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Can environmental nebulization of lavender essential oil (*L. angustifolia*) improve welfare and modulate nasal microbiota of growing pigs?

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ABSTRACT

The use of phytoextracts has been proposed as a method to improve animal welfare, also in pigs, by reducing stress and anxiety and improving performances. *Lavandula angustifolia (Miller)* essential oil (LaEO) is an interesting calming phytoextract that could be administered by inhalation for prolonged periods of time to help pigs coping with on-farm conditions. The aim of this study was to assess the effects of daily inhalation of vaporized LaEO on pigs' welfare and health indicators, and nasal microbiota, trying to understand whether this phytoextract represents a feasible tool to improve animal welfare under intensive farming conditions. Eighty-four crossbred barrows were randomly divided into 3 experimental groups: control (C); lavender (L): 3 vaporization sessions of 10 min each of a custom made 1% solution of LaEO; sham (S): same vaporization sessions of L group but only using the solution vehicle. Experimental readouts included growth parameters, behavioural traits, tail and skin lesions, hair steroids and nasal microbiota. L group animals did not show altered growth performance and seemed calmer (increased recumbency time), with decreased amount of skin lesions also associated with lower severity class for tail lesions. They also showed decreased CORT/DHEA ratio, potentially suggesting a beneficial effect of LaEO. Inhalation of LaEO significantly affected the nasal pig microbiome by reducing its diversity. Overall, the study suggests how inhalation of Lavender essential oil may be capable of improving welfare in growing pigs, yet it is pivotal to consider the microbial modulatory capabilities of essential oils before exploiting them on larger scale.

1. Introduction

Raising fattening pigs under commercial, intensive farming conditions exposes them to several challenging factors, including overcrowding, indoors confinement, lack of stimulating environment, and limited (or absent) possibility to express natural behaviours such as rooting. Despite the provisions of the EU legislation in terms of animal protection (EC, 2008), these farming conditions may not allow animals sufficient comfort to fully cope with the farm environment, and may result in increased levels of stress, high aggression, expression of redirected oral activities (including tail biting) and other behavioural alterations including apathy and stereotypies. In particular, tail biting is widely acknowledged as a severe and multifactorial problem in pig farming, with causes mostly related to intensive farming conditions (Sonoda et al., 2013).

Notwithstanding, the importance of providing adequate care and resources to farmed pigs (also by adopting housing and management practices that go beyond the minimum legislation requirements), and pending the revision of the legislation expected by the end of 2023 (EFSA Panel on Animal Health and Welfare (AHAW) et al., 2022), the use of phytoextracts and active botanical ingredients has been proposed as a method to improve animal welfare by reducing stress and anxiety in pigs, improving performance, and promoting better animal welfare. Initially, these herbal products were proposed to reduce stress during

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transportation. For example, Peeters and colleagues suggested that the administration in the drinking water of a commercial product containing *Valeriana officinalis* and *Passiflora incarnata* to pigs of approximately 24 kg of body weight (BW) before transportation, mediated a reduction in the increase in some cardiac-related physiological parameters (minimum heart rate, ventricular ectopic beats, ST elevation), suggesting sedative and antianxiety effects (Peeters et al., 2004).

The same product, containing *V. officinalis* and *P. incarnata*, was administered to growers (16 to 24 weeks of age) with the feed, leading to slightly increased BW at the end of the trial, and reduced hair cortisol and salivary Chromogranin A (a marker of acute stress co-released with catecholamines as a consequence of the activation of the sympathetic adrenomedullary system) (Casal et al., 2017).

Pastorelli and colleagues tested the administration in post-weaning piglets of a supplement containing only *P. incarnata* powder extract delivered with feed and found no difference in growth parameters, but a slight improvement in wellbeing (lower skin lesions, higher skin temperature detected thermographically in the ears and back regions) and immune parameters (cytokine activation); authors argued that the effects on animal wellbeing could be due to the calming and anti-anxiety effects of this plant extract (Pastorelli et al., 2020).

At present, commercial products containing botanical extracts are available for use on all productive categories, including sows, weaners, growers and fattening pigs. Most products contain a mixture of plant extracts (e.g. rosemary, sage, lavender, valerian, passiflora, etc.) despite the lack of scientific evidence of the distinct effects of individual extracts in improving animal welfare. In addition, most products are formulated for oral administration either as complementary feed or to be added to drinking water, while other delivery methods such as inhalation, potentially less invasive and more standardisable, are much less investigated.

Lavender (*Lavandula angustifolia, Miller*) essential oil (LaEO) is a potentially interesting calming phytoextract that could be administered by inhalation and for prolonged periods of time to help pigs coping with on-farm conditions. Its calming and even sedative effects are well known and have been tested in laboratory animals such as gerbils (Bradley et al., 2007), rats (Shaw et al., 2011) and rabbits (Haverbeke et al., 2022), with anxiolytic effects sometimes similar to those obtained with benzodiazepines. In humans, the use of lavender-based products inhalation is well established for different purposes such as cognitive function empowerment and decrease of depression, anxiety, and stress levels (Ebrahimi et al., 2022; Malloggi et al., 2022; Yoo and Park, 2023).

As for the use of LaEO in pigs, only few experiments are available according to literature. Bradshaw transported finisher pigs using either regular straw or lavender straw, and observed that pigs exposed to the latter were more active and showed lesser signs of motion sickness (Bradshaw et al., 1998). This is, to the best of the authors' knowledge, the first attempt in pigs to obtain a calming effect by administering LaEO by inhalation. A more recent study tested the effects of LaEO inhalation on aggressive behaviour of weaned pigs and found that smelling lavender oil for 4 h after transportation possibly mitigated motion sickness, allowing the pigs to adapting sooner to the new environment compared to the control group. Additionally, according to the authors, LaEO did not prevent aggressive behaviour (frequencies of fighting in the lavender group being significantly higher than those of control pigs) but stimulated the hierarchy to settle sooner (Direksin et al., 2017).

It appears from these two studies that LaEO does not overall reduce animal activity but promotes their adaptability to the environment. However, to the best of our knowledge, no study has investigated the effects of prolonged administration of LaEO by inhalation in growing pigs, during the entire growing phase.

