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The *in ovo* screening of 27 single essential oils showed selective effects on hatchability, performance and gene expression relevant to gut functions in broilers at hatch

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ABSTRACT

The early post-hatching phase remains to be one of the most vulnerable phases in broiler production. Some essential oils have been reported to improve gut health and growth in broiler chickens when applied to post-hatching diets. However, in-feed applications are unable to prevent the health challenges observed immediately after hatching. Thus, pre-hatch interventions need to be considered. A research project was developed with the aim of investigating the impact of *in ovo* application of 27 selected essential oils **(EOs)** on foetal development with emphasis on gut integrity in broiler hatchlings. The eggs were incubated under standard conditions until day 17.5, when 1 mL of each EO preparation (5 μL EO + 5 μL polysorbate-80 + 990 μL saline) was injected into the amnion. Hatchability, body weight and organ weights (residual yolk, gizzard-proventriculus, intestines, liver, and heart) were measured at hatch. Five essential oils eugenol, clove, tea tree, lemongrass, and thyme, significantly ($P < 0.05$) reduced hatchability (66.67 %, 58.33, 83.30 and 83.30 %) compared to the saline (96.80 %), were discarded from the rest of the study. The other 22 essential oils were investigated in a second phase to assess their impact on expression of gut biomarkers including: a) jejunum integrity; b) digestive enzymes and nutrient transporters; and c) immune system. The results indicated that lemon myrtle significantly increased and oregano EO decreased body weight at hatch **(BW0)** compared to the saline ($P < 0.05$). Ylang ylang, clary sage, bergamot, lemon myrtle, and black pepper upregulated the expressions of biomarkers regulating gut integrity and barrier functions (ZO-1, ZO-2, CLDN1, MARVELD2, EGFR and EGF), nutrients transporters (EAAT3, PEPT1, I-FABP1, SGLT1), and digestive enzymes (APN, SI). Ylang ylang, turmeric acid, star anise, clary sage, and black pepper upregulated the expression of gut immunity biomarkers IL1B, IL10, IGMH, CD3D, and BU1 compared to the saline. In conclusion, *in ovo* delivery of selected EOs has the potential to improve embryonic development relevant to nutrient digestion and absorption, gut integrity and immunity in broilers.

Introduction

The development of antimicrobial resistance **(AMR)** is one of the challenges affecting livestock and human health (Marshall and Levy, 2011). The emergence and perpetuation of AMR genes in bacterial pathogens have been associated with the use of antibiotics in animal production for therapeutic purposes (e.g., fluoroquinolones, enrofloxacin, aureomycin etc.) or for growth promotion (e.g., bacitracin, virginiamycin, etc). These AMR genes can be transferred to human bacterial pathogens becoming a public health concern (Hoelzer et al., 2017). Thus, restricting the use of antibiotics has been one of the main priorities in animal production, including poultry, for a few decades (Roura et al., 1992).

In broiler chickens the reliance on antimicrobial treatments is highly dependent on the robustness of the day-old chicken which, in turn, reflects embryonic development (Kornasio et al., 2011; Noy and Uni, 2010). Thus, strategies to improve digestive and immune development during foetal development have the potential to increase chicken

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robustness at hatch and decrease the need of antimicrobial treatments. Some of the critical aspects in gut development include the integrity and functional properties of the epithelia such as the full asset of digestive enzyme secretions, nutrient sensors and transporters, and gut associated lymphoid tissues (Niknafs and Roura, 2018).

The *in ovo* technology allows the delivery of biological substances such as vaccines to the developing foetus. *In ovo* technologies emerged with the original application patented on Marek's disease vaccination (Johnston et al., 1997). More recently, *in ovo* manipulation has been adapted to the concept of "*in ovo* feeding", which has been extensively researched and includes the delivery of some of the most limiting nutrients such as carbohydrates, proteins, amino acids, vitamins and minerals into the egg through the amniotic compartment (Ajayi et al., 2022; Das et al., 2024; Li et at., 2024; Wang et al., 2020; Zhu et al., 2021). *In ovo* interventions have the potential to be more effective than post-hatching interventions since it facilitates access in the late stages of developing embryo when nutrients are needed the most (Uni & Ferket, 2004).

Since the ban on antibiotic growth promoters in European countries, essential oils **(EOs)** have received significant attention as potential alternatives in livestock production. EOs are complex mixtures of plant metabolites often lipophilic in nature and cover a wide array of properties including antimicrobial and antioxidant activities, appetite stimulation, and enhancement of digestion and lipid metabolism (Brenes and Roura, 2010). The potential application of EOs *in ovo* has received little attention to this date. On the one hand, Niknafs et al (2024) reported toxicity effects of oregano EO when applied above 10 μL/egg. On the other hand, the flow dynamics of carvacrol (the main component of oregano essential oil) after *in ovo* administration in the amniotic fluid at embryonic day 17.5 showed a quick migration towards the yolk thus may serve as a route for delivery into the gastrointestinal tract (via the yolk stalk) potentially influencing gut development during the peri-hatching phase (Meijer et al., 2024). However, Meijer and co-workers (2024) did not report potential effects post-hatching chicks which remain to be studied. Thus, a better understanding of the effects of oregano and other EOs on gut functionality in post-hatched chicks warrants further investigation. The objective of this investigation was to study the *in ovo* application of a wide array of selected EOs. It was hypothesised that the *in ovo* application of selected EOs has the potential to improve digestive and immune development in the small intestine during the peri-hatching period in broiler chicks.

Materials and methods

All the experimental procedures were approved by the Animal Ethics Committee of the University of Queensland (UQ AEC, St Lucia, Queensland, Australia) (approval certificate 2019/AE000463). The UQ AEC complies with the Australian code for use of animals for scientific purposes.

Fertile eggs and incubation

Fertile eggs (Ross 308, $n = 672$) with an average weight of 61.6 g were supplied by Darwalla (Allora, Queensland, Australia) from a 39 to 49-week-old broiler breeder flock and brought to The University of Queensland experimental hatchery (St Lucia, Queensland, Australia). Eggs with 5 % heavier and lighter weights than the average and/or with abnormal shapes and shells were discarded prior to the start of the experiment. Eggs were incubated for 17.5 days in a setter (Ova-Easy 580 Advance Series II, Brinsea, FL, USA) distributed over six levels and two trays per level (total 12 trays) with a temperature of 37.8°C, relative humidity of 57 %, and turning interval of 1h.

