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Effect of powdered rosemary (*Rosmarinus officinalis*) essential oil and phenolic compounds on broiler chickens zootechnical parameters

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Abstract

This work aims at exploiting the essential oil (EO) and phenolic compounds (PC) of rosemary (*Rosmarinus officinalis*) in a formulation containing leaf powder stabilized with chitin (1%, w/w) to improve chicken meat and investigate their effect on qualitatively and quantitatively broiler chickens' growth performances. To such a purpose, chicks, at 1 day of age, were distributed in pens on ground divided into four batches. The two control batches were fed with conventional feed, but the positive control contained flavomycin (0.5 g/kg) as growth factor. The two treated batches received a conventional feed supplemented with two doses of 20 and 50 g/kg of the developed formulation. Essential oil and phenolic compound contents in powdered leaves were, respectively, 1.20 and 22.86% in ethanolic extract. 1,8-Cineole (46.88%), followed by camphor (19.20%), α-pinene (9.56%), L.α.-terpineol (5.91%) and β-pinene (4.40%) were the main compounds of the used EO. The two batches of treated chicks showed a significant improvement in body weight (281.88 and 283.75 g, respectively), a decrease in feed conversion and a reduction in mortality (8%), when compared to the control batches. In addition, the elaborated formulation induced catalase activity used as an indicator of cellular antioxidant activity modulation.

Keywords: catalase activity; chitin; consumption index; essential oil; phenolic compounds; poultry; *Rosmarinus officinalis*; zootechnical parameters

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Introduction

Antibiotics employed as growth promoters in chicken feed has been reported to cause undesirable side effects (Ouedraogo *et al*., 2021). This alarming situation has led the European Union (EU) to completely ban the use of antibiotics in poultry feed formulations since 2006. This suppression has caused a deterioration in chicken status health, leading to an increase in mortality rate, index consumption drops of body weight and, consequently, a decrease in poultry farming economic profitability (Abd El-Hack *et al*., 2022).

Many alternatives to antibiotics are proposed in broilers, such as essential oil (EO) of medicinal plants (Diaz-Sanchez *et al.*, 2015). Indeed, these products have many biological effects: antibacterial without resistance development phenomenon, antioxidant activators of immune system and stimulators of digestion process (Shamma *et al*., 2019). Rosemary (*R. officinalis*), belonging to Lamiaceae family, and it is a medicinal plant well known for its beneficial properties like food flavoring and preserving agent. Thus, its components are widely used as antimicrobial agents in poultry feed formulation, allowing digestive functions stimulation (Khaouchene *et al*., 2016). However, the essential oil used as antimicrobial mediators comes up against technical problems, namely their solubility and stability in food matrix (Ruben *et al*., 2017). Besides, it should be noted that pure EO used in poultry feed can cause microbiological composition disturbance or even a loss of a large part of non-pathogenic bacteria intestinal microbiota (Rowland *et al*., 2018). Previous studies have shown that live weight, average daily gain, amount of feed intake and feed conversion were not improved after the addition of supplements (Gadde *et al*., 2017). Recently, chitin is attracting more attention from researchers due to its biological qualities for chicken rearing (Subbarayudu *et al*., 2020). The latter can break it down thanks to the enzyme acid chitinase (Tabata *et al.*, 2018). Chickens, after being fed with chitin, had best feed conversion rates while consuming less feed (Lokman *et al*., 2019). In addition to this, chitin also exhibits antimicrobial activities (El-araby *et al*., 2022). The intestinal microbiota is also enriched when this component is fed to chickens, as it induces the quantitative increase of lactic acid bacteria (LAB) and the decrease of pathogenic bacteria in the digestive tract (Ibitoye *et al*., 2018). Although the presence of chitin in animal feed offers many benefits, some inconsistencies have been spotted by some authors. Namely, chitin can lead to reduced feed digestibility and reduced growth performances of chickens at certain doses (Krysiak *et al*., 2021). To our knowledge, most growth performance in chickens has been based on chitin alone but there is no data on their effect in chicken production in combination with EO of medicinal plants such as rosemary.

Considering the antimicrobial properties of natural compounds, the aspect of using chitin as a food component obtained from by-products is a sustainable solution. The approach of the present study is the exploitation of rosemary EO in their natural matrix in association with other bioactive compounds within the same matrix, which targets an organic broiler feed formulation without antibiotics use.