Additionally, since it is well known that phytoextracts and essential oils can influence bacterial growth, nasal microbiota was characterized to gain further knowledge regarding the direct effects of prolong inhalation of lavender phytoextracts. The presence of a diverse and dynamic microbial community in the nasal cavity is crucial for the overall health of animals. Within the respiratory tract, this microbiota plays a significant role in maintaining pulmonary health by activating the immune response and offering protection against pathogens through colonization resistance. Common management practices such as transportation, mixing unfamiliar pigs, antibiotic use, and social stress can disrupt this respiratory microbiome, potentially compromising its ability to defend against infectious threats (Correa-Fiz et al., 2016, 2019). Therefore, the use of LaEO or different essential oils can be a valid strategy to selectively inhibit pathogenic bacteria while promoting the growth of beneficial endogenous members, aiming to restore homeostasis in the respiratory microbiota and reduce respiratory issues. Currently, studies investigating the impact of sprayed essential oils on nasal microbiota are lacking. Nonetheless, a recent study demonstrated the influence of a single intranasal administration of a spray containing various essential oils on the nasal microbiota of cattle (Magossi et al., 2023).

The aim of the present study was to assess the effects of daily inhalation of vaporized LaEO on pigs' welfare and health indicators, including growth parameters, skin and tail lesions, overall behaviour and hair cortisol and dehydroepiandrosterone, and also on nasal microbiota, trying to understand whether this phytoextract represents a feasible tool to improve animal welfare under intensive farming conditions.

2. Materials and methods

2.1. Ethics

The trials were carried out at the facilities of the Department of Veterinary Medical Sciences (DIMEVET) of the University of Bologna, Italy. The experiment did not include any invasive in vivo procedure, as defined by the D.Lgs 26/2014, therefore the research project was authorized as an observational study by the Ethical committee of the University of Bologna, with approval number 3610/2023. Rearing was carried out according to the EU legislation and pigs were inspected at least once a day (EC, 2008).

2.2. Reagents and Lavandula angustifolia essential oil (LaEO)

All reagents, unless otherwise specified, were purchased from Sigma-Aldrich (Saint Louis, MO, USA). The *Lavandula angustifolia* essential oil (LaEO) used for the trial was provided by APA-CT (Forlì, FC, Italy); its chemo-characterization is reported in Table S1 (see Appendix A: Supplementary material). For the experimental purposes, the EO was diluted, at the final concentration of 1%, into a water-based mixture containing polyoxyethylene sorbitan monooleate and propylen glycol.

2.3. Animals, housing and experimental trial

A total of 84 ($N = 84$) crossbred (Goland × Large White) barrows with undocked tails were enrolled in the trial. Animals were bought from a commercial farm. Pigs were individually identified by means of ear tags and homogeneously allotted to the experimental groups based on their initial BW. The average BW at the beginning of the trial was 44 kg and animals were 90 ± 2 days old. Pigs were kept in collective pens with partially slatted floor (7 pigs/pen) at a floor space availability of 80 kg/m^2 BW at the end of the trial. Groups were maintained the same during all the trial period (72 days). Each pen was equipped with a nipple drinker, a collective stainless-steel feeder (50 cm/pig of feeder space) and an environmental enrichment tool (soft wooden logs hanging from a wall). Pens were located in temperature- and humidity-controlled 3 different rooms equipped with a forced-air ventilation system (RH was set at 65% and T at 20 °C). The three rooms had the same volume. Artificial lighting was provided by neon tubes with a 12:12 light/dark cycle (light hours from 07:00 to 19:00). Animals were fed using two commercial feed formulations (first phase, up to 65 kg BW: 3200 kcal DE/kg DM, CP 16.6% DM; second phase, from 65 kg BW to the end of the

trial: 3195 kcal DE/kg DM, CP 14.50% DM). Feed was offered twice a day (at 8:00 and 15:00) as a meal, rationed at 9.5% of the metabolic BW ($BW^{0.75}$). The daily rations were adjusted every 2 weeks on the basis of the expected growth and of intermediate weighing.

Animals were randomly divided into 3 experimental groups, each composed of 4 pens (all pens belonging to the same treatment were located in the same room each treatment was allocated to a different room):

- Control group (C; $n = 28$): standard experimental conditions above described;
- Lavender group (L; n = 28): standard experimental conditions, with the addition of 3 vaporization sessions (7:00, 12:00, 17:00) of 10 min each (approx. 200 mL of product), of the custom made 1% solution of LaEO (dispersed in the above-mentioned commercial vehicle);
- Sham group (S; n = 28): standard experimental conditions and same vaporization sessions of L group (3 times a day for 10 min), but only using the solution vehicle without LaEO.

The duration of the vaporization session was set based on the recommendations of the machine constructor for the size of the rooms on preliminary nebulization tests, to ensure that all animals were equally exposed to the treatments.

Animals were divided into the different groups 10 days prior to the beginning of the trial, which lasted 72 days (from May to July). Growth parameters and lesions scores were assessed the day before the beginning of the trial (d0), halfway (d37) and at its end (d72).

Behavioural traits were video-recorded approximately every three weeks (d0, d24, d48, d72). Cortisol (CORT) and dehydroepiandrosterone (DHEA) were quantified in bristles samples collected at d72, with preliminary shaving of the animals performed at t0 (with collection of samples). Nasal microbiome was only characterized in animals belonging to groups C and L (20 randomly-sampled animals, 10 per experimental group) on swabs collected at d0 and d72.

2.4. Growth parameters

All pigs were individually weighed at d0, d37 and d72, and the individual average daily gain (ADG) was calculated for each period. Feed intake of each replication (pen) was recorded to calculate the feed conversion ratio (FCR) for each period.

2.5. Behavioural traits

A digital closed-circuit system (DSE, Turin, Italy) was used to videotape the diurnal behaviour of all pigs (from 06:30 to 18:30). One camera was installed above each pen (attached to the ceiling, at approximately three metres above the floor). Videotaping sessions were scheduled every three weeks, for a total of four sessions. In order to carry out individual behavioural observations, on the evening before each videotaping session, a coloured spot was painted on the back of each pig with a pre-determined combination of colour/position. Commercial marking sticks (blue, green, red and purple colours —RAIDEX GmBH, Dettingenan der Erms, Germany) were used. One pig in each pen was left uncoloured. After the end of the trial, a single trained observer analysed the videos using the scan sampling technique at 10-min intervals. The behaviours were observed according to a predetermined ethogram for pigs (Nannoni et al., 2019) including the following behaviours: sitting inactive, standing inactive, lateral recumbency, sternal recumbency, rooting/exploring the floor, eating, drinking, walking, tail biting, social interactions, interaction with the enrichment device, interaction with pen fixtures. Results will be expressed as proportion of observed time spent performing each behaviour.

