Control (Not treated animals)	10.26 ± 0.18	39.02 ± 0.52
Vehicle control (Tween 80/Span 60)	13.04 ± 1.65	39.10 ± 0.48
EO ^b (10 mg/kg/day)	16.38 ± 0.80*	39.00 ± 0.50
NEO ^c (2.5 mg/kg/day)	12.44 ± 0.93	39.05 ± 0.61
NEO (5 mg/kg/day)	11.65 ± 0.25	39.03 ± 0.55
NEO (10 mg/kg/day)	15.26 ± 0.97* [^]	39.01 ± 0.66
P-value	* < 0.05 ^ < 0.05	N/O ^d

^a Weight gain is calculated as the mean difference between the final and initial weight. Data are represented as means (n = 6) ± standard deviation (SD).

^b EO: free *Minthostachys verticillata* essential oil.

^c NEO: nanoencapsulated *M. verticillata* essential oil.

^d N/O: not observed.

*indicate P-value for statistical significance compared to control group.

[^]indicate P-value for statistical significance compared to NEO (2.5 and 5 mg/kg/day) groups.

One-way analysis of variance (ANOVA) and the Tukey multiple comparison test, GraphPad Prism software.

Histopathological analysis

The histopathological study revealed that piglets orally supplemented with EO (10 mg/kg/day) presented histological alterations in stomach, jejunum liver and kidney. These organs were found to be mildly inflamed and damaged, with presence of hyperemia, oos of epithelial and connective tissues around the inflammatory sites, and presence of inflammatory cell infiltration (Fig. 2). In contrast, it was observed that piglets orally supplemented orally with NEO (2.5, 5 and 10 mg/kg/day) did not exhibit histological alteration in the organs evaluated (stomach, jejunum, colon, liver and kidney) (Fig. 2).

TBARS determination

Malondialdehyde (MDA) levels were increased in stomach ($p < 0.01$), kidney ($p < 0.05$), and liver ($p < 0.001$) of animals orally supplemented whit EO compared to control group. MDA levels in stomach and kidney of all NEO groups were similar to control and lower than EO ($p < 0.05$). Interestingly, MDA levels were decreased in liver of all NEO groups compared to control ($p < 0.05$) or EO ($p < 0.01$, $p < 0.01$ and $p < 0.05$, respectively) groups. In addition, MDA levels were decreased in liver of vehicle control group, compared to control ($p < 0.01$) or EO ($p < 0.05$) groups as well as in kidney compared to EO group ($p < 0.05$). MDA contents in jejunum, colon and muscle was not altered by oral supplementation with EO or NEO (Table 2).

Table 2
Malondialdehyde (MDA), nmol/g of tissue in digestive organs and muscle of weaned piglets

Groups (n = 6)	Stomach	Jejunum	Colon	Kidney	Liver	Muscle
Control (Not treated animals)	32.24 ± 2.13	40.58 ± 2.89	31.43 ± 5.39	19.87 ± 7.037	125.8 ± 10.91	60.20 ± 2.94
Vehicle control (Tween 80/Span 60)	30.57 ± 4.24	32.22 ± 4.05	28.65 ± 3.81	20.11 ± 2.35#	20.23 ± 1.93**#	54.85 ± 2.859
EO ^a (10 mg/kg/day)	52.32 ± 6.52**	33.94 ± 1.06	29.55 ± 2.20	71.07 ± 17.95*	217.2 ± 56.15***	60.31 ± 7.385
NEO ^b (2.5 mg/kg/day)	32.70 ± 1.32#	39.33 ± 7.67	31.55 ± 2.06	26.33 ± 1.90#	38.18 ± 7.27*##	59.60 ± 6.917
NEO (5 mg/kg/day)	36.53 ± 4.11#	40.69 ± 9.83	30.96 ± 4.37	26.11 ± 5.87#	29.50 ± 9.05*##	59.89 ± 5.480
NEO (10 mg/kg/day)	36.15 ± 1.09#	37.57 ± 2.05	28.46 ± 3.19	31.38 ± 5.87#	63.17 ± 15.14*#	59.99 ± 12.14
P-value	** < 0.01 # < 0.05	N/O ³	N/O	* < 0.05 # < 0.05	*** < 0.001 ** < 0.01 * < 0.05 # < 0.05	N/O ^c

^a EO: free *Minthostachys verticillata* essential oil.

^b NEO: nanoencapsulated *M. verticillata* essential oil.

^c N/O: not observed.

*indicate P-value for statistical significance compared to control group

#indicate P-value for statistical significance compared to EO group.

Data are represented as means (n = 6) ± standard deviation (SD).

One-way analysis of variance (ANOVA) and the Tukey multiple comparison test, GraphPad Prism software.

White blood cell counts

Table 3
Total leukocytes count in weaned piglets

Groups (n = 6)	Leukocytes, 10 ³ cells/ μ L
Control (Not treated animals)	14.50 $\pm$ 0.20
Vehicle control (Tween 80/Span 60)	15.25 $\pm$ 0.95
EO ^a (10 mg/kg/day)	14.50 $\pm$ 1.30
NEO ^b (2.5 mg/kg/day)	16.37 $\pm$ 1.67
NEO (5 mg/kg/day)	14.53 $\pm$ 0.63
NEO (10 mg/kg/day)	16.70 $\pm$ 0.20
P-value	N/O ^c
^a EO: free <i>Minthostachys verticillata</i> essential oil. ^b NEO: nanoencapsulated <i>M. verticillata</i> essential oil. ^c N/O: not observed. Data are represented as means (n = 6) $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) and the Tukey multiple comparison test, GraphPad Prism software.	

No significant differences were observed in the total leukocyte counts of weaned piglets orally supplemented with EO (10 mg/kg/day) or NEO (2.5, 5 or 10 mg/kg/day) compared to control animals (Table 3).

CD4⁺ and CD8⁺ T cells quantification

CD4⁺ T cells percentage was significantly higher in NEO (10 mg/kg/day) group compared to control, EO (10 mg/kg/day) and NEO (2.5 and 5 mg/kg/day) groups ($p < 0.001$, $p < 0.05$ and $p < 0.001$, respectively) (Fig. 3a and 3c). No differences in CD8⁺ T cells number was observed in all groups (Fig. 3b and 3c).

TNF- α , IL-1 β and IL-10 measurement

TNF- α and IL-1 β levels were not altered by oral supplementation of EO (10 mg/kg/day) or NEO (2.5, 5 or 10 mg/kg/day) (Fig. 4a and 4b). However, a significant increase in the levels of the anti-inflammatory cytokine IL-10 was observed in the group that received EO (10 mg/kg/day) compared to the control group ($p < 0.05$) (Fig. 4c). In addition, the IL-10 levels were increased in the groups that received NEO (5 and 10 mg/kg/day) compared to the control group ($p < 0.05$ and $p < 0.01$, respectively) (Fig. 4c). Interestingly, the highest levels of IL-10 were observed in the animals group treated with NEO (10 mg/kg/day) compared to NEO 2.5 mg/kg/day ($p < 0.001$), NEO 5 mg/kg/day ($p < 0.01$) and EO 10 mg/kg/day ($p < 0.05$) (Fig. 4c).