Abo Ghanima *et al*. (2020) reported that supplementation of rosemary EO (300 mg/Kg) in the diet of laying hens had significant positive effects on the performance and egg production of the hens. Cholesterol, liver and kidney functions, immunity and antioxidant parameters improved with rosemary and cinnamon supplementation compared to the control. Furthermore, the different housing systems had no positive or negative impact on these characteristics. In the same context, Adaszyńska-Skwirzyńska and Szczerbińska (2017) presented the advantageous characteristics of EO, with particular emphasis on their antimicrobial and immune-stimulating properties. Interest in plant phenolic compounds as a potential source of natural antioxidants has been gaining attention in the research community due to their predictable potential role as feed additives in poultry production. Mahfuz *et al*. (2021) reported that supplementation of phenolic compounds as natural feed additives can play a role on antioxidant, immune, antimicrobial, and overall production performance in poultry. Previous research indicated that different insect species could be safely used as nutraceuticals in poultry farming to improve broiler growth performance and laying hen egg production. Various products and extracts (chitin and chitosan) obtained from these insect species, can be sustainably used in poultry feed (Khalifah *et al*., 2023).

In the northeastern part, Morocco has a large natural rosemary plantation characterized by its very rich eucalyptol EO (1-8 cineol). Morocco is also a major fishing country, and the export of frozen shrimps in of the country generates huge quantities of shells. With a view to sustainable development, it would be useful to valorise these products in an innovative agri-food process. Thus, the purposes of this study were to improve poultry meat quality by valorising agri-food wastes and natural resources, identify and characterize extracted EO from *R. officinalis* by using a gas-chromatography coupled with mass spectrometry (GC-MS), determine the total phenolic compounds (PC) of the extracted EO, as well as to evaluate the effect of chitin in combination with rosemary powder (rich in EO) on broiler chickens growth performances.

Materials and Methods

Rosemary powder preparation

Rosemary leaves powder was prepared according to (Labban *et al*., 2014). Plant material was collected during November 2021 from an organic farm in Fez city (Morocco), washed of residual soil and dried under-vacuum as follows: temperature at 50 °C, vacuum of 0.3 bar and drying time of 3 hours until complete drying. Fine powdered rosemary leaves (0.1 mm) were stored in airtight plastic containers until use

Essential oil extraction

Extraction of EO from *R. officinalis* was done by hydro-distillation by using a Clevenger type apparatus. Plant sample was placed in contact with distilled water and boiled at 100 °C for 3 h as reported by (Lauridsen, 2019). The obtained EO was separated from water and stored at 4 °C until analysis. Extraction of the EO was performed in triplicate.

Phenolic compounds extraction by organic solvents

Rosemary leaves powder (10 g) was mixed with 100 mL solvents of different polarity [hexane, dichloromethane, ethanol, and acetone/hexane mixture (1:1, v/v)] for 24 h with one repetition (2 × 24 h). Extracts were filtered with a Whatman paper n°4 and then evaporated with using a BUCHI Rotavapor™ R-300 (BÜCHI Labortechnik AG, Flawil/Switzerland) at a temperature not exceeding 40 °C.

GC-MS analysis

The identification and characterization of *R. officinalis* EO was conducted by a gas chromatography (Trace GC ULTRA) coupled to mass spectrometer (Polaris Q-Ion Trap MS (Thermo Electron Corporation, USA). Analyses were performed by injecting 1 µL of the EO, using high-purity helium (99.99% purity) as carrier gas, and the linear velocity was 35 cm/s. Initial programming of injection temperature column is 40 °C for 2 min, then rises in steps of 5 °C/min to 280 °C for 10 min. Volatile compounds were identified by their mass spectra and their relative retention index (IR) calculated from separated compounds and linear alkane retention time (RT).

Infrared spectroscopy

Fourier-transform infrared spectroscopy (FTIR) of the studied extracts was performed with a Nicolat avatar 320 spectrophotometer (Zuzi 4255/50, Auxilab S.L., Navarra, Spain). Samples were placed in attenuated total reflectance (ATR) crystal cell and covered with a glass lid to avoid evaporation. The plate was placed in the sample holder and the spectra were obtained in the range from 400 to 4000 cm^{-1} in transmission mode with a resolution on 4 cm^{-1}.