2.6. Tail and skin lesions

Lesions on the body (skin and tail) were assessed according to the Welfare Quality® protocol for growing and finishing pigs (Welfare Quality®, 2009). Lesions were scored at the beginning, in the middle, and at the end of the trial by a trained person, which remained the same throughout the trial. For each body region (ears, front, middle, hindquarters, and legs), the number of lesions on one side of the animal was counted and used for classification according to the Welfare Quality protocol. The region was scored as: a, if it had up to 4 lesions; b, if showing 5 to 10 lesions; c, when 11 to 15 lesions were observed. Each pig was then classified as 0 (all body regions classified as a), 1 (any body region classified as b and/or a maximum of one region scored as c), or 2 (at least two body regions or more classified as c, or at least one body region showing >15 lesions). Tail lesions were scored as 0 if the tail was intact, 1 when superficial biting was found, without evidence of fresh blood or swelling, and 2 when we observed fresh blood, evidence of swelling or infection, or tissue missing with the formation of a crust.

2.7. Bristle sampling and steroids extraction

All animals enrolled in the trial were shaved off manually, using an electric trimmer, on the day before the beginning of the trial (d0), in the rump area (approximately 20 × 30 cm.) At the end of the trial (d72), the re-grown bristles were shaved from the same area and collected into small plastic bags, individually marked, and stored at 4C. Samples were handled and analysed as previously described (Bacci et al., 2014). Briefly, 250 mg of each sample were washed with tap water first, and isopropanol after, in order to remove any organic residue from their surface. Once fully dried, samples were finely pulverized and 120 mg of each sample were incubated overnight with 4 mL of methanol for steroids extraction at room temperature in glass tube. Upon centrifugation, 3 mL of supernatant methanol were collected and evaporated to dryness under an air-stream suction hood.

2.8. CORT and DHEA quantification

The dry extracts were reconstituted in assay buffer (phosphate buffered saline, 0.1% BSA, pH 7.4) for measurement of CORT (6 mg hair equivalent) and DHEA (4 mg hair equivalent) by radioimmunoassay (RIA) as previously described (Bacci et al., 2014); tritiated cortisol (30 pg/tube; 94.6 Ci/mmol; PerkinElmer, USA) or tritiated DHEA (30 pg/tube; 76.1 Ci/mmol; PerkinElmer, USA) were added, followed by rabbit anti-cortisol serum (0.1 mL, 1:20000; produced in our laboratory) or rabbit anti-DHEA serum (0.1 mL, 1:10,000; produced in our laboratory) respectively. After incubation and separation of antibody-bound and –unbound steroid by charcoal-dextran solution (charcoal 0.25%, dextran 0.02% in phosphate buffer), tubes were centrifuged (15 min, 3000 *g*), the supernatant was decanted and radioactivity immediately measured using a β-scintillation counter (Packard C1600, Perkin Elmer, USA). The sensitivity of the cortisol assay was 4.3 pg/tube, the intra- and inter-assay coefficients of variation were 4.7% and 10.2% respectively. The sensitivity of the DHEA assay was 3.3 pg/tube, the intra- and inter-assay coefficients of variation were 5.5% and 9.7% respectively. Cross reactions of various steroids with antiserum raised against cortisol were: cortisol 100%, cortisone 5.3%, 11α-deoxycortisol 5.0%, corticosterone 9.5%, 20α-dihydrocortisone 0.4%, prednisolone 4.60%, progesterone <0.001%, testosterone <0.001%. Cross reactions of various steroids with antiserum raised against DHEA were: dehydroepiandrosterone 100%, dehydroepiandrosterone sulfate 39%, androstenedione 10%, testosterone 0.25%, progesterone and cortisol <0.001%. The assay results were expressed as pg/mg hair.

In order to determine the parallelism between hormone standards and endogenous hormones in hair, a pool sample containing high concentrations in CORT and DHEA was serially diluted (1:1–1:8) with assay buffer. A regression analysis was used to determine parallelism between

the two hormone concentrations in the same assay and a high degree of parallelism was confirmed (r2 = 0.98).

2.9. Nasal swab sampling, bacterial DNA extraction and bioinformatic analysis

Sterile swabs (Sterile swab wooden applicator cotton tipped, APTACA Spa, Italy) were used to collect samples for nasal microbiota analyses. On d0 and d72, 10 animals per experimental group L and 10 per group C were manually restrained and swabs were inserted for approximately 5 cm into each nostril and rotated for 5 s. In case of accidental touch with the snout or other surfaces, swabs were discharged and procedure was repeated. Swabs were store at −80 °C until analyses. For the microbiota analysis, bacterial DNA extraction from nasal swabs was carried out using FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, Ca, USA) adding an initial step where dry swabs were soaked in a Sodium Phosphate Buffer solution and vortexed for 5′ in order to resuspend microbial cells, the following steps were carried out according to the manufacturer's instructions. DNA concentration and purity (absorbance ratio 260/280 and 260/230) of the isolated DNA were checked by spectrophotometry on the NanoDrop (Fisher Scientific, 13 Schwerte, Germany).

The V3-V4 region of the 16S rRNA gene (~460 bp) was amplified, amplicons were produced using the universal primers Pro341F: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNBGCA SCAG-3′ and Pro805R: 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACNVGGGTATCTAATCC-3′ (Takahashi et al., 2014) using the Platinum™ Taq DNA Polymerase High Fidelity (Termo Fisher Scientific, Italy) and sequenced using the Illumina MisSeq platform 300x2bp. The libraries were prepared using the standard protocol for MiSeq Reagent Kit V3 and sequenced on MiSeq platform (Illumina Inc., San Diego, Ca, USA). For the bioinformatics analysis, the DADA2 pipeline was used (Callahan et al., 2016) considering the Silva database (Quast et al., 2013) (version 138.1) as reference for the taxonomic assignment.