Biochemical parameters

Serum triglycerides levels were significantly lower in animals orally supplemented with EO (10 mg/kg/day) ($p < 0.001$) or NEO (2.5, 5 or 10 mg/kg/day) ($p < 0.01$, $p < 0.001$ and $p < 0.001$, respectively) compared to the control group (Table 4). An increasing trend in albumin levels of weaned piglets supplemented with NEO (5 and 10 mg/kg/day), without statistically significant differences compared to the control was observed (Table 4). A decreasing trend in total cholesterol levels of weaned piglets supplemented with EO (10 mg/kg/day) or NEO (2.5, 5 and 10 mg/kg/day) without statistically significant differences compared to control, also was observed. No differences in glucose, GPT and GOT levels were observed between the animals of control group and those supplemented with EO or NEO (Table 4).

Table 4
Biochemical parameters in weaned piglets

Groups (n = 6)	Triglycerides, g/L	Total cholesterol, g/L	Albumin, g/dL	Glucose, g/dL	GPT ^a , UI/L	GOT ^b , UI/L
Control (Not treated animals)	5.92 ± 0.05	1.33 ± 0.08	3.15 ± 0.64	0.76 ± 0.10	71.92 ± 2.81	45.16 ± 3.99
Vehicle control (Tween 80/Span 60)	5.76 ± 0.39	1.32 ± 0.24	4.66 ± 0.00	0.50 ± 0.00	73.97 ± 6.15	43.44 ± 0.87
EO ^c (10 mg/kg/day)	3.60 ± 0.08***	0.98 ± 0.10	3.60 ± 0.34	0.69 ± 0.21	57.81 ± 3.85	44.39 ± 2.46
NEO ^d (2.5 mg/kg/day)	4.53 ± 0.26**	1.01 ± 0.08	3.86 ± 0.25	0.82 ± 0.05	67.60 ± 2.62	47.94 ± 5.53
NEO (5 mg/kg/day)	4.05 ± 0.02***	1.18 ± 0.14	4.53 ± 0.15	0.70 ± 0.05	69.33 ± 1.88	51.09 ± 3.52
NEO (10 mg/kg/day)	3.79 ± 0.22***	1.13 ± 0.05	4.49 ± 0.25	0.89 ± 0.09	62.56 ± 2.43	50.93 ± 2.17
P-value	*** < 0.001 ** < 0.01	N/O ^e	N/O	N/O	N/O	N/O

^a GPT: glutamic-pyruvic transaminase.

^b GOT: glutamic-oxalacetic transaminase.

^c EO: free *Minthostachys verticillata* essential oil.

^d NEO: nanoencapsulated *M. verticillata* essential oil.

^e N/O: not observed.

*indicate P-value for statistical significance compared to control group.

Data are represented as means (n = 6) ± standard deviation (SD).

One-way analysis of variance (ANOVA) and the Tukey multiple comparison test, GraphPad Prism software.

Effect of EO and NEO on gut microbiota

Analysis of sequencing data to monitor changes in the gut microbiota of piglets not supplemented or supplemented with EO (10 mg/kg/day) or nanoencapsulated EO (10 mg/kg/day) was performed. It should be noted that a sample from the control group (not supplemented) gave very few readings, which is why it was excluded from the analysis.

Alpha diversity analyzes revealed that supplementation with EO decreased microbial diversity compared to the control and NEO ($p < 0.05$) (Faith index) (Fig. 5a). Furthermore, the Shannon index showed that the lowest diversity was observed in the group of animals supplemented with EO, compared to the control and the nanoencapsulated EO, although without statistical differences (Fig. 5b).

The β -diversity was determined by principal component analysis (PCA) and represented as a cluster analysis. The unweighted (Fig. 6a) and weighted (Fig. 6b) UniFrac analyzes, the PCA results based on the Bray Curtis distance (Fig. 6c) and the Jaccard similarity index (Fig. 6d), revealed the presence of different clusters between the three groups (control, EO and NEO), showing that the microbial communities of each group are clearly separated from each other, and being the EO group the furthest from the control group.

Comparison of the taxonomic composition by 16S rRNA sequencing analysis revealed the highest relative abundance of the phyla Firmicutes, Proteobacteria and Actinobacteriota in the three groups evaluated (Fig. 7a). The analysis at the genus level showed that in control group, the abundance of the phylum Firmicutes was related to the genera *Coprococcus* ($5.05 \pm 0.25\%$), *Ruminococcus* ($4.37 \pm 0.89\%$) and *Blautia* ($3.68 \pm 0.63\%$); the abundance of the Proteobacteria phylum was related to the genus *Sphingomonas* ($9.05 \pm 5.40\%$), and the abundance of the phylum Actinobacteriota was related to the genera *Rothia* ($16.49 \pm 1.75\%$) and *Corynebacterium* ($3.07 \pm 0.42\%$) (Fig. 7a).

In the group supplemented with EO, the abundance of the phylum Firmicutes was related to the genera *Terrisporobacter* ($44.74 \pm 31.22\%$) and *Clostridium* ($1.49 \pm 1.09\%$); while the abundance of the phylum Proteobacteria was related to the genus *Escherichia/Shigella* ($37.81 \pm 11.51\%$) (Fig. 7a).

In the group supplemented with NEO, the abundance of the phylum Firmicutes was related to the genera *Clostridium sensu stricto* 1 and 6 (*C. bornimense*) ($26.53 \pm 13.55\%$), *Terrisporobacter* ($5.05 \pm 1.63\%$), *Blautia* ($2.39 \pm 0.52\%$), and *Romboutsia* ($2.48 \pm 0.89\%$). The abundance of the phylum Proteobacteria was related to the genus *Sphingomonas* ($16.42 \pm 9.77\%$), and the abundance of the phylum Actinobacteriota to the genera *Rothia* ($2.68 \pm 1.39\%$) and *Corynebacterium* ($4.54 \pm 0.03\%$) (Fig. 7a).

On the other hand, in the NEO group a trend towards an increase in the *Sphingomonas* genus was observed compared to the control group ($P = 0.58$) (Fig. 7b). In addition, a significant decrease in the *Rothia* genus also was observed compared to the control group ($p < 0.05$) (Fig. 7b). Furthermore, a trend towards an increase in the *Clostridium* genus and a decrease in the *Terrisporobacter* genus was

observed compared to the group supplemented with free EO ($P = 0.14$ and $P = 0.07$, respectively) (Fig. 7c).