In Ovo injection

The experiment was performed following a complete randomized blocked design consisting of 28 experimental groups: the control group was injected with 0.9 % saline, and the rest were injected with 27 different EOs selected on the principle of enhancing gut digestive, absorption and immune function. A full list of the selected EOs and source is shown in Table 1. On embryonic day 17.5 **(E17.5)** eggs were candled, and infertile eggs were replaced with spare eggs. After sterilization with a 70 % ethanol swap a hole was drilled at the larger end of eggs using a multipurpose rotary tool (Ryobi EHT150, Ryobi, Hiroshima, Japan) with an arrow-shaped insert (Dremel High-Speed Cutter 6.4 mm, Dremel, Mount Prospect, IL) ensuring the internal membranes remained intact. Using the disposable needle (23G 1 ¼" (0.6mm × 32mm)) and syringe (1 mL) (ZebraVet Pty. Ltd., Qld, Australia), 1 mL of the saline solutions without or with EOs were injected into the amniotic fluid. The solutions injected consisted of 1000 μL of a 0.9 % saline solution (control group), or saline solutions of the selected EOs consisting of 5 μL EO + 5 μL polysorbate 80 (Sigma-Aldrich Pty. Ltd., Australia, CAS:9005-65-6) + 990 μL saline, based on the procedure developed in a series of studies in our group (Niknafs et al., 2024). Immediately after injection at E17.5, the hole was sealed with a droplet of beeswax and all eggs were transferred from the setters to the hatchers (GREATLANDER, Taabinga, Australia) with the temperature set at 37.8°C and the relative humidity at 70 %. Hatchability and individual body weight (BW0) were recorded at days 20, 21, and 22.

Post-hatch tissue sampling

At hatch, 6 ($n = 6$) chicks per treatment were sacrificed and organ weights of residual yolk, proventriculus-gizzard, intestine, liver, and heart were recorded. Tissue samples obtained from the jejunum were excised and flushed with PBS (20°C) before transferring to cryogenic storage vials containing RNAlater solution (1.5 mL). The samples were maintained at room temperature for 24 hours and stored at −80°C until required for RNA extraction. A total of five EOs showed significantly ($P < 0.05$) lower hatchability and were discarded at this point while the rest of EOs were selected for studying the expression of biomarkers associated with gut integrity (OCLN, ZO-1, ZO-2, CLDN1, MARVELD2, EGF and EGFR), digestive enzymes (APN and SI), nutrient transport (EAAT3, PEPT1, I-FABP1 and SGLT1) and the immune system (IL1B, IL10, IGMH,

Table 1
List of twenty-seven essential oils (EOs) tested *in ovo* including supplier and catalogue reference number.

Local name	Scientific name	Catalogue number	Supplier
Bergamot	*Citrus bergamia*	30791507	doTERRA
Black Pepper	*Piper nigrum*	41041507	doTERRA
Cassia	*Cinnamomum cassia*	FR-345	Delacon
Cinnamon	*Cinnamomum zeylanicum*	30031507	doTERRA
Clary Sage	*Salvia sclarea*	30421507	doTERRA
Clove	*Eugenia caryophyllata*	30041507	doTERRA
Coriander	*Coriandrum sativum*	30781507	doTERRA
Eucalyptus	*Eucalyptus radiata*	FR-334	Delacon
Eugenol	*Eugenia caryophyllata*	FR-351	Delacon
Geranium	*Pelargonium graveolens*	30091507	doTERRA
Ginger	*Zingiber officinale*	60216144	doTERRA
Grapefruit	*Citrus x paradisi*	30101507	doTERRA
Lavender	*Lavandula angustifolia*	30111507	doTERRA
Lemon	*Citrus limon*	FR-306	Delacon
Lemongrass	*Cymbopogon flexuosus*	30131507	doTERRA
Oregano	*Origanum vulgare*	30181507	doTERRA
Patchouli	*Pogostemon cablin*	30891507	doTERRA
Peppermint	*Mentha piperita oil*	60200228	doTERRA
Rosemary	*Rosmarinus officinalis*	FR-342	Delacon
Sandalwood	*Santalum paniculatum*	41861507	doTERRA
Spearmint	*Mentha spicata*	31611507	doTERRA
Star Anise	*Illicium verum*	FR-305	Delacon
Tea Tree	*Melaleuca alternifolia*	30151507	doTERRA
Thyme	*Thymus vulgaris*	60206102	doTERRA
Turmeric	*Curcuma longa*	60206102	doTERRA
Ylang Ylang	*Cananga odorata*	30241507	doTERRA

CD3D, BU1). A full description of the acronyms and genes is shown in Table 2.

RNA isolation and real-time quantitative PCR (qRT-PCR)

Total RNA was extracted from each tissue after homogenization with Scilogex D160 homogenizer following the manufacturer's instructions. RNA was extracted using a RNeasy Mini Kit, QIAGEN (Cat. No. 74104) following the manufacturer's protocol. Total RNA quantity and purity was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA) at an optical density of 260nm. The isolated RNA was reverse transcribed with the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) following manufacturer's instructions (gDNA elimination step performed).

The primers utilised in the current study were sourced from previously published studies in chickens (Supplementary Table 1). The quantitative PCR was performed in duplicate using a SYBR green mix (QuantiNova, Qiagen, Hilden, Germany) on an ABI QuantStudio 6 real-time PCR machine. The PCR reaction was performed in a volume of 10 μL containing 1 μL forward and reverse primers (4 μM), 5 μL SYBR, 3 μL RNase free water and 1 μl cDNA. All reactions were analysed in duplicate. PCR conditions included 2 minutes 95°C, then 45 cycles of 5 sec 95°C and 20 sec 60°C. For determining single product amplification, a melt curve was produced at the end of the run. The delta-delta Ct procedure was applied for quantifying results of qPCR. (Livak & Schmittgen, 2001).

Table 2
List of genes of interest studied including the acronyms, the full name and their functions according to UniProtKB database.

Acronym	Gene name	Functions of encoded proteins
Epithelial barrier and gut integrity / tight junctions		
OCLN	Occludin	Stability and barrier functions in tight junctions.
ZO-1	Zonula Occludens 1	Tight junction adaptor and adherence in junctions.
ZO-2	Zonula Occludens 2	Tight junction barrier and assembly of junctions.
CLDN1	Claudin 1	Barrier forming abilities and tight junction integrity.
MARVELD2	MARVEL Domain Containing 2	Tricellular tight junctions' formation and epithelial barriers.
EGFR	Epidermal Growth Factor Receptor	Growth, proliferation, and differentiation of numerous cell types.
EGF	Epidermal Growth Factor	Proliferation of different types of cells, especially fibroblasts and epithelial cells.
Nutrient digestion and transportation		
EAAT3	Excitatory Amino Acid Transporter 3	Glutamate uptake
PEPT1	Peptide Transporter 1	Uptake of di- and tripeptides.
I-FABP1	Intestinal-Type Fatty Acid-Binding Protein	Transport of long-chain fatty acids.
SGLT1	Sodium/Glucose Cotransporter 1	Glucose and galactose uptake
APN	Aminopeptidase N	Protein digestion
SI	Sucrase-Isomaltase	Carbohydrate digestion.
Gut immune system		
IL1B	Interleukin 1β	Pro-inflammatory cytokine
IL10	Interleukin 10	Anti-inflammatory cytokine
IGMH	Immunoglobulin M	Antibody that can neutralize antigens and activate the complement system
CD3D	T-cell surface glycoprotein CD3 delta chain	T-cell development and signal transduction.
BU1	Transmembrane protein of B-cells and a subset of macrophages	B-cell development