2.10. Statistical methods

Statistical analyses were performed using SAS Inst. Inc. (Cary, NC; release 8.0, 2014), R version 3.6 (The R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism v.9 (GraphPad Software Inc., San Diego, CA, USA) for graphic representation. Descriptive statistics were reported as Mean ± standard deviation (SD). Normal distribution was assessed by means of the Shapiro-Wilk test ($p < 0.05$). Growth parameters were analysed with analysis of variance using the mixed procedure of SAS, where pen was used as the experimental unit. Differences between groups in behavioural analysis, lesion score and hair hormones were tested upon Kruskal-Wallis test (non-parametric ANOVA), followed by Tukey or Bonferroni post-hoc tests. Chi-squared test was used to evaluate the distribution of skin and tail lesions into the three severity classes. Alpha diversity, Chao1, Shannon, and Simpson diversity indices where measured and differences between groups were tested using a linear model including sequencing depth, treatment (control and lavender), timepoint (d0 and d72) and their interaction in the model. For the Beta diversity a dissimilarity matrix using Euclidean distances of centred log ratio (clr) transformed data was constructed, results were plotted using a NMDS plot. Differences were tested using a PERMANOVA model with 10,000 permutations, including the effect of Treatment (control and lavender), timepoint (d0 and d72) and their interaction. The differential abundance analysis was performed using Linear discriminant analysis Effect Size (LEfSe) (Segata et al., 2011) implemented in the wrapper function included in the package microbiome Marker (Yang, 2020), aggregating the data at Genus level. Using a LDA cut-off of 3.5 and *P*.adj <0.05. The significance level was set at $P < 0.05$ for all tests.

3. Results

3.1. Growth parameters

The descriptive results of weight, ADG and FC, expressed as mean and SD, alongside with *P* values of the analysis of variance, are reported in Table 1. No statistically relevant differences were highlighted across the experimental groups during the experimental period.

3.2. Behavioural traits

The results of the analysis of the diurnal behaviour are reported in Table 2.

The level of activity resulted statically different among the three groups ($P = 0.016$), with pigs treated with LaEO (L) spending more time in recumbency compared with the Sham (S) group, with Control (C) group being intermediate. The difference in the activity level was due to the fact that C and L animals spent significantly more time resting in lateral recumbency compared to S ($P < 0.01$). Similarly, S animals spent more time sitting inactive when compared to L animals, but not when compared to C. Exploring behaviour and walking did not seem to be different among groups.

When looking at feeding activities, L and S groups spent less time eating in comparison to control (C) animals ($P < 0.001$) and S spent less time drinking than C animals ($P = 0.019$).

Neutral social interactions were significantly higher in S animals when compared to C and L ones, whereas no differences in tail biting and aggressive interactions were recorded. However, tail biting showed a tendential difference, with S animals spending more time carrying out tail biting behaviour compared to the other groups ($P = 0.093$). Similarly, interactions with environmental enrichments and pen fixtures did not seem to differ among different groups ($P = 0.778$).

3.3. Tail and skin lesions

The results of the lesion score (lesion counts and severity classes) are reported in Tables 3 and 4, respectively.

Animals treated with LaEO showed less lesions on the body than C and S groups, in particular on the shoulder and thigh regions. In the flank and legs regions, S had a slightly higher number of lesions. Looking at the severity classes, in particular for the tail, data highlighted how lavender treated animals had the highest percentage of score 0 (i.e., intact tails).

Table 1
Growth parameters of the animals, divided per experimental group, reported as mean and standard deviation in brackets (SD).

		Control (*n* = 28)	Lavender (n = 28)	Sham (n = 28)	*P* value
Weight (kg)	d0	44.57 (7.16)	43.03 (5.56)	43.05 (5.79)	0.985
	d37	59.71 (8.48)	57.22 (5.59)	59.73 (6.52)	0.953
	d72	80.29 (10.25)	77.55 (6.63)	78.38 (7.72)	0.978
ADG (g)	d0-d37	0.54 (0.05)	0.51 (0.02)	0.60 (0.03)	0.096
	d37-d72	0.59 (0.05)	0.58 (0.04)	0.53 (0.04)	0.692
	d0-d72	0.57 (0.05)	0.55 (0.03)	0.56 (0.03)	0.933
FCR	d0-d37	2.54 (0.11)	2.66 (0.23)	2.30 (0.08)	0.229
	d37-d72	4.02 (0.18)	3.99 (0.20)	4.45 (0.17)	0.226
	d0-d72	3.39 (0.14)	3.43 (0.20)	3.42 (0.06)	0.980

ADG, Average Daily Gain; FCR, Feed Conversion Ratio.

Table 2
Diurnal behaviour (06:30–18:30) of animals, divided per experimental group, expressed as a percentage of observed time and standard deviation in brackets (SD).

	Control (n = 28)	Lavender (n = 28)	Sham (n = 28)	*P* value
Standing inactive	2.57 (0.17)	2.72 (0.18)	2.80 (0.20)	0.674
Sitting Inactive	0.88 (0.12) ^{ab}	0.82 (0.11) ^{b}	1.26 (0.16) ^{a}	0.030
Lateral recumbency	27.53 (0.73) ^{a}	29.17 (0.82) ^{a}	23.98 (0.87) ^{b}	<0.001
Sternal recumbency	29.21 (0.76)	29.00 (0.80)	30.78 (0.89)	0.244
Total Recumbency	56.75 (0.71) ^{ab}	58.16 (0.91) ^{a}	54.76 (0.88) ^{b}	0.016
Walking	2.18 (0.19)	1.88 (0.13)	1.90 (0.17)	0.365
Exploring the floor	15.21 (0.61)	15.14 (0.80)	16.30 (0.72)	0.440
Eating	9.57 (0.18) ^{a}	8.96 (0.19) ^{b}	8.78 (0.16) ^{b}	0.004
Drinking	1.10 (0.10) ^{a}	1.02 (0.11) ^{ab}	0.71 (0.10) ^{b}	0.019
Neutral social interaction	3.15 (0.24) ^{b}	2.38 (0.20) ^{b}	4.20 (0.32) ^{a}	<0.001
Aggressive social interaction	0.50 (0.08)	0.4 (0.07)	0.73 (0.13)	0.112
Tail biting	0.04 (0.02)	0.05 (0.02)	0.14 (0.05)	0.093
Exploration of the enrichment	1.28 (0.21)	1.47 (0.17)	1.63 (0.25)	0.512
Exploration of pen fixtures	6.77 (0.35)	6.97 (0.37)	6.79 (0.39)	0.919

P values refer to the Kruskal Wallis outcome (significant with $p < 0.05$), while superscript letters indicate statistically relevant differences between groups upon post-hoc comparisons.