Total phenolic compounds

One milliliter of the investigated extract was mixed with 0.5 mL of Folin-Ciocalteu phenol reagent (1:1, v/v, in water). Then 2.5 mL of sodium carbonate solution (20%, w/v) was added. The mixture was mixed and then allowed to stand for 40 min in the dark. Absorbance was measured with a spectrophotometer at a wavelength of 725 nm. Calibration curve was prepared with standard gallic acid.

Extraction of chitin from shrimp shells solid waste

Shrimp (*Parapenaeus longirostris*) shell waste was obtained from a local fish market in Fez city (Morocco). Shells were washed several times with warm distilled water to remove impurities, dried in an oven at 100 °C for 2 h, and then crushed into powder. Shrimp shell powder was deproteinized with 1.25 N sodium hydroxide (NaOH) at a ratio of 1:8 (g/mL). The reaction was performed at 70 °C for 3 h. The product was collected, washed with distilled water until neutral pH, and then dried at 80 °C overnight to reach a constant weight. Afterwards, discoloration was done with pure acetone at a ratio of 1:12 (g/mL) for 24 h under constant stirring of 100 rpm. The resulting product was recovered, washed to neutrality and, then, dried at 80 °C (El-araby *et al*., 2022). Demineralization was performed using 4% (w/v) citric acid ($C_6H_8O_7$) at a ratio of 1:10 (g/mL) for 24 h at room temperature and under constant stirring (250 rpm). The obtained chitin was collected, washed with distilled water several times until neutral pH, and then dried at 80 °C until use.

Broiler chickens rearing

This study was conducted in accordance with the recommendations of an internal validated guide. The protocol was approved by the Committee on the Ethics of Animal Experiments of the USMBA University. Two hundred male broiler chicks (Hubbard F37), 1 day old, were placed in adequate conditions for their growth. Chicks were divided into four experimental groups with five replicates (12 individuals/m^2) in floor pens. Rearing temperature and lighting were fixed at 30 °C and 23 hours, respectively, during start-up phase and at 22 °C and 18 hours, respectively, during growth phase. Chickens were housed in floored pens (1 × 2 m) with bedding at a depth of 5-6 cm. Chicks were given water and experimental diets (Table 1) that varied with fledgling phase ranging from 1 to 10 days (non-pelleted form) and growth phase ranging from 11 to 37 days (pelleted form). Administered feed composition of different batches of chicks during start-up and growth phases was as follows (Table 1).

Table 1. Composition of basic diets ant its supplements used in this study

Ingredients (g/kg)	**Starter feed**				**Growth Feed**			
Batches	Control [c]	Flavomycin [d]	Treatment [e]	Treatment [2f]	Control	Flavomycin	Treatment [1]	Treatment [2]
Corn	574.5	574	574.5	574.5	617.5	617	617.5	617.5
Soybean meal	310	310	310	310	276	276	276	276
Corn gluten meal	44	44	44	44	30	30	30	30
Calcium carbonate	15	15	15	15	17	17	17	17
Dicalcium phosphate	20	20	20	20	18	18	18	18
Premix [a]	3	3	3	3	3	3	3	3
Salt	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Soy oil	30	30	30	30	35	35	35	35
Flavomycin	-	0,5	-	-	-	0.5	-	-
R. officinalis	-	-	19.8	49.5	-	-	19.8	49.5
Chitin	-	-	0.2	0.5	-	-	0.2	0.5

Nutriment composition [b]								
Crude protein (%)	21.5	21.5	21.5	21.5	19.3	19.3	19.3	19.3
Metabolic energy (kcal/kg)	12.15	12.15	12.15	12.15	12.45	12.45	12.45	12.45
Na (%)	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22
Ca (%)	1.1	1.1	1.1	1.1	0.95	0.95	0.95	0.95
P (%)	0.46	0.46	0.46	0.46	0.45	0.45	0.45	0.45

[a] Included 3.0 g/kg of vitamin and mineral mix suppling the following per kg diet: retinyl acetate 11 000 IU, cholecalciferol 1810 IU, dl-a-tocopheryl acetate 10.8 mg, menadione sodium bisulfate 2 mg, riboflavin 5.7 mg, pyridoxine hydrochloride 2 mg, cyanocobalamin 0.025 mg, nicotinic acid 27 mg, folic acid 0.48 mg, pantothenic acid 13 mg, choline chloride 252 mg, Mn 100 mg, Zn 64 mg, Cu 5 mg, Se 0.23 mg, I 0.5 mg, Co 0.5 mg ; [b] According to NRC (1994) ; [c] Control batch without supplementation (CBWS) ; [d] Control batch supplemented with Flavomycin (CBSA) ; [e] Batch treated with 20g/kg of rosemary powder and chitin (TB1) ; [f] Batch treated with 50g/kg of rosemary powder and chitin (TB2).