Statistical analyses

Since hatchability is a categorical binary variable (hatched or not hatched), a logistic model was fitted to compare hatchability treatments and control. The PROC LOGISTIC in SAS9.4 was used, and 'hatchability=no' was set as the reference. The effect of 'Hatcher' and 'Setter' was added to the model as a fixed effect. The *P* value for the overall effect of treatment on hatchability was calculated using the Chi-squared test, and pair-wise comparisons were used to compare treatment groups. A $P < 0.05$ was considered as the threshold for significant differences between treatments. Organ weights were analysed using the General Linear Model (Univariate) procedure in IBM SPSS Statistics version 27. The statistical model consisted of the residual yolk, proventriculus-gizzard, intestines, liver, and heart weights as dependent variables and the treatment as fixed factor with the tray as random factor. Body weight at hatch **(BW0)** was analysed using ANCOVA where, initial egg weight was used as a covariate in the model. Least square means were adjusted for the covariate effect and pairwise comparison of treatments was performed using Tukey HSD test ($P < 0.05$). The data on gene expression was analysed using the General Linear Model (Univariate) procedure. The statistical model considered the genes OCLN, ZO-1, ZO-2, CLDN-1, MARVELD2, EGF, EGFR, APN, SI, EAAT3, PEPT1, I-FABP1, SGLT1, IL1B, IL10, IGMH, CD3D and BU1 as dependent variables, the treatment as fixed factor, and the qPCR plate as random factor. The Tukey HSD test was used to perform pairwise comparisons. Means were considered significantly different at $P < 0.05$.

The correlate => bivariate function in IBM SPSS was used to assess Pearson's correlation coefficients between all the genes within each gut function: integrity (OCLN, ZO-1, ZO-2, CLDN-1, MARVELD2, EGF, and EGFR); digestion and nutrient transport (APN, SI, EAAT3, PEPT1, I-FABP1 and SGLT1); and immune system (IL1B, IL10, IGMH, CD3D and BU1). Correlation with a $P < 0.05$ were considered as statistically significant.

The Principal Component Analysis **(PCA)** was performed using SPSS version 27.0. Data of gut expression integrity genes OCLN (Occludin), ZO-1 (Zonula occludens 1), ZO-2 (Zonula occludens 2), CLDN1 (Claudin 1), MARVELD2 (MARVEL Domain Containing 2), EGFR (Epidermal Growth Factor Receptor) and EGF (Epidermal Growth Factor), nutrients transporters and digestive enzymes EAAT3 (Excitatory Amino Acid Transporter 3), PEPT1 (Peptide Transporter 1), I-FABP1 (Intestinal-Type Fatty Acid-Binding Protein), SGLT1 (Sodium/Glucose Cotransporter 1), APN (Aminopeptidase N), SI (Sucrase-Isomaltase), and gut immunity genes IL1B (Interleukin 1β), IL10 (Interleukin 10), IGMH (Immunoglobulin M), CD3D (CD3 δ subunit of T-cell Receptor Complex, present on T-cells and NK-cells) and BU1 (Transmembrane protein of B-cells and a subset of macrophages) were subjected to Kaiser-Meyer-Olkin (KMO) and Bathlett's tests to assess the validity of the factor analysis of each of the datasets and to test whether the treatments had any effects on the expression of the genes evaluated. Eigenvalues greater than 1.0 ("Kaiser rule") were considered adequate.

Results

Impact of EOs on hatchability, body and organ weights at hatch

The *in ovo* injection of lemongrass, thyme, tea tree, clove, and eugenol EOs significantly ($P < 0.05$) reduced hatchability to 83.3, 83.3, 75.00, 66.67 and 58.33 %, respectively, when compared to the 96.80 % rate of the saline control (Fig. 1). In addition, significant ($P < 0.05$) differences were observed in BW0 indicating that lemon myrtle increased (47.68 g), and oregano decreased (41.30 g) the body weight of the chicks at hatch when compared to the saline control (45.11g) (Fig. 1). Moreover, the tea tree EO injection significantly ($P < 0.05$) increased liver weight compared to the saline control (1.31 vs 1.01 g, respectively) (Table 3). No significant ($P > 0.05$) differences were observed for residual yolk, gizzard-proventriculus, intestines and heart