Table 3
Count of skin lesions per body area of the animals, divided per experimental group, reported as mean and standard deviation in brackets (SD).

	Control (n = 28)	Lavender (n = 28)	Sham (n = 28)	*P* value
Ear	2.10 (0.188)	1.78 (0.162)	1.87 (0.166)	0.394
Shoulder	3.73 (0.335) ^{a}	2.71 (0.256) ^{b}	3.01 (0.241) ^{ab}	0.031
Flank	1.44 (0.150) ^{a}	0.96 (0.122) ^{a}	2.09 (0.216) ^{b}	0.001
Thigh	1.53 (0.151) ^{a}	0.78 (0.096) ^{b}	1.30 (0.141) ^{a}	0.001
Legs	0.23 (0.055) ^{a}	0.28 (0.081) ^{ab}	0.54 (0.100) ^{b}	0.015
Total Lesions	9.30 (0.633) ^{a}	6.64 (0.435) ^{b}	8.94 (0.562) ^{a}	0.001

Different superscript letters indicate statistically relevant differences between groups ($P < 0.05$).

Table 4
Tail and skin severity classes distribution, expressed in %, in the experimental groups.

	Control (n = 28)	Lavender (n = 28)	Sham (n = 28)
Tail			
0 (intact tail)	53	76	70
1 (moderate damage)	39	19	24
2 (severe damage)	8	6	6
$P = 0.0254$	a	b	ab
Overall skin score			
0 (low damage)	64	79	71
1 (intermediate damage)	33	21	29
2 (severe damage)	2	0	0
$P = 0.0995$	/	/	/

3.4. CORT and DHEA

The level of hair CORT and DHEA are represented in Fig. 1 (panels A and B respectively), alongside the CORT/DHEA ratio (panel C). The analysis of variance did not show any statistically significant differences among groups for both hormones, despite hair CORT mean being visibly lower in L group. As for the CORT/DHEA ratio, the analysis of variance was statistically significant ($P = 0.022$), with L animals showing lower values than S ones ($P = 0.018$).

3.5. Nasal microbiota

Overall, the sequencing run produced an average of 54,811 sequence reads per sample, which resulted to an average of 27,392 sequences after the bioinformatics analysis. These sequences were assigned to 2461 amplicon sequence variants (ASVs). For the taxonomic composition a total of 24 Phyla were identified: Proteobacteria 40.11 ± 3.82%, Firmicutes 39.54 ± 2.08%, Bacteroidota 13.73 ± 1.48%, Actinobacteriota 5.69 ± 1.09% and Euryarchaeota 0.21 ± 0.15% were the most represented. At family level a total of 135 families were identified: Moraxellaceae 31.99 ± 4.9%, Streptococcaceae 10.00 ± 5.3%, Weeksellaceae 8.84 ± 2.5%, Clostridiaceae 6.03 ± 1.9% and Leuconostocaceae 4.87 ± 5.0% were the most abundant. At Genus level a total of 323 genera were identified: *Moraxella* 24.91 ± 7.81%, *Streptococcus* 9.97 ± 5.54%, *Acinetobacter* 6.65 ± 1.33%, *Clostridium_sensu_stricto_1* 5.92 ± 2.1% and *Weissella* 4.83 ± 5.23% were the most abundant. To assess if faecal contamination could affect the overall bacterial composition, we included a contamination score for each swab (0,1), as a safety check. Beta diversity was not influenced suggesting that the results obtained reflect the actual nasal microbiota. This is also confirmed by the fact that our results agree with what observed in previous study in terms of taxonomic composition at Phylum level (Wang et al., 2019; Weese et al., 2014).

3.6. Alpha and beta diversity

Alpha diversity measures are represented in Fig. 2. Chao1 was not influenced by any of the factors considered, instead Shannon diversity was significantly affected by sampling time (Timepoint, $P = 0.01$), with higher values recorded in the second sampling point. InvSimpson was affected by Timepoint ($P = 0.01$), with higher values recorded at the second sampling point, and by the interaction between Timepoint and Treatment ($P = 0.03$), with a reduction of the alpha diversity observed in the L group at the second sampling timepoint.

For beta diversity, PCoA plots generated using the Euclidean distance matrix between samples are reported in Fig. 3. The Adonis test evidenced that Treatment ($R2 = 0.058$, $P = 0.01$), Timepoint ($R2 = 0.13$, $P < 0.001$) and their interaction ($R2 = 0.057$, $P < 0.01$) significantly affected the microbial structure.

3.7. LEfSe analysis

Results for the LEfSe analysis are reported in Fig. 4. At d0 no differences were observed between the groups while at d72 subjects of the control group (C) were characterized by a higher abundance of bacteria *Empedobacter* (LDA_score = 5.35, *P*.adj =0.02), *Acinetobacter* (LDA_score = 5.21, *P*.adj =0.04), *Flavobacterium* (LDA_score = 4.46, *P*.adj <0.05), *Comamonas* (LDA_score = 4.43, *P*.adj =0.01), *Pseudomonas* (LDA_score = 4.24, *P*.adj <0.01), *Chryseobacterium* (LDA_score = 4.00, *P*.adj =0.02), *Escherichia/Shigella* (LDA_score = 4.00, *P*.adj =0.02), Enterobacteriaceae (uncultured genera) (LDA_score = 3.85, *P*.adj =0.03) and *Enterobacter* (LDA_score = 3.69, *P*.adj =0.01). Subjects from the lavender group were characterized by a higher abundance of *Corynebacterium* (LDA_score = 5.03, *P*.adj =0.03), *Aerococcus* (LDA_score = 5.03, *P*.adj =0.02) *Turicibacter* (LDA_score = 4.23, *P*.adj =0.02), *Terrisporobacter* (LDA_score = 4.11, *P*.adj =0.02), *Romboutsia* (LDA_score =