Discussion

Minthostachys verticillata EO is a natural product with immunomodulatory and antioxidant effects scientifically verified by our research group. In the last study we have reported that *M. verticillata* EO modulated inflammatory and oxidative parameters improving the gastrointestinal and immune health in orally supplemented mice (Montironi et al. 2022). The present study is the first in which the effects of free and nanoencapsulated EO are evaluated in weaned piglets by oral supplementation.

The identification and relative percentage of compounds by GC-MS showed that NEO chemical profile was not altered compared to EO, and the pulegone-menthone chemotype was maintained in agreement with reported in our previous studies (Cariddi et al. 2011; Montironi et al. 2019, 2022; Campa et al. 2021, 2022; Cecchini et al. 2021). This result demonstrated that the high-energy method and the surfactants used did not alter the chemical profile of *M. verticillata* EO.

Previous to the *in vivo* study, the NEO safety was evaluated in Caco-2 cells and compared to EO. EO was safety on Caco-2 cells. This result was as expected due to in recent study we have demonstrated that high concentrations of EO (up to 5000 $\mu\text{g/mL}$) did not alter Caco-2 cells viability (Montironi et al. 2022). However, the behavior of NEO does not agree with results previously reported by our group where a nanoemulsion based on *M. verticillata* EO formulated with Tween 20 as surfactant was not toxic in a higher range of concentrations (50 to 450 mg/mL) on bovine and equine erythrocytes (Cecchini et al. 2021). This could be due not only to the use of different surfactants, but also to the interaction of NEOs with different cells. Some factors can increase the cytotoxicity of nanoemulsions based on essential oils. They may have direct release or micelles-mediated release of surfactant to cell membranes or electrostatic or affinity interactions of charged membranes with nanoemulsion droplets (Marchese et al. 2020). In this way, and as observed in the present work, different researchers report a greater toxicity of essential oil nanoemulsions than free essential oils on different cell types (Caco-2, Vero, HepG-2, MCF-7 or THP-1 cells, human lymphocytes) in a wide range of concentrations (23, 36, 100, 1200 or 2000 $\mu\text{g/mL}$) (Jerobin et al. 2015; Marchese et al. 2020; Mansour et al. 2022). Taking count these reports and that a drug is toxic when it induces damage to more than 50% of treated cells, it is slightly toxic when this percentage is between 50–70% and it is not toxic when the viability percentage is greater than 70% (Abdillahi et al. 2012), the NEO evaluated in this work, present acceptable toxicity levels on Caco-2 cells.

In the *in vivo* assay, weaned piglets were supplemented orally with EO (10 mg/kg/day) or NEO (2.5, 5 and 10 mg/kg/day) for 30 consecutive days and showed no signs of toxicity evidenced by the normal fecal consistency and the body weight gain. These results were similar to those obtained in a previous study in which the oral administration of free *M. verticillata* EO (5 or 10 mg/kg/day) to Balb/c mice for 10 consecutive days increased the body weight gain of animals and did not alter the fecal consistency (Montironi et al. 2022).

Essential oils can increase gastric secretions and hydrochloric acid (HCl) concentrations, and thus improve protein digestion. This could be due to the presence of terpenoid substances which improve the feed conversion rate and enhance the absorption of nutrients (de Oliveira Monteschio et al. 2017; Kazemi-Bonchenari et al. 2018; Tian and Piao 2019; Kalaitzidis et al. 2021; Abd El-Hack et al. 2022). In this sense, *M. verticillata* EO is a mixture of monoterpenes and sesquiterpenes which could be responsible for the weight gain observed in piglets.

The histological analysis showed significant changes in stomach, liver, jejunum and kidney of animals that received EO (10 mg/kg/day). This is in part agreed with a previous study in which we observed leukocyte infiltration in the small intestine of Balb/c mice orally supplemented with the same dosis of EO (Montironi et al. 2022). Interestingly, NEO (2.5, 5 and 10 mg/kg/day) did not induce alterations in the evaluated digestive organs, being safer than EO. These results are not consistent with those obtained *in vitro* in Caco-2 cells. In this sense, recent literature offers contradictory findings on the *in vitro* and *in vivo* toxicity of free and nanoencapsulated essential oils (Horky et al. 2019).

The weaning is a stressor period that increases the production of free radicals resulting in a lipid peroxidation in different organs (Wei et al. 2017). The lipid peroxidation and free radical formation breakdown cell membrane structure, disrupt transportation processes, and cause cell organelle dysfunction (Pisoschi and Pop 2015). Here, we measurement the MDA concentrations due to it is the end product of lipid peroxidation (Romero et al. 1998). Oral supplementation with EO (10 mg/kg/day) did not alter the lipid peroxidation in jejunum, colon and muscle; however, increased MDA concentration in stomach, kidney and liver. These results agreed with the histopathology alterations observed in these organs of piglets supplemented with EO. In a previous study, we reported a decrease in MDA levels in jejunum of Balb/c mice orally supplemented with EO (10 mg/kg/day), although did not observe any effect on others organs (Montironi et al. 2022). It is known that the essential oils can also act as pro-oxidants by destroying the integrity of cells and organelles, depending on the dose, main actives metabolites and cell susceptibility (Bakkali et al. 2008). The active metabolite found in the highest percentage in the *M. verticillata* EO is the monoterpene pulegone. The R-pulegone metabolism has been widely studied in experimental animals and the products of its metabolism, S-pulegone and menthofuran, are considered to be mainly responsible for the toxic effects in various organs, mainly in liver (Zárybnický1 et al. 2018). Therefore, it is probably that the *M. verticillata* EO administered orally to weaned pigs has been metabolized to more toxic products, resulting in lipid peroxidation in stomach, liver and kidney. Interestingly, NEO supplementation (2.5, 5 or 10 mg/kg/day) decreased MDA levels in liver and kidney and did not affect the lipid peroxidation in other organs and tissues. These results allow us to hypothesize that the nanoencapsulation process protects EO from the metabolism and prevent the production of toxic products in important organs such as liver and kidney.

In the present work, we observed that oral supplementation of EO (10 mg/kg/day) or NEO (2.5, 5 and 10 mg/kg/day) did not alter the white blood cell counts. However, in a previous study we observed an increase in white blood cells after oral administration of EO (5 and 10 mg/kg/day) in Balb/c mice

(Montironi et al. 2022). These differences could not only be due to the animal species used but also to the number of days they received the supplements.