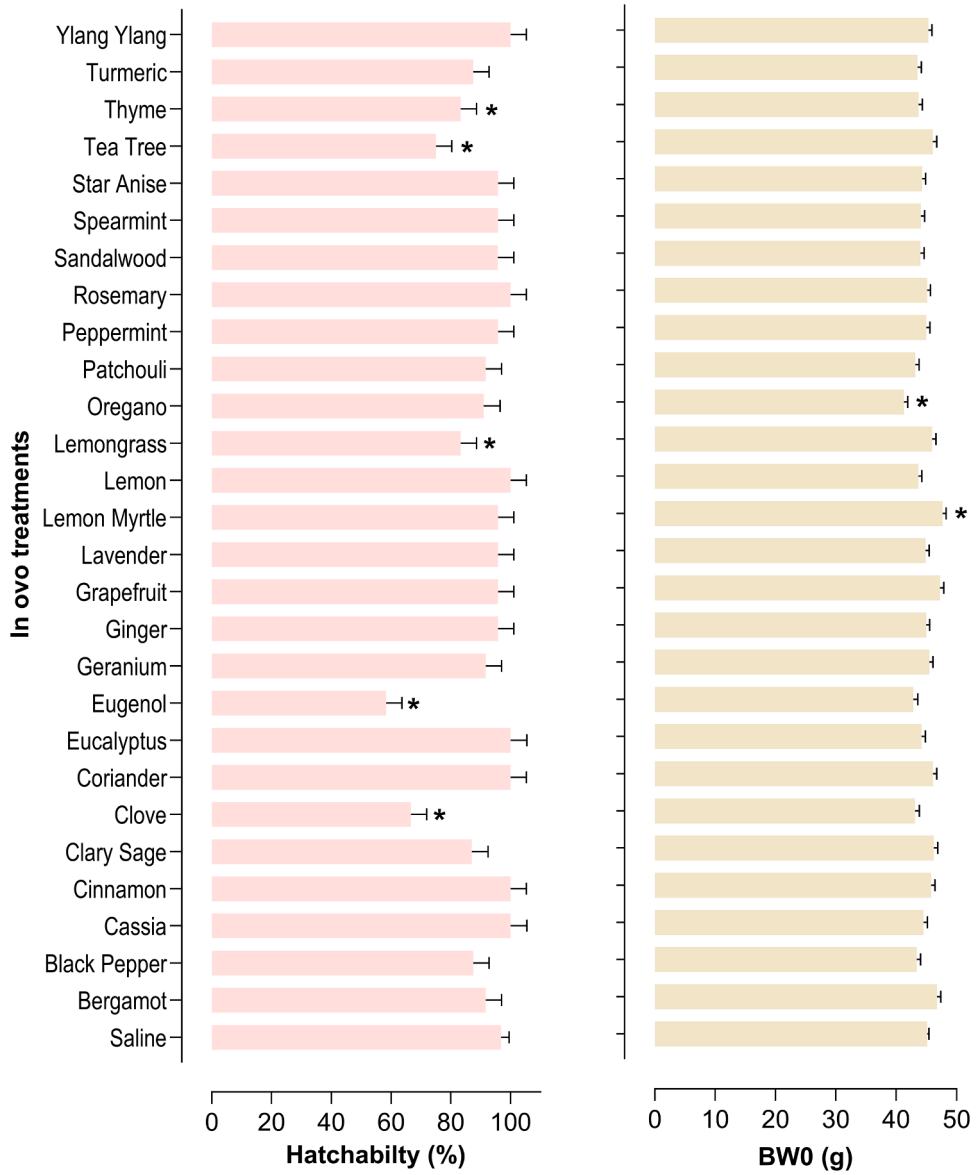

Fig. 1. Results of experiment 1 showing the effects of *in ovo* injection of 27 essential oils (EOs) in saline solution and a saline control on hatchability and body weight of broiler chickens on day 0 (BW0) post hatch ($n = 24$). Means with an asterisk (*) are significantly different compared to the saline control at a $P < 0.05$.

weights.

Impact of EOs on the expression of genes related to gut integrity, digestion, and immunity

The effects of *in ovo* application of all the EOs selected on genes relevant to gut integrity (OCLN, ZO-1, ZO-2, CLDN1, MARVELD2, EGF, and EGFR), nutrients transporters (EAAT3, PEPT1, I-FABP1, SGLT1), digestive enzymes (APN and SI), and immune biomarkers (ILB, IL10, IgMH, CD3D, and BU1) are presented in the Figs. 2A, 2B, and 2C. *In ovo* injection of rosemary, eucalyptus, and cassia EOs significantly ($P < 0.05$) downregulated the expression of the OCLN gene compared to saline. Sandalwood and rosemary EOs significantly ($P < 0.05$) downregulated expression of ZO-2 and EGFR compared to saline. None of the EOs tested influenced expressions of ZO-1, CLDN1, MVLD2 and EGF genes (Fig. 2A). *In ovo* injection of spearmint, patchouli, turmeric EOs significantly ($P < 0.05$) downregulated the expression of EAAT3 compared to saline (Fig. 2B). Bergamot EO significantly ($P < 0.05$) upregulated the expression of SI compared to saline. In contrast, the *in ovo* injection of EOs showed no significant effect ($P > 0.05$) on expression PEPT1, I-FABP1, SGLT1 and APN. The effects of *in ovo* injection of different EOs on immune related biomarkers are shown in Fig. 2C. *In ovo* feeding of rosemary, eucalyptus, cassia, lemon, patchouli, turmeric, ginger, sandalwood, peppermint, lavender, lemon myrtle, and grapefruit EOs significantly ($P < 0.05$) downregulated CD3D compared to the control.

Correlation matrices assessing the expression of genes of interest affected by EOs

Correlation analyses were performed amongst biomarkers regulating gut integrity (Fig. 3A). Expression of OCLN was positively correlated with ZO1 ($r = 0.694$, $P < 0.001$), ZO2 ($r = 0.717$, $P < 0.001$), CLDN1 ($r = 0.530$, $P < 0.001$), MARVELD2 ($r = 0.583$, $P < 0.001$), EGFR ($r = 0.687$, $P < 0.001$) and EGF ($r = 0.426$, $P < 0.001$). Correlation of ZO1 with ZO2 ($r = 0.749$), CLDN1 ($r = 0.495$), MVLD2 ($r = 0.575$), EGFR ($r = 0.584$) and EGF ($r = 0.439$), correlation of ZO2 with CLDN1 ($r = 0.514$), MVLD2 ($r = 0.425$), EGFR ($r = 0.600$) and EGF ($r = 0.345$), correlation of CLDN with MVLD2 ($r = 0.454$), EGFR ($r = 0.479$) and EGF ($r = 0.292$), correlation of MARVELD2 with EGFR ($r = 0.688$), EGF ($r =$

Table 3
Results of experiment 1 showing the effects of *in ovo* injection of 27 essential oils (EOs) in saline solution and a saline control on organs weights of broiler chickens on day 0 post hatch ($n = 6$). The results are expressed as the average ± standard deviation.