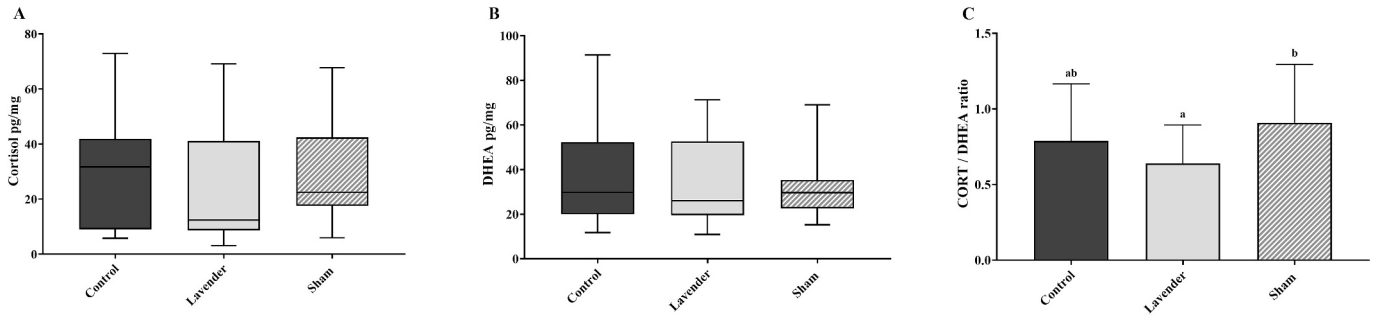

Fig. 1. Concentration of hair cortisol (A), dehydroepiandrosterone (DHEA) (B) and Cortisol/DHEA ratio (C) at the end of the trial.

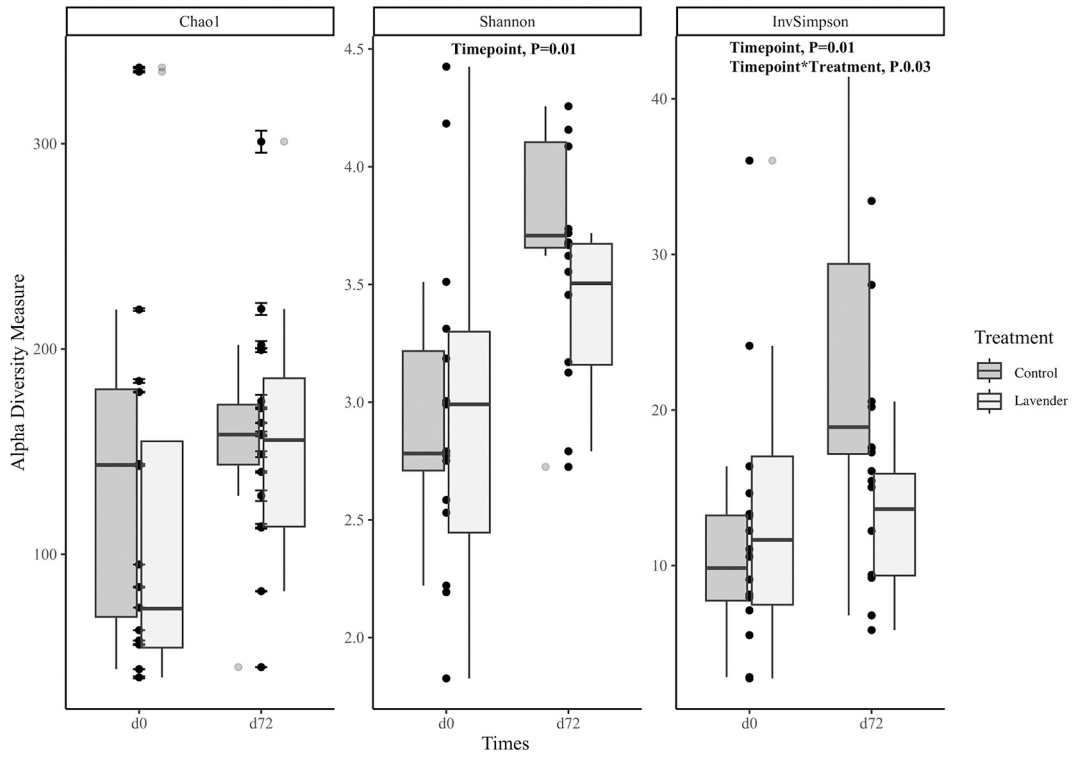

Fig. 2. Effect of lavender essential oil (LaEO) treatment and timepoint on the alpha diversity measure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.72, *P*.adj =0.02) and *Bifidobacterium* (LDA_score = 3.62, *P*.adj =0.01).

4. Discussion

In the last decades, the use of essential oils in veterinary medicine, including for livestock, has seen a constant growth, most likely due to the increased sensitivity toward the One Health approach, and the awareness of the need to promote more sustainable farming conditions and support the fight against antibiotic resistance (Mariotti et al., 2022; Nehme et al., 2021). Indeed, essential oils have proven biological properties that could be exploited in a vast variety of fields. Nonetheless, due to their high variability, in terms of composition and effects, a systematic approach aimed at assessing safety and efficacy is needed.

As reported by several studies, lavender essential oil affects the central nervous system and behavioural patterns, both in humans and animals, with sedative, analgesic, and neuroprotective properties, and is one of the best-selling natural remedies to treat central nervous system symptoms, such as anxiety, stress, and sleep disorders (Koulivand et al., 2013; López et al., 2017). Its direct contact toxicity has been assessed in vitro on different cell populations including porcine spermatozoa (Troisio et al., 2024) and human endothelial cells and fibroblasts (Prashar et al., 2004), but specific data for inhalation are still lacking. Nonetheless, it was proved that inhalation of LaEO induces protective pharmacological effects at doses 400 fold lower than those toxic for its main components (Barocelli et al., 2004). Therefore, it seemed the best candidate for a study aimed at finding a natural solution to positively modulate pigs' behaviour and improve their coping capabilities toward farming conditions.

Looking at the results of the present study, the exposure to LaEO did not affect the growth performance of the animals, as shown by the absence of statistically significant differences. Growth-related parameters recorded in the present trial are perfectly in line with the national data for Italian heavy pig belonging to the same productive phase (Montanari, 2020).