Different *in vitro* and *in vivo* studies carried out by our research group have demonstrated the ability of free *M. verticillata* EO to stimulate T cells (Cariddi et al. 2011; Montironi et al. 2019, 2022; Campira et al. 2021, 2022). Here, we showed that the oral supplementation of EO (10 mg/kg/day) or NEO (10 mg/kg/day) increased the percentage of CD4⁺ T cells. Moreover, the highest percentage of CD4⁺ T cells was observed in weaned piglets treated with NEO, suggesting that the nanoencapsulation process increased the immunomodulatory effect of EO. The number of CD8⁺ T cells was not altered by EO or NEO supplementation. These results agreed with those obtained in a recent study in which an increase in CD4⁺/CD8⁺ T cells ratio in Balb/c mice treated orally with EO (5 or 10 mg/kg/day) was observed (Montironi et al. 2022).

We also measured the immunomodulatory effects of EO and NEO by evaluating the levels of pro-inflammatory (TNF- α and IL-1 β) and anti-inflammatory (IL-10) cytokines in plasma. TNF- α and IL-1 β play an important role in the inflammatory process during innate immune response (Fainboim and Geffner 2017). On the other hand, IL-10 is synthesized by CD4⁺ T cells of Tfh or Treg profile and M2 macrophages at the end of the inflammatory process (Fainboim and Geffner 2017). We observed that oral supplementation with EO (10 mg/kg/day) or NEO (5 and 10 mg/kg/day) did not show an effect on proinflammatory cytokines, but, interestingly, up-regulated the IL-10 plasma levels, showing an anti-inflammatory profile. These results are correlated in part with those obtained in a previous study in which we observed a decrease of pro-inflammatory cytokine (IL-6) levels and an increase of anti-inflammatory cytokines (IL-4 and IL-10) levels in the intestine of Balb/c mice orally supplemented with EO (5 and 10 mg/kg/day) (Montironi et al. 2022). The results are interesting because oral supplementation with free *M. verticillata* EO or NEO has probably acted as a gastrointestinal immunoregulator, maintaining normal conditions of the gut-associated lymphoid tissue (GALT) and this has been reflected in the serum levels of IL-10.

The weaned piglets orally supplemented with EO (10 mg/kg/day) or NEO (2.5, 5 and 10 mg/kg/day) did not show alterations in the serum levels of glucose or the GPT and GOT enzymes. However, we observed an increasing trend in albumin levels of weaned piglets supplemented with NEO (5 and 10 mg/kg/day), without statistically significant differences compared to the control. Albumin is one of the most important predictors of performance, especially average daily weight gain (Wei et al. 2021). This result could be partly related to the increase in body weight gain observed in piglets supplemented with NEO (10 mg/kg/day). We also observed a decreasing trend in total cholesterol levels of weaned piglets supplemented with EO (10 mg/kg/day) or NEO (2.5, 5 and 10 mg/kg/day) without statistically significant differences compared to control. Interestingly, both EO and NEO significantly decreased the serum triglycerides levels. It is possible that EO and NEO affect lipid metabolism because they could be rapidly absorbed after ingestion, increasing saliva and bile secretions, which play a key role in the metabolism of lipids (Su et al. 2020). These results are important due to the reduction of cholesterol and triglycerides

can be beneficial both for piglets and for the final consumer of pork meat, being able to reduce the incidence of cardiovascular diseases in humans (Patiño et al. 2019).

The gut microbiota analysis showed that the microbial diversity was higher in weaned piglets orally supplemented with NEO (10 mg/kg/day) than those of control or EO (10 mg/kg/day) groups. The genera *Clostridium sensu stricto* 1 and 6 (*C. bornimense*) were predominant in the NEO group. These bacteria are able to produce antibacterial substances and can ferment polysaccharides leading to the production of short-chain fatty acids that reduce gut inflammation (Kim et al. 2014; Dang et al. 2022; Niu et al. 2023). *Sphingomonas* was other of the predominant genus in the NEO group. This genus is potentially gut-protective and their abundance is related with an anti-inflammatory gastric environment (Sung et al. 2020). Other predominant genera in the NEO group were *Rothia* and *Corynebacterium*. *Rothia* genus participates in the degradation of proteins present in animals' feed and contributes to nutrients absorption in the intestine (Wei et al. 2020). *Corynebacterium* genus is related with immunomodulatory properties (Satora et al. 2020). The microbial diversity found in the intestine of the NEO group was related to the results obtained in the present study and suggests that NEO would favor the development of beneficial intestinal microorganisms to maintain an anti-inflammatory microenvironment.

On the other hand, in EO group the predominant genera were *Terrisporobacter* and *Escherichia/Shigella*. *Terrisporobacter* genus is related with the generation of lactic acid and is able to synthesize butyric acid by exploiting lactic acid and acetic acid under anaerobic conditions. This process prevents lactic acid accumulation and stabilizes the intestinal environment (Niu et al., 2023). However, the presence of *Escherichia-Shigella* genus in gut content may be associated to enterocolitis (Fan et al. 2017). In previous studies, we have demonstrated the antimicrobial activity of free *M. verticillata* EO against gram-positive and gram-negative bacteria (Cariddi et al. 2013; Montironi et al. 2016; Cerioli et al. 2018; Moliva et al. 2023). The antimicrobial potential of EO could be related to the low microbial diversity found in the gut of piglets from this group. Furthermore, free EO was more toxic than NEO, causing damage to some organs, included jejunum. All this could have favored the development of opportunistic species. In conclusion, the results obtained showed that although free EO as well as NEO improved parameters altered by early weaning in piglets, NEO was safer and powerful. The nanoencapsulation using Tween 80 and Span 60 as surfactants enhanced the biological activity of *M. verticillata* EO. NEO supplemented orally to weaned piglets improved growth performance, decreased triglycerides levels, showed antioxidant effect by decreasing pro-inflammatory mediators such as MDA, and modulated the systemic adaptive immune response by increasing the percentage of CD4⁺ T cells, showing an anti-inflammatory profile with increased levels of IL-10. In relation to this, NEO favored the development of beneficial microbial genera that promote an anti-inflammatory gut microenvironment. This safe and eco-friendly nanoproduct based on *M. verticillata* essential oil should be further studied to be applied in swine health.

Declarations

Author Contributions

Dr. L. Cariddi and Dr. R. Bellingeri conceived and designed the experiments. Dr. F. Alustiza, Med. Vet. F. Bessone and Med. Vet. A. Pinnoti handled the animal experiments, blood sampling and organ procurement. Mic. S. Arsaute performed the essential oil extraction and chemical analysis. Dr. I. Montironi and Lic. E. Cecchini performed the NEO synthesis. Dr. I. Montironi and Lic. E. Cecchini carried out the cytotoxicity assays and statistical analysis. Dr. D. Roma and Dr. F. Mañas carried out the antioxidant measurement and statistical analysis. Dr. F. Mañas performed the biochemical analysis. Dr. I. Montironi, Mic. S. Arsaute and Dr. D. Roma performed the ELISA assays and statistical analysis. Dr. L. Cariddi and Dr. R. Bellingeri performed the analysis of blood parameters and histology. Dr. I. Montironi and Mic. S. Arsaute carried out the quantification of CD4⁺ and CD8⁺ T cells and statistical analysis. Dr. I. Montironi, Mic. S. Arsaute, Dr. A. de Moreno de LeBlanc and Dr. L. Cariddi performed the gut microbiota analysis and statistical analysis. Dr. L. Cariddi, Dr. R. Bellingeri, Dr. I. Montironi and Mic. S. Arsaute wrote the manuscript. All the authors contributed with revision of manuscript. They read and approved the final manuscript.