Treatments	Residual Yolk (g)	Gizzard-Proventriculus (g)	Intestines (g)	Liver (g)	Heart (g)
Saline	6.67 ± 1.34	2.19 ± 0.26	1.80 ± 0.37	1.01^{bc} ± 0.10	0.33 ± 0.05
Bergamot	7.04 ± 1.73	2.26 ± 0.17	2.04 ± 0.38	1.03^{bc} ± 0.06	0.32 ± 0.08
Black Pepper	5.68 ± 1.07	2.1 ± 0.24	1.94 ± 0.19	1.02^{bc} ± 0.11	0.32 ± 0.03
Cassia	6.42 ± 1.16	2.25 ± 0.30	1.75 ± 0.24	1.07^{bc} ± 0.08	0.31 ± 0.04
Cinnamon	6.75 ± 1.30	2.43 ± 0.23	1.80 ± 0.21	1.09^{ab} ± 0.07	0.33 ± 0.05
Clary Sage	6.61 ± 0.93	2.28 ± 0.18	2.09 ± 0.23	1.17^{abc} ± 0.11	0.38 ± 0.04
Clove	5.98 ± 1.52	2.35 ± 0.45	2.22 ± 0.29	1.09^{abc} ± 0.14	0.35 ± 0.02
Coriander	5.97 ± 1.65	2.19 ± 0.22	1.98 ± 0.40	1.11^{bc} ± 0.12	0.33 ± 0.04
Eucalyptus	6.39 ± 1.55	2.16 ± 0.10	1.67 ± 0.17	1.00^{abc} ± 0.05	0.33 ± 0.03
Eugenol	5.93 ± 1.46	2.25 ± 0.34	1.97 ± 0.31	1.11^{bc} ± 0.06	0.38 ± 0.05
Geranium	6.23 ± 0.51	2.35 ± 0.23	1.98 ± 0.30	1.06^{bc} ± 0.06	0.32 ± 0.03
Ginger	7.19 ± 1.61	2.19 ± 0.21	1.72 ± 0.38	1.04^{abc} ± 0.09	0.34 ± 0.05
Grapefruit	7.33 ± 1.68	2.26 ± 0.28	2.14 ± 0.35	1.12^{c} ± 0.09	0.32 ± 0.04
Lavender	7.80 ± 1.50	2.16 ± 0.33	1.71 ± 0.45	0.93^{abc} ± 0.09	0.3 ± 0.03
Lemon Myrtle	6.08 ± 0.91	2.26 ± 0.13	1.99 ± 0.24	1.12^{bc} ± 0.08	0.35 ± 0.04
Lemon Oil	7.13 ± 0.43	2.11 ± 0.21	1.63 ± 0.24	0.97^{bc} ± 0.09	0.33 ± 0.04
Lemongrass	7.87 ± 1.28	2.24 ± 0.17	1.95 ± 0.35	1.08^{bc} ± 0.08	0.32 ± 0.04
Oregano	5.57 ± 1.35	2.16 ± 0.20	2.19 ± 0.30	1.02^{bc} ± 0.08	0.33 ± 0.03
Patchouli	5.85 ± 1.46	2.17 ± 0.21	2.13 ± 0.45	1.02^{bc} ± 0.11	0.31 ± 0.02
Peppermint	6.35 ± 0.98	2.26 ± 0.22	1.77 ± 0.20	1.00^{bc} ± 0.05	0.33 ± 0.04
Rosemary	6.98 ± 0.55	2.26 ± 0.27	1.60 ± 0.27	0.99^{bc} ± 0.08	0.31 ± 0.04
Sandalwood	6.55 ± 1.13	2.17 ± 0.22	2.15 ± 0.39	1.00^{bc} ± 0.10	0.33 ± 0.04
Spearmint	5.91 ± 0.88	2.28 ± 0.24	2.00 ± 0.34	0.97^{bc} ± 0.11	0.34 ± 0.05
Star Anise	6.18 ± 0.69	2.22 ± 0.29	1.78 ± 0.27	1.00^{bc} ± 0.08	0.31 ± 0.06
Tea Tree	7.21 ± 1.25	2.00 ± 0.19	1.70 ± 0.20	1.31^{a} ± 0.25	0.29 ± 0.02
Thyme	6.87 ± 0.75	2.14 ± 0.21	1.78 ± 0.31	1.05^{bc} ± 0.11	0.29 ± 0.03
Turmeric	6.63 ± 2.20	2.15 ± 0.37	1.99 ± 0.33	0.97^{bc} ± 0.12	0.33 ± 0.06
Ylang Ylang	6.13 ± 1.13	2.11 ± 0.17	1.88 ± 0.25	1.06^{bc} ± 0.09	0.30 ± 0.05
P values	0.047	0.825	0.149	<0.001	0.088

Means not sharing common superscripts $^{a\text{-}c}$ represent to significant differences ($P < 0.05$).

0.619) and correlation of EGFR with EGF (r = 0.638) were significantly ($P < 0.001$) positive.

Correlation of EAAT3 with PEPT1 (r = 0.227), SGLT1 (r = 0.305), APN (r = 0.377), SI (r = 0.311), correlation of PEPT1 with I-FABP1 (r = 0.480), SGLT1 (r = 0.692), APN (r = 0.751) and SI (r = 0.305), correlation of I-FABP1 with SGLT1 (r = 0.737), APN (r = 0.318), SI (r = 0.554), correlation of SGLT1 with APN (r = 0.623), SI (r = 0.656) and correlation of APN with SI (r = 0.396) were significantly ($P < 0.001$) positive (Fig. 3B).

The expression of IL1B was positively correlated with the expression of IL10 (r = 0.337), IgMH (r = 0.203), CD3D (r = 0.291) and BU1 (r = 0.210), expression of IL10 was positively correlated with the expression of CD3D (r = 0.337) and BU1 (r = 0.155), expression of IgMH was positively correlated with the expression of CD3D (r = 0.501) and BU1 (r = 0.456), however the expression of CD3D was positively correlated with the expression of BU1 (r = 0.338) ($P < 0.001$) (Fig. 3C).

In addition, the expression of genes in different functional groups were also correlated. The expression of OCLN was positively correlated with PEPT1 (r = 0.641), SGLT1 (r = 0.584) and CD3D (r = 0.509); EGF was positively correlated with PEPT1 (r = 0.260), SGLT1 (r = 0.273), IL1b (r = 0.338) and CD3D (r = 0.229); PEPT1 was positively correlated with CD3D (r = 0.565); however SGLT1 was positively correlated with IL1b (r = 0.200) and CD3D (r = 0.487) ($P < 0.05$) (Fig. 3D).

Principal component analysis of gut functions following in ovo application of EOs

The principal component analysis (PCA) was performed among EOs treatments for gut integrity/epithelial barrier/tight junctions, digestion, nutrients transporters and immune system biomarkers to get a two-dimensional (2D) representation. Using Kaiser rule for Eigenvalues, 9 Principal Components were extracted that accounted for 75.51 % of the total variance in the experiment. Fig. 4 shows loadings of different variables (EOs) on PC1 and PC2. Clary sage followed by black pepper, oregano, turmeric, peppermint, coriander, and bergamot EOs had higher loadings on PC1 with coefficient values 0.755, 0.698, 0.693, 0.684, 0.645, 0.612 and 0.601, respectively. In addition, cassia followed by saline, patchouli, lemon, ginger, and ylang ylang EOs had higher loadings on PC2 with coefficient values 0.717, 0.533, 0.523, 0.483, 0.468 and 0.362, respectively.