As concerns pig behaviour, animals treated with environmental nebulisation of LaEO seemed calmer (increased recumbency time). Although no differences in aggressive behaviour were observed at the analysis of time budgets, L animals showed a decrease in the amount of skin lesions, in particular in the flank and thigh areas, also associated with lower severity class for tail lesions. This finding agrees with the

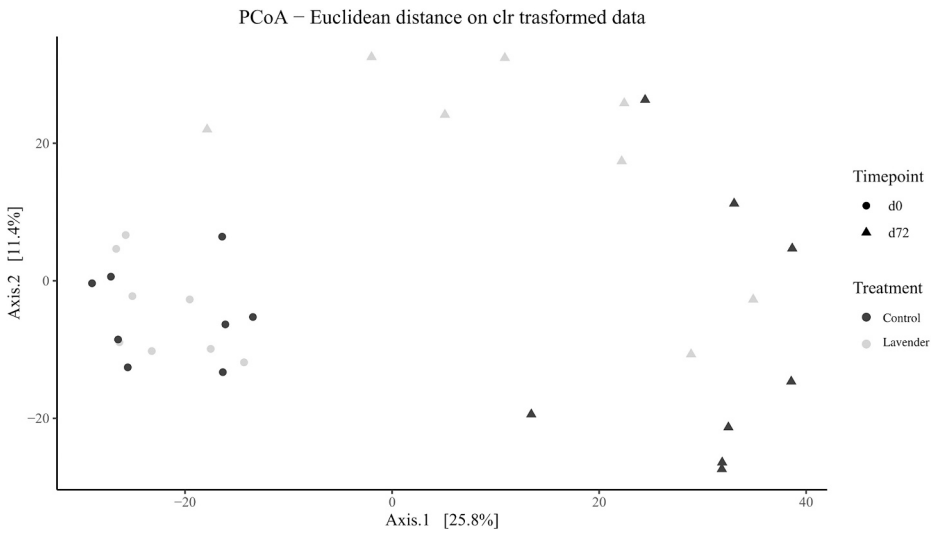

Fig. 3. Effect of lavender essential oil (LaEO) treatment and timepoint on the beta diversity measured with Euclidean distance between 'clr' transformed abundance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

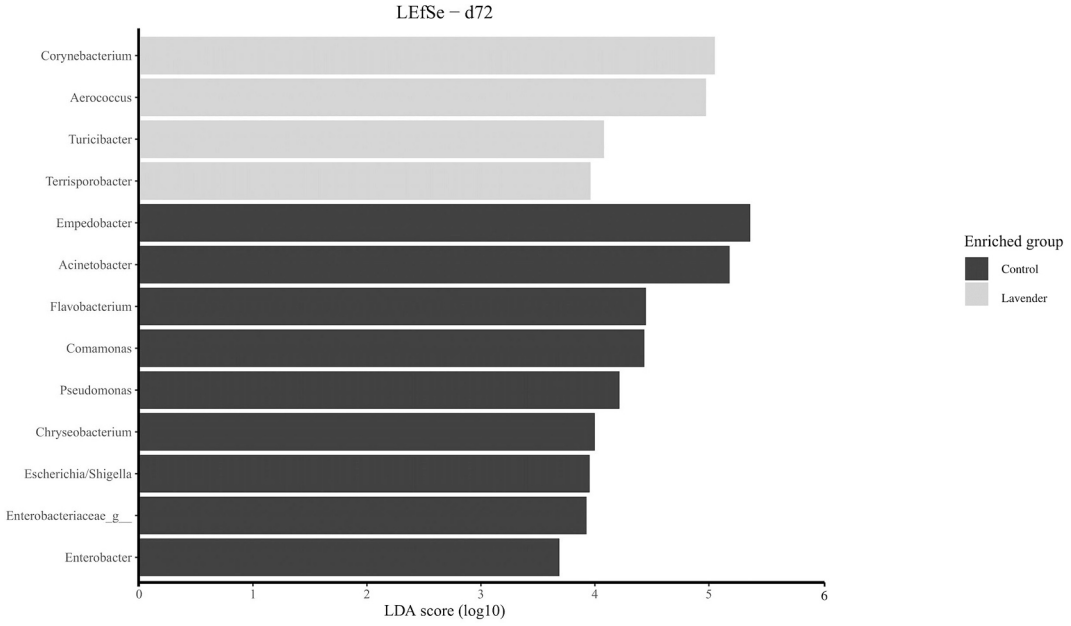

Fig. 4. Linear discriminant analysis effect size (LEfSe) plots of the biomarker taxa for each experimental group at Genus level.

tendentially higher time spent carrying out tail biting behaviour by the S group. This could indicate that the effects of lavender nebulization may have been limited to the period immediately after the administration, possibly affecting aggression levels in the minutes after each administration but without affecting the overall time budgets of the animals. Interestingly, results previously recorded in fattening pigs treated with the same LaEO delivery system (Nannoni et al., 2023), showed that older animals exposed to lavender twice a day showed higher level of skin lesions than the control group. This may indicate that younger animals might be more sensitive to the positive effects of lavender treatment. Furthermore, other studies showed higher levels of activity in pigs transported with lavender straw (Bradshaw et al., 1998) and higher frequencies of fighting in weaned pig exposed to lavender after transportation (Direksin et al., 2017), yet the overall evaluation of animal welfare did not seem to be affected in both cases. When trying to justify or at least contextualize such differences, it is important to highlight how the present experiment did not include acute stressful event such as mixing or transportation. In addition, supporting the hypothesis of a relative lack of major stressors during the experiment and overall good welfare level, both the number and the severity of the lesions hereby reported were lower than what already described by literature (Norring et al., 2023; van Staaveren et al., 2019), also in similar farming conditions (Vitali et al., 2019). With respect to the sham group, S animals seemed to be more active compared to C (lower time spent in lateral recumbency, higher time spent in dog-sitting behaviour) and their lesion count in the flank area were higher compared to the control group. Minor differences were also observed in the time animals spent eating and drinking, however, since they were fed on a rationed diet, this seems to be related to their overall activity level (exploring the empty feeder or the drinker after the meal was over) rather than to an increased feed and water intake. For example, L animals were the less active and they also spent less time eating.