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Data Availability The data included are available on request from the corresponding author.

Statements and Declarations

Consent for publication All authors gave their consent for research publication.

Competing Interests The authors declare no competing interest.

Compliance with Ethical Standards

All animals used in this study were handled and cared in accordance with the “Guide for the Care and Use of Laboratory Animals” (Albus, 2012), following the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guideline. In addition, the experimental and animal care protocols have been approved by the Comité Institucional para el Cuidado y Uso de Animales en Experimentación (CICUAE) of the Instituto Nacional de Tecnología Agropecuaria (INTA) (protocol number: E04-21, November 1st 2022).

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Figures

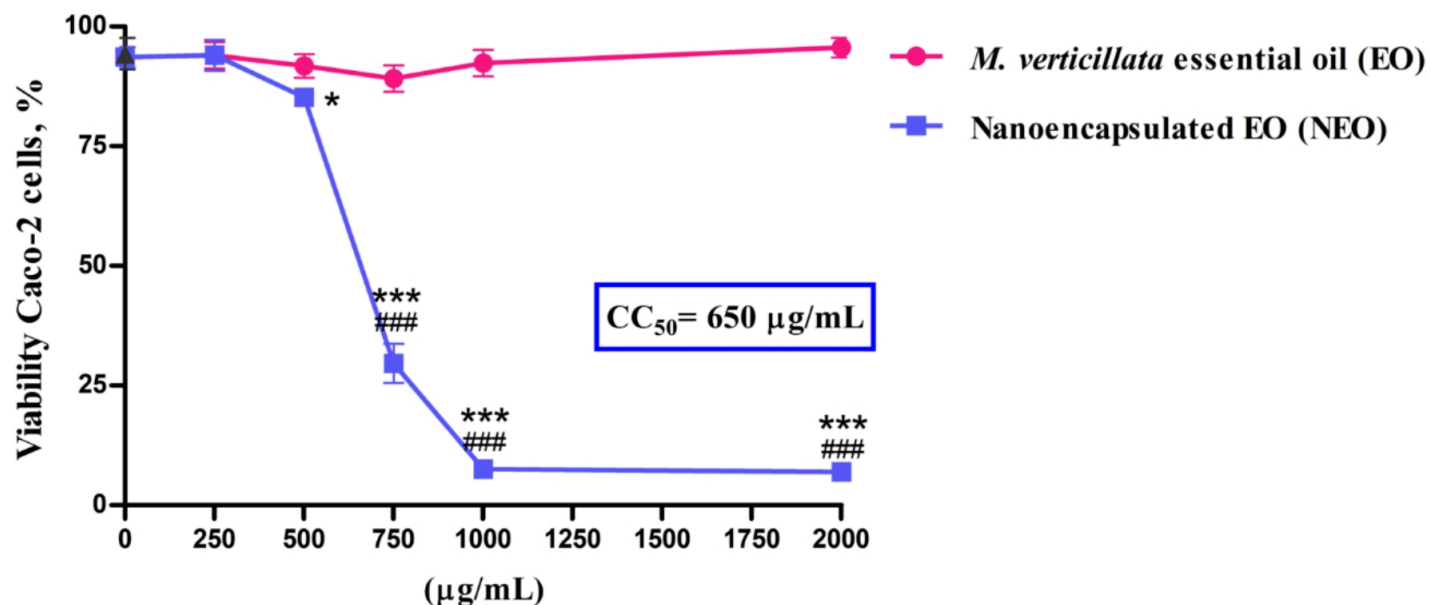


Figure 1

Viability percentage of human colorectal adenocarcinoma (Caco-2) cell line exposed to different concentrations of free *M. verticillata* essential oil (EO) or nanoencapsulated EO (NEO) during 24 h. The results are presented as percentage (mean ± SD). Cell viability was evaluated by MTT assay. For each concentration, quadruplicate wells in three independent assays were performed. *p < 0.05 and ***p < 0.001 compared to control (cells cultured with complete DMEM medium alone); ###p < 0.001 compared to EO. One-way analysis of variance (ANOVA) and the Tukey multiple comparison test, GraphPad Prism software.