Discussion

The main indicator of a healthy embryonic development is the hatchability rate (Narushin et al., 2016). Thus, it is fundamental that any *in ovo* manipulation does not compromise the survivability of the chicken embryo to hatching. This was the case for 22 of the 27 EOs tested in this study. However, the delivery of eugenol, clove, tea tree, thyme, and lemongrass EOs in the fertile egg significantly compromised hatchability rates for which they were discarded for the rest of the experiment. Another crucial indicator is body weight at hatch which has been highly correlated to chick robustness early in life (Molenaar et al., 2008). The *in ovo* application of lemon myrtle EO resulted in a significant increase in body weight at hatch. This increase in body weight at hatch might be related to an increase in gut development as shown in the upregulation of gut integrity indicators OCLN, CLDN-1, MARVELD2, ZO-1, and ZO-2, and epithelial growth promoters EGF and EGFR in the jejunum at hatch. In addition, the injection of lemon myrtle EO was also associated with the higher expression of glucose, fatty acid and amino acid transporters in the jejunum. In fact, gut integrity and nutrient transporters were highly correlated possibly indicating an overall impact on gut development and gut health (Suzuki, 2020). Altogether, the results observed after lemon myrtle application *in ovo* makes this EO a candidate for industry adoption that warrants further investigation. In contrast, oregano EO resulted in a significant decrease in body weight at hatch compared to the saline control. This is partially in contrast with the popularity of oregano supported by a significant body of literature highlighting positive impact on gut antioxidative capacity, morphology, immunity and microbiota, and growth performance (Peng et al., 2016; Sarıca et al., 2014; Ruan et al., 2021).

In addition, the *in ovo* delivery of ylang ylang, clary sage, bergamot, and black pepper EOs compared to other essential oils upregulated the expressions of several gene biomarkers regulating gut integrity and

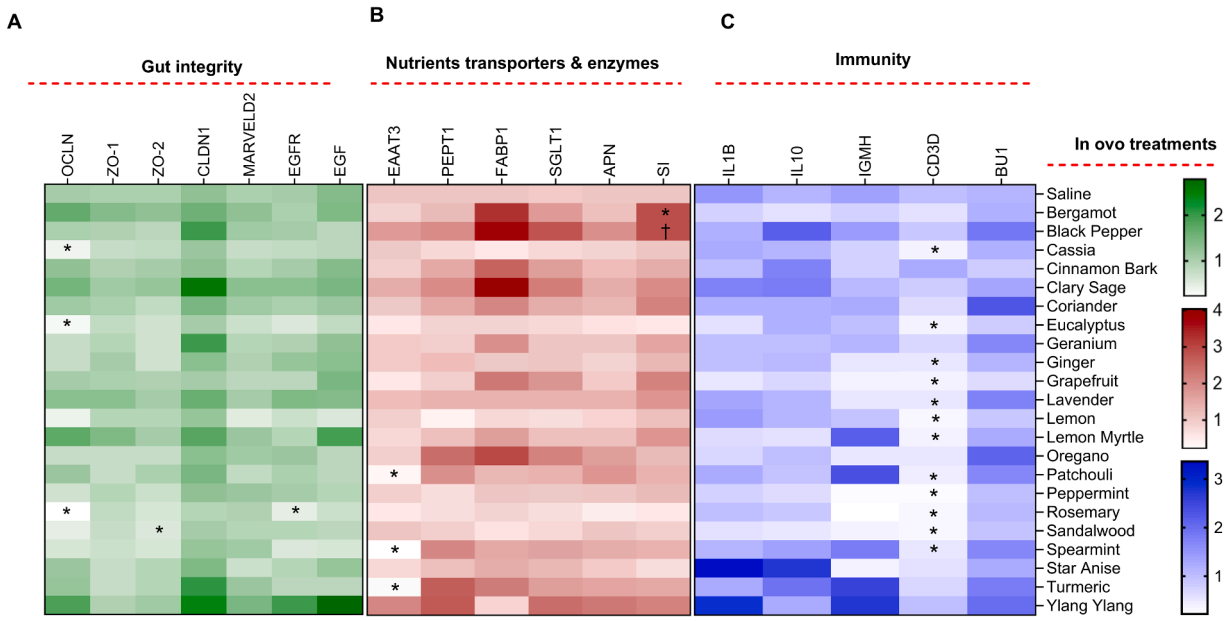

Fig. 2. Results of experiment 2 showing the effects of *in ovo* injection of 22 essential oils (EOs) on gene expression of biomarkers related to epithelial barrier, gut integrity, permeability, tight junctions, nutrient digestion and transportation and immune system in the jejunum of broiler chicken at day 0 post hatch ($n = 6$). Asterisks (*) represent significant differences within the same gene compared to the saline control at $P < 0.05$. The symbol † represents a trend associated to a $P < 0.1$. Acronyms: OCLN = Occludin, ZO-1 = Zonula occludens 1, ZO-2 = Zonula occludens 2, CLDN1 = Claudin 1, MARVELD2 = MARVEL Domain Containing 2, EGFR = Epidermal Growth Factor Receptor, EGF = Epidermal Growth Factor, EAAT3 = Excitatory Amino Acid Transporter 3, PEPT1 = Peptide Transporter 1, I-FABP1 = Intestinal-Type Fatty Acid-Binding Protein, SGLT1 = Sodium/Glucose Cotransporter 1, APN = Aminopeptidase N, SI = Sucrase-Isomaltase, IL1B = Interleukin 1β, IL10 = Interleukin 10, IGMH = Immunoglobulin M, CD3D = CD3 δ subunit of T-cell Receptor Complex, present on T-cells and NK-cells, BU1 = Transmembrane protein of B-cells and a subset of macrophages.

barrier functions (OCLN, CLDN-1, EGF and EGFR), as shown in Fig. 2A, and of nutrients transporters and digestive enzymes (I-FABP1, SGLT1, PEPT1, and SI) (Fig. 2B). CLDN1 and OCLN are the main tight junction proteins reported in chickens (Criado-Mesas et al., 2021; Proszkowiec-Weglarz et al., 2020). These proteins are crucial for maintaining the selective permeability of the intestinal epithelium forming tight seals between epithelial cells (Chen et al., 2015). The epithelial growth factor (EGF) and receptor (EGFR) activate signalling pathways involved in epithelial cell proliferation, differentiation, and survival. Thus, the upregulation of EGF and EGFR can promote the growth and/or repair of the intestinal mucosa. This may enhance the absorptive capacity of the gut and improves its resilience to damage and stress (Kim et al., 2020). Enhanced epithelial cell turnover and overall gut health can lead to more efficient nutrient utilization, supporting growth and production efficiency in poultry (Bai et al., 2021; kim et al., 2017). Improved tight junctions contribute to preventing the translocation of harmful pathogens and toxins from the gut lumen into the bloodstream (Du et al., 2016). This may reduce the risk of infections and inflammatory responses (Hollemans et al., 2020; Karcher and Applegate, 2008). In addition, proper gut integrity facilitates better absorption of nutrients, contributing to improving growth and feed efficiency in chickens (Barekatain et al., 2019).