Overall, it seems that the sham treatment may have resulted in a slightly worse welfare of the animals, probably due to the fact that these

animals were somehow disturbed by the activation of the nebulizing system without having any positive effect of the lavender treatment.

Looking at the results of hair hormones quantification, the concentrations of CORT, DHEA and their ratio are in agreement with what reported in the same species (Bergamin et al., 2019; Montillo et al., 2020). Despite the lack of differences across groups for both hormones, the lower CORT/DHEA ratio recorded in L animals supports the beneficial effect of LaEO on pig. Indeed, it is reported that the CORT/DHEA ratio more clearly represents the hypothalamic-pituitary-adrenocortical (HPA) axis function when compared to single hormones quantification, with higher values occurring in some psychopathology disorders as depression (Kamin and Kertes, 2017; Netherton et al., 2004). In pigs, a higher CORT/DHEA ratio has been associated with naturally occurring *Taenia solium* infection, probably due to an elevated metabolic effort trying to cope with the environment in term of housing, feeding, and regrouping (Trevisan et al., 2017). Once again, despite the lack of differences in both CORT and DHEA levels among groups, the mean cortisol value was lower in animals nebulized with LaEO. According to Casal and colleagues, the effects of *V. officinalis* and *P. incarnata* on hair cortisol were only remarkable upon 12 weeks of treatment, while the present study only covered 10 weeks, potentially explaining the lack of statistically relevant differences (Casal et al., 2017).

Regarding the analysis of the interaction between the essential oil and the microbial environment of the initial airways tract, the results suggest that LaEO inhalation significantly impacted the overall structure of the nasal pig microbiome and reduced its diversity. Moreover, the InvSimpson index was notably lower in pigs exposed to LaEO inhalation, while the other alpha diversity indexes were unaffected. This implies that the reduced diversity occurred at the expense of more 'common' bacteria, as this measure is more influenced by them. This was further confirmed by the significant reduction of genera like *Comamonas* and *Acinetobacter*, which are considered normal inhabitants of the swine nasal microbiota (Yue et al., 2011), in pigs exposed to LaEO. Additionally, LaEO decreased the presence of *Pseudomonas*, that despite some species possessing known pathogenic capabilities, is typically found in higher proportions in the nasal microbiome of swine, (Sadikot et al., 2005; Wang et al., 2019).

Conversely, LaEO promoted the proliferation of bacterial genera less commonly found in the nasal microbiome of pigs, including *Terrisporobacter*, *Turicibacter*, and *Aerococcus*, which have been associated with particulate matter inside finishing pig units (Hong et al., 2021), and were favored by lavender exposure. Furthermore, Actinobacteria, including *Bifidobacterium* and *Corynebacterium*, appeared to be favored by the treatment. *Corynebacterium* plays an important protective role in the human nasal microbiota due to its ability to produce siderophores to compete for limited iron in the human nasal cavity, thus limiting the growth of coagulase-negative staphylococci (Stubbendieck et al., 2019). Currently, there is no study in the literature evaluating the effect of essential oils in pig's nasal microbiota. However, in the study conducted by Magossi and colleagues, where they employed a spray containing a blend of essential oils (ajowan, thyme, fennel, citronella, and cinnamon leaf) on weaned cattle a comparable impact on nasal microbiota was noted (Magossi et al., 2023). In particular, they observed a reduction in microbial diversity after a single dose administered within the initial 24 to 48 h post-application. Nonetheless, this effect was transient, as the nasal microbiota returned to a composition similar to that of the control group in the subsequent days. In our study, prolonged exposure to LaEO likely induced long-lasting alterations in the nasal microbiological environment.

Since this was, to the best of the author's knowledge, one of the first study using nebulizing machines in a pig facility, we decided to include a Sham group, still exposed to nebulization but without any EO, to assess the potential effects of the machines and the solution vehicle. The results of this experimental group, and in particular the lesion score and CORT/DHEA ratio, allow the authors to hypothesize that the used custom-made delivery device has somehow affected the animals, potentially masking the lavender beneficial effects. In particular, the key stressing event may be represented by the noise and vibrations produced by the device during the 10 min nebulization sessions. Other LaEO delivery methods may be evaluated to avoid such issue, such as lavender infusion of environmental enrichment (Casal et al., 2017), addition to the bedding material (when present) and manual operated environmental sprays (Bradshaw et al., 1998) or administration in feed and drinking water (Zhai et al., 2018). Nonetheless, in light of the obtained results and the high standardization potential of nebulization procedures, it would be extremely interesting to modify the devices in order to minimize noise and vibrations.

5. Conclusions

In conclusion, this study represents one of the few reports trying to comprehensively assess the effects of repeated inhalation sessions of *Lavandula angustifolia* essential oil in growing pigs. Growth parameters were not negatively affected, and the overall welfare status of the animals seemed to be slightly improved, as supported by behavioural traits, skin lesions and CORT/DHEA ratio. Improvement in the delivery system, probably too noisy as it is, may help uncovering more straightforward positive effects on welfare. Finally, the evident effects on nasal microbiota, suggest how it is pivotal to take into account and characterize the microbial modulatory capabilities of essential oils before exploiting them on larger scale.

Institutional review board statement

The research project was authorized as an observational study by the Ethical committee of the University of Bologna, with approval number 3610/2023.

CRediT authorship contribution statement

Alberto Elmi: Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Federico Correa:** Writing – original draft, Methodology, Investigation. **Domenico Ventrella:** Methodology, Formal analysis, Data curation. **Maurizio Scozzoli:** Supervision, Conceptualization. **Niccolò Ian Vannetti:** Methodology, Investigation. **Nadia Govoni:** Methodology, Formal analysis. **Eleonora Truzzi:** Methodology, Data curation. **Simona Belperio:** Methodology. **Paolo Trevisi:** Supervision, Resources, Formal analysis. **Maria Laura Bacci:** Supervision, Resources, Formal analysis. **Eleonora Nannoni:** Writing – review & editing, Investigation, Data curation, Conceptualization.

Declaration of competing interest

APA-CT kindly supplied the materials, without interfering with both the experimental design and the interpretation of the results.

Data availability

Data will be made available on request. Raw reads are publicly available at Sequence Research Archive (SRA) under the accession number PRJNA1048305.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2024.105251.

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