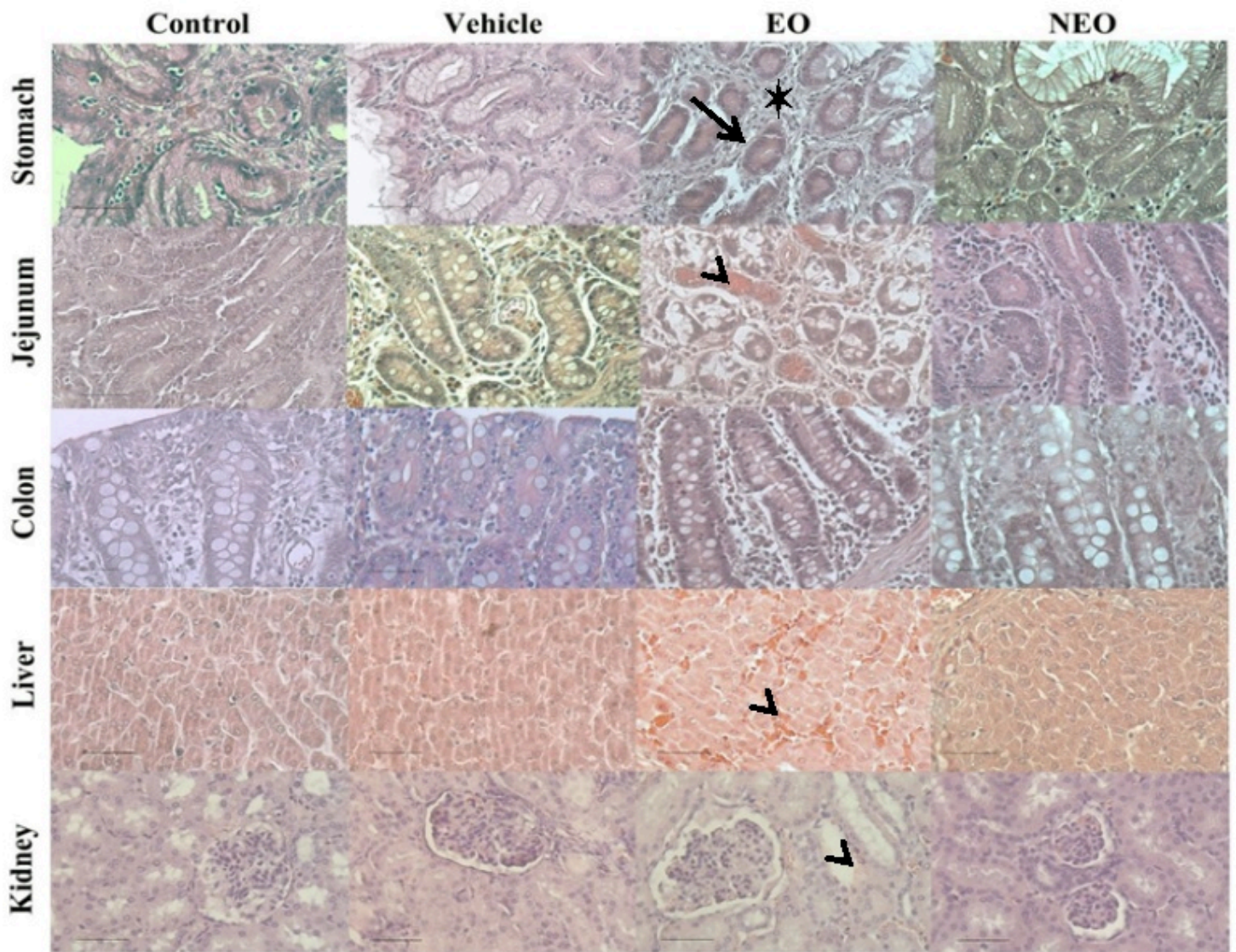


Figure 2

Photomicrographs of stomach, jejunum, colon, liver and kidney representative sections of weaned piglets orally treated with free *M. verticillata* essential oil (EO) (10 mg/kg/day) or nanoencapsulated EO (NEO) (10 mg/kg/day) during 30 consecutive days. H-E staining, 40× (Scale bar = 50 µm). The arrowheads indicate hyperemia, stars indicate inflammatory cell infiltration and the black arrows indicate loosening of epithelial and connective tissues.

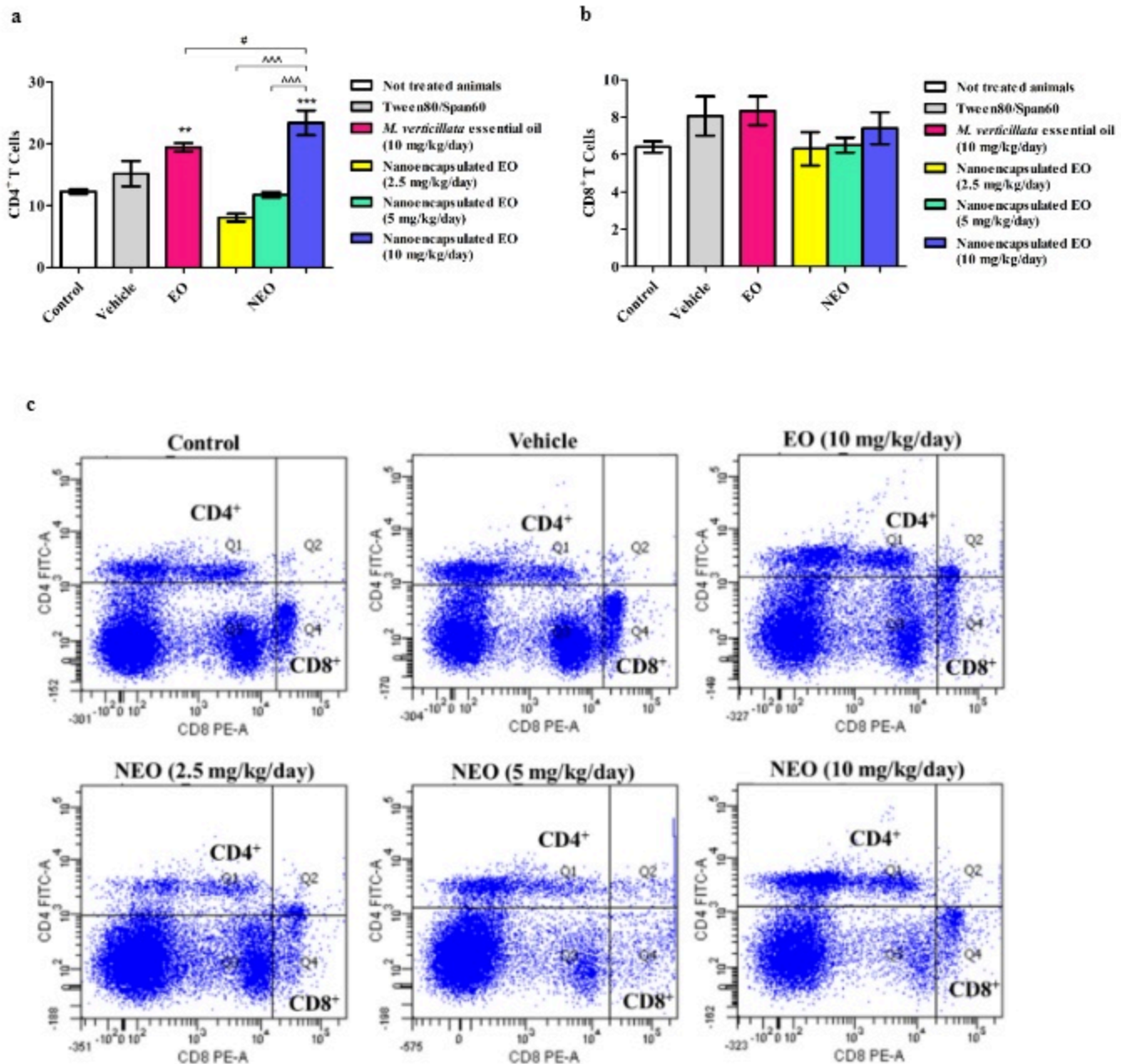


Figure 3

CD4⁺ (a) and CD8⁺ (b) T cells percentage. Weaned piglets were treated orally with free *M. verticillata* essential oil (EO) (10 mg/kg/day) or nanoencapsulated EO (NEO) (2.5, 5 or 10 mg/kg/day) during 30 consecutive days. Each bar represents the mean $\pm$ SD (n= 6 animals/group). ** p < 0.01, *** p < 0.001 compared to control. p < 0.05 compared to EO (10 mg/kg/day), ^^^ p < 0.001 compared to NEO (2.5 and 5 mg/kg/day) groups. Dot plot graphs (c). One-way analysis of variance (ANOVA) and the Tukey multiple comparison test, GraphPad Prism software. Note that double positives T cells populations (Q2) observed in dot plot graphs is due to the monoclonal antibodies (anti-CD4*FITC, anti-CD8*PE) (Invitrogen, USA) used.