The jejunum of broiler chickens plays a crucial role in nutrient absorption, facilitated by a complex network of nutrient transporters and digestive enzymes. Nutrient transporters are integral membrane proteins responsible for the absorption of specific nutrients across the epithelial cells of the jejunum (Kaminski and Wong, 2018; Kheravii et al., 2018). Some key glucose, amino acids and peptides, and fatty acid transporters have been identified as biomarkers of digestive function including SGLT1, EAAT3, PEPT1, and I-FABP1. In addition, APN is a catabolic enzyme digesting peptides releasing free amino acids, while SI is a glycoprotein that regulates the final steps in carbohydrate catabolism (Ferrer et al., 2003; Hundal and Taylor, 2009; Niknafs and Roura, 2018). Regardless of the EO treatment, the results of gene expression within (Fig. 3A, 3B, and 3C) and between (Fig. 3D) functions showed a high correlation indicating that all the selected gene biomarkers were similarly affected by the treatments. This is consistent with the selection criteria of the biomarkers and validates the genes selected being representative of gut integrity, epithelial development and nutrient digestion and absorption (Barekatain et al., 2021; Kheravii et al., 2018; Song et al., 2021; Su et al., 2015). In addition, the cross-correlation between gut integrity biomarkers and nutrient transporters indicate that these two functional parameters are also associated. While speculative, the cross-correlation between the two functions when taken together may indicate an overall status of gut health.

The jejunum is also a critical site for immune surveillance and defence mechanisms against environmental pathogens. We found that the *in ovo* delivery of ylang ylang, turmeric, star anise, clary sage, and black pepper EOs upregulated immune system biomarkers IL1B, IL10, IgMH and BU1 when compared to other EOs or the control group (Fig. 2C). Interleukins 1β and 10 (IL1B and IL10) were chosen as the main pro- and anti-inflammatory cytokines, respectively (Roura et al., 1992; Withanage et al., 2004). Upregulation of IL1B can enhance the initial immune response to infections, improving the chicken's ability to fight off pathogens quickly (Del Vesco et al., 2020). In contrast, IL10 is an anti-inflammatory cytokine that regulates immune responses by inhibiting the production of pro-inflammatory cytokines. Higher expression of IL10 has been associated with the control of inflammatory responses to prevent undesirable tissue damage and improve overall health and tissue repair processes (Arendt et al., 2019; Zhang et al., 2022). IgMH is the first antibody produced during an immune response. The upregulation of IgMH can enhance the production of IgM antibodies, leading to improved efficiency of pathogen clearance (Li et al., 2020; Luo et al., 2013). BU1 is a marker of B cells in chickens and is involved in the development and function of the humoral immune response. Upregulation of BU1 can enhance B-cell development and maturation, which could be critical for robust antibody responses against pathogens (LePage et al., 2000). Finally, gut integrity and