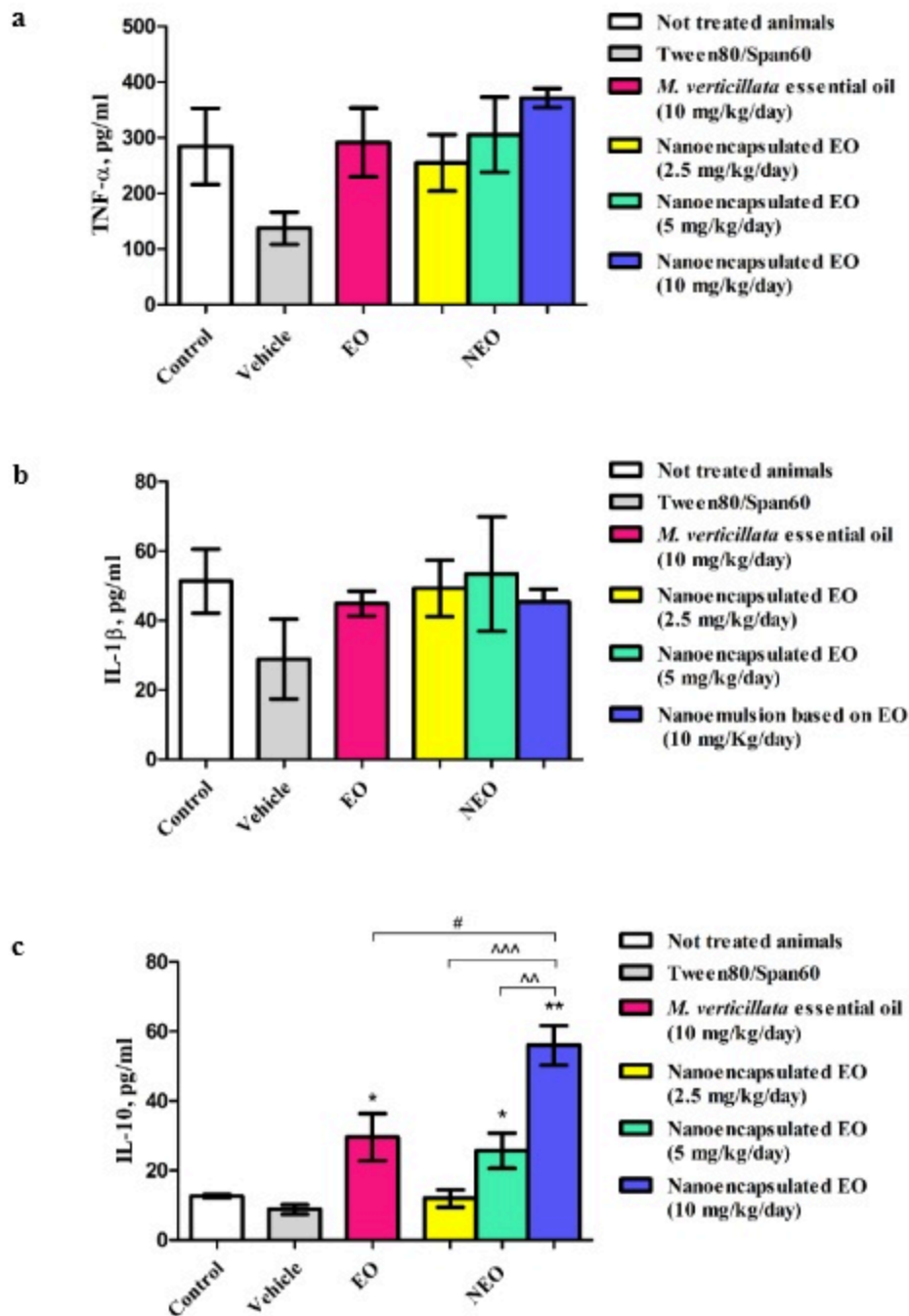


Figure 4

Pro-inflammatory ([TNF- α] **(a)** and [IL-1 β] **(b)**) and anti-inflammatory ([IL-10] **(c)**) cytokines measurement in plasma of weaned piglets treated orally with free *M. verticillata* essential oil (EO) (10 mg/kg/day) or nanoencapsulated EO (NEO) during 30 consecutive days. Each bar represents the mean $\pm$ SD (n= 6 animals/group). *p < 0.05, **p < 0.01 compared to control. #p < 0.05 compared to EO (10 mg/kg/day), ^p < 0.01, ^^^p < 0.001 compared to NEO (5 and 2.5 mg/kg/day, respectively) groups. One-way analysis of variance (ANOVA) and the Tukey multiple comparison test, GraphPad Prism software.