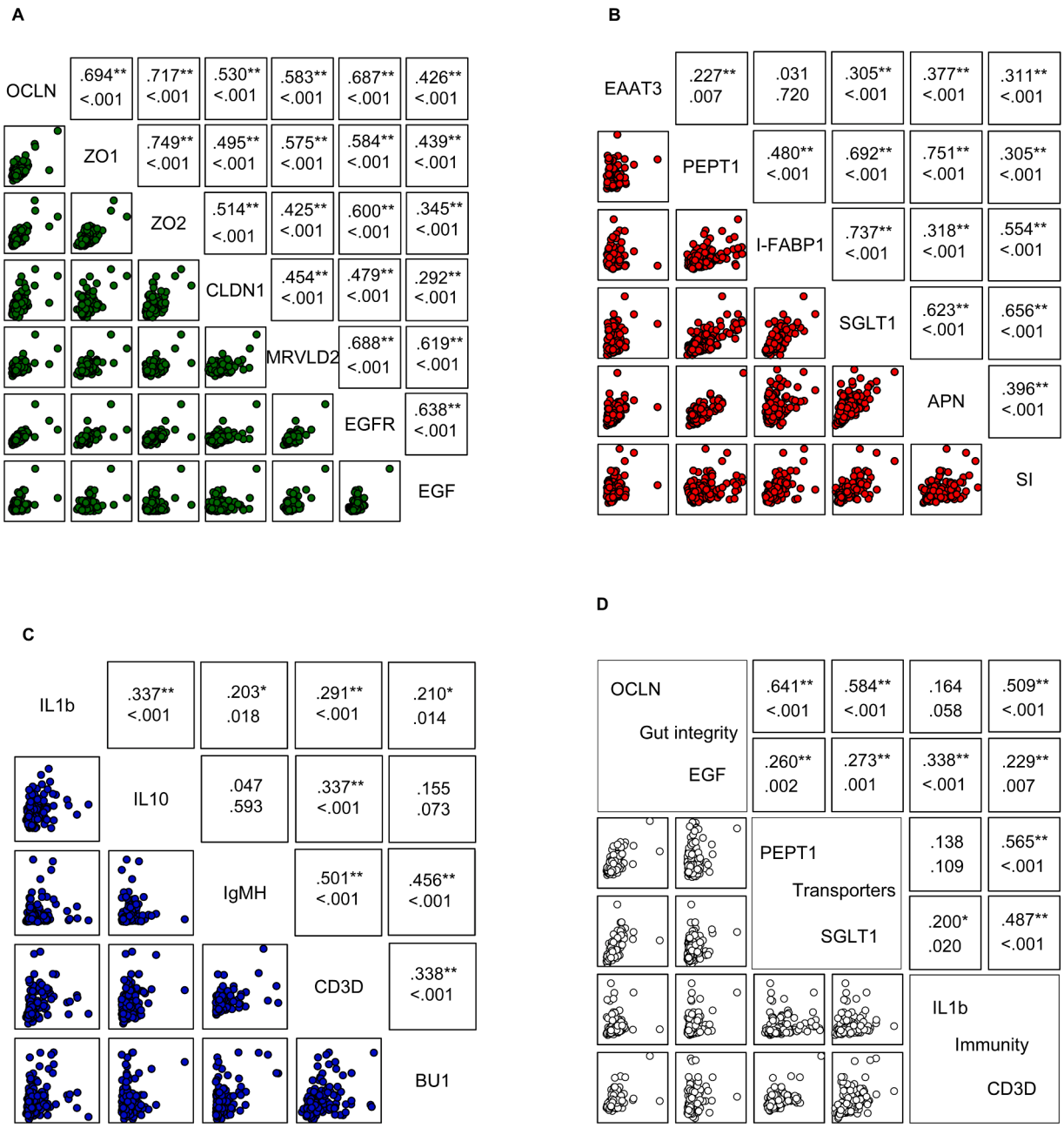

Fig. 3. Results of experiment 2 showing the correlation matrix relating the expression levels of biomarkers related to a) gut integrity (green), b) nutrient transporters and digestive enzymes (burgundy), and c) immune biomarkers (blue) in the jejunum of broiler chicken on day 0 post hatch ($n = 6$). Figure d) shows the correlation between two selected genes from each of the groups in a), b), and c). Acronyms: OCLN = Occludin, ZO-1 = Zonula occludens 1, ZO-2 = Zonula occludens 2, CLDN1 = Claudin 1, MARVELD2 = MARVEL Domain Containing 2, EGFR = Epidermal Growth Factor Receptor, EGF = Epidermal Growth Factor, EAAT3 = Excitatory Amino Acid Transporter 3, PEPT1 = Peptide Transporter 1, I-FABP1 = Intestinal-Type Fatty Acid-Binding Protein, SGLT1 = Sodium/Glucose Cotransporter 1, APN = Aminopeptidase N, SI = Sucrase-Isomaltase, IL1B = Interleukin 1β, IL10 = Interleukin 10, IGMH = Immunoglobulin M, CD3D = CD3 δ subunit of T-cell Receptor Complex, present on T-cells and NK-cells, BU1 = Transmembrane protein of B-cells and a subset of macrophages. **. Correlation is significant at the $P < 0.01$ level. *. Correlation is significant at the $P < 0.05$ level.

nutrient digestion and transporters interact closely with the gut-associated immune system as indicated by the significant correlations between them shown in Fig. 3D. The CD3D, a glycoprotein signalling T-cell development, was positively correlated with gut integrity and nutrient transporter genes and was upregulated by several EOs. This would indicate that EOs improving gut integrity would also be increasing T-cell development in the gut (Amer et al., 2023; Revajova et al., 2010). In contrast, pro-inflammatory cytokine IL-1B was not correlated with gut integrity or amino acid/peptide transporters. A milder correlation was observed between IL-1B and the epithelial growth factor (EGF) and glucose transporter (SGLT1). While purely speculative, these results seem to indicate that EOs improve expression of EGF and SGLT1 have the potential to enhance the development, maturation and functionality of immune cells, and production of immunoglobulins IgA and IgM (Bhanja et al., 2015; Cheng et al., 2004; Humphrey and Rudrappa, 2008; Zhou et al., 2021).

Conclusions

Twenty-seven EOs were tested in an *in ovo* experiment injected at day

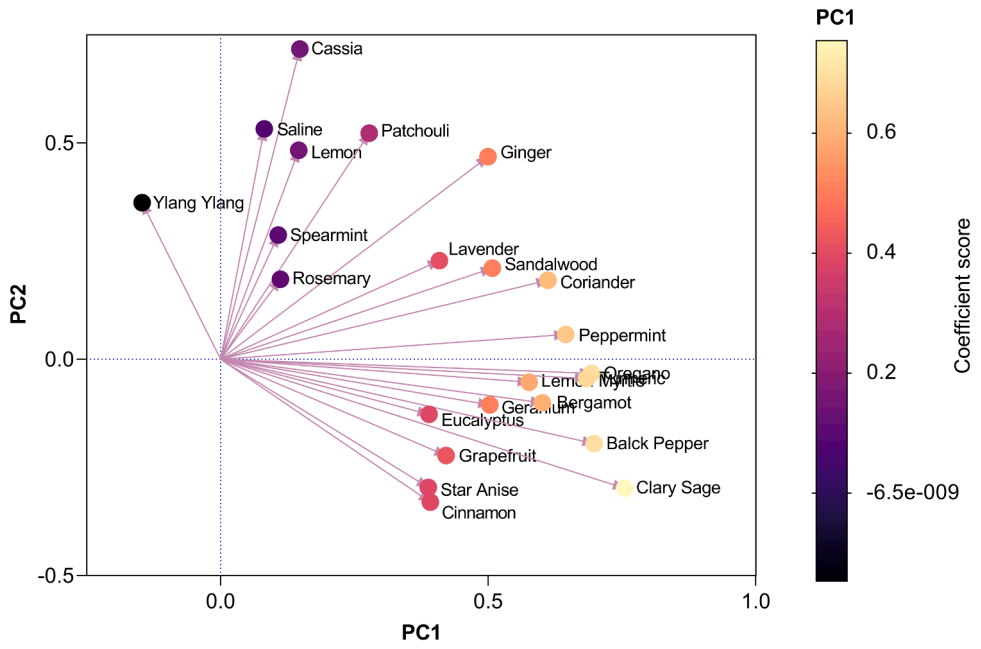

Fig. 4. Principal Component Analysis (PCA) representing the effect of 22 essential oils on the gene expression of biomarkers of gut integrity, nutrient transporter and digestive enzymes, and immune system in the jejunum in broiler chickens. The genes OCLN (Occludin), ZO-1 (Zonula occludens 1), ZO-2 (Zonula occludens 2), CLDN1 (Claudin 1), MARVELD2 (MARVEL Domain Containing 2), EGFR (Epidermal Growth Factor Receptor) and EGF (Epidermal Growth Factor) were used in the statistical model as biomarkers of gut integrity. The genes EAAT3 (Excitatory Amino Acid Transporter 3), PEPT1 (Peptide Transporter 1), I-FABP1 (Intestinal-Type Fatty Acid-Binding Protein), SGLT1 (Sodium/Glucose Cotransporter 1), APN (Aminopeptidase N), and SI (Sucrase-Isomaltase) were used as biomarkers for nutrient transporters and digestive enzymes, while genes IL1B (Interleukin 1β), IL10 (Interleukin 10), IGMH (Immunoglobulin M), CD3D (CD3 δ subunit of T-cell Receptor Complex, present on T-cells and NK-cells) and BU1 (Transmembrane protein of B-cells and a subset of macrophages) as biomarkers for gut immunity.

17.5 of embryonic development of which 5 decreased hatchability and were discarded, and 22 were selected to assess their impact on gut and immune development in the jejunum of broiler chicks at hatch. Lemon myrtle EO increased body weight at hatch. In addition, injection of lemon myrtle and also black pepper, clary sage, ylang ylang, and bergamot EOs upregulated the key biomarker genes of interest involved in the maintenance and function of tight junctions, epithelial growth promotion, glucose, amino acid and fatty acid transportation, and digestive enzymes in the jejunum. Moreover, jejunum-associated immune biomarkers IL1B, IL10, IgMH and BU1 were upregulated by the *in ovo* injection of ylang ylang, turmeric, star anise, clary sage, and black pepper. Overall, the study presents robust evidence of the benefits of *in ovo* application of EOs on embryonic development in broiler chicken worth further investigation.

Declaration of competing interest

Authors declare that no conflict of interest exist for this research manuscript.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2024.104670.

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