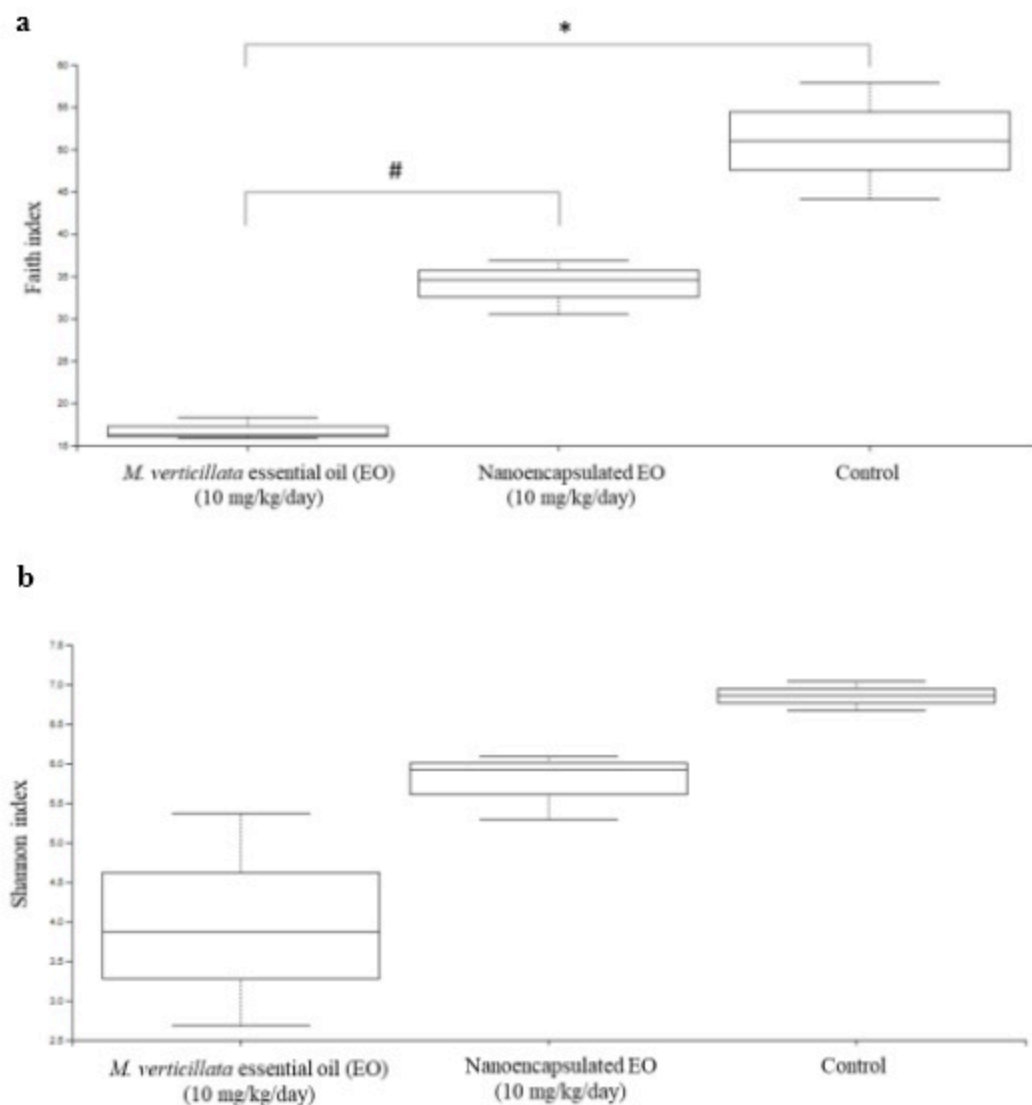


Figure 5

Microbial α -diversity in intestinal content from the middle colon of weaned piglets treated orally with free *M. verticillata* essential oil (EO) (10 mg/kg/day) or nanoencapsulated EO (NEO) (10 mg/kg/day) during 30 consecutive days. Faith index (**a**) and Shannon index (**b**). * $p < 0.05$ and # $p < 0.05$ compared to control or EO (10 mg/kg/day) groups respectively. Kruskal-Wallis test in QIIME2 program.

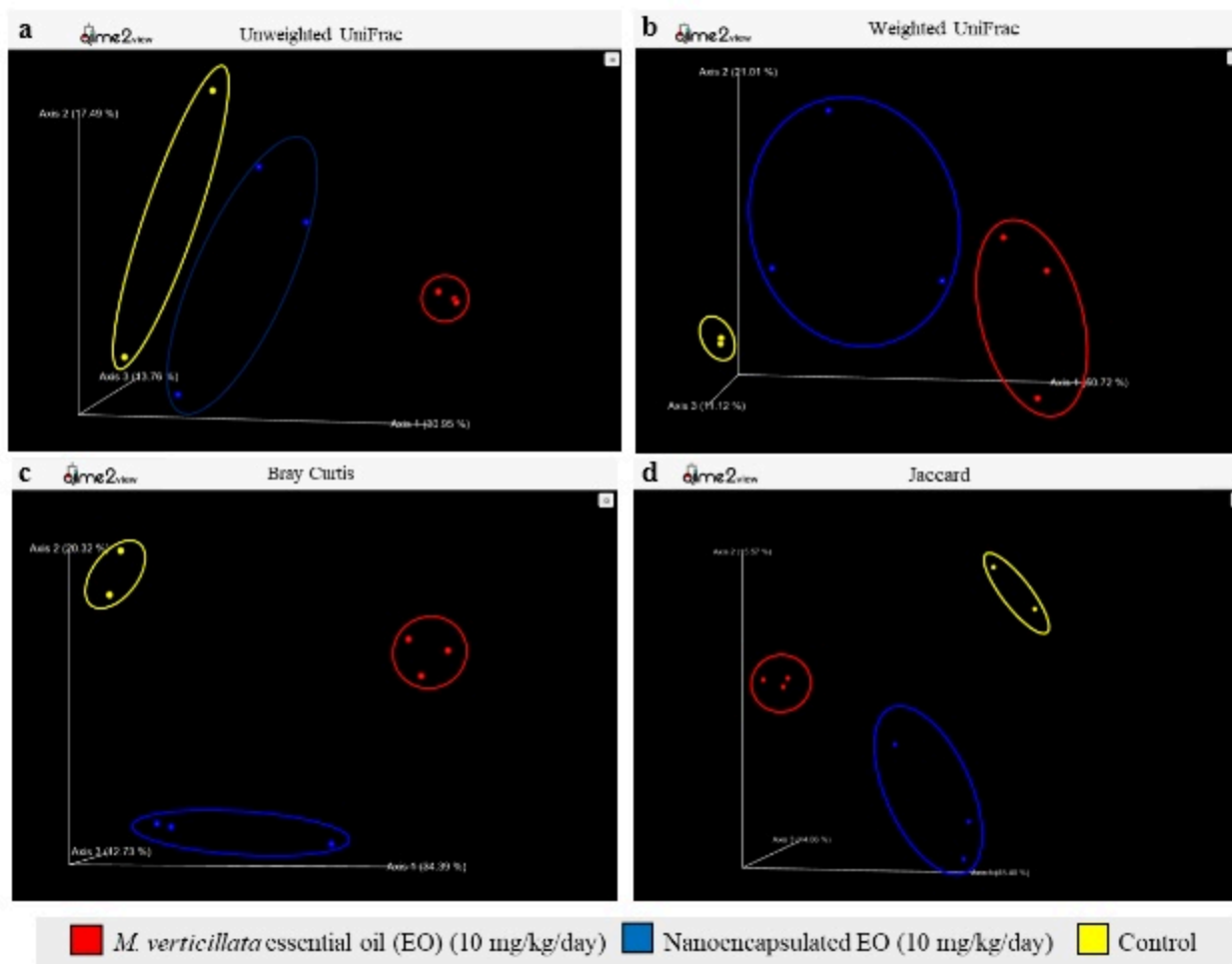


Figure 6

Principal component analysis (PCA) of the microbial β -diversity in intestinal content from the middle colon of weaned piglets treated orally with free *M. verticillata* essential oil (EO) (10 mg/kg/day) or nanoencapsulated EO (NEO) (10 mg/kg/day) for 30 consecutive days. Unweighted UniFrac analysis (a). Weighted UniFrac analysis (b). PCA based on the Bray-Curtis distance (c). Jaccard similarity index (d).