- A negative control (CBWS) group consisting of chicks fed with a basic diet without any supplementation;

- A positive control (CBSA) batch made up of chicks fed with a staple food supplemented with an antibiotic (Flavomycin 0.5 g/kg);

- A treated batch (TB1) consisting of chicks fed with a basic diet supplemented with 20 g (per kg) of rosemary powder containing 1% (w/w) of chitin;

- A treated batch (TB2) consisting of chicks fed with a basic diet supplemented with 50 g (per kg) of rosemary powder containing 1% (w/w) chitin, the batches of chicks were housed individually and had access to food and water *ad libitum*. Regarding rosemary EO levels used in the study, it should be noted that in the first treated batch TB1 one Kg of the formulation contains 0.238 g of rosemary EO and in the second treated batch TB2 one Kg of the formulation contains 0.2594 g/kg of rosemary EO.

Rearing evaluation

Zootechnical parameters

Data relating to zootechnical performances were collected and recorded at the age of 10, 21 days, and at the end of each experimental period (37 days).

To evaluate live weight (g), 24 broilers were taken at random. It was measured every day of rearing. The growth kinetics of the chicks were monitored every day by individual weighing and calculation of the average.

Food consumption was determined every three days during the starter phase and daily during the growth phase. And this, by determining the quantity of food consumed (difference between the quantities deposited in the feeders between two periodic moments). The consumption index (CI) was calculated according to the following equation 1:

$$\boldsymbol{CI} = \frac{\text{quantity of food ingested during a period (g)}}{\text{Live weight per chicken during a period (g)}} \quad \textbf{(1)}$$

Mortality during the study period was expressed as a percentage (equation 2).

$$\text{Mortality (\%)} = \frac{\text{Number of dead subjects}}{\text{Initial number of subjects}} \times 100 \quad \textbf{(2)}$$

The daily mortality record was carried out on the day of the event, by removing the dead broilers found.

Physiological parameters

Catalase activity is a good indicator of the physiological state of the body's cells. In this objective, chicken blood sampling, a 2 mL disposable syringe needle was inserted into the wing vein. Approximately 1 mL of venous blood was collected and then transferred to a 10 mL glass test tube. After blood coagulation, test tube containing blood was subjected to centrifugation at 2500 rpm for 5 minutes at 4 °C. Serum layer was pipetted into a vial, which was then stored at 4 °C in a refrigerator in the dark at 4 °C until use.

Protein content was determined by colorimetric assay using Folin–Ciocalteu reagent according to the method described by (Lowry *et al*., 1951). Protein concentrations were determined using a standard curve constructed from Bovine Serum Albumin (BSA) stock solution of 0.1 g/L. Catalase activity was determined by the colorimetric method reported by Sinha (1972) using the dichromate/ acetic acid reagent. The latter was prepared by mixing 5% $K_2Cr_2O_7$ aqueous solution with acetic acid (1 : 3; v : v).). Catalase assay was performed by adding 100 µL of snail extract (crushed and centrifuged in the phosphate buffer) with 300 µL of H_2O_2 (2 mM), and the phosphate buffer solution (pH = 7.4; 20 mM). After 5 min, 2 mL of the dichromate/ acetic acid reagent were added to stop the reaction. After 10 min, Catalase activity measurement was performed at 570 nm, and a calibration curve for hydrogen peroxide (H_2O_2) was run in order to calculate the enzymatic units.

Statistical analysis

All experiments were done at least three times, and results are shown as mean values and standard deviations (mean ± standard deviation). Statistical significance of experimental results (significance level of $P < 0.05$) was calculated using ANOVA analysis. Numerical tool for performing the statistical analysis was the Excel 2013 software (Microsoft).

Results

GC-MS analysis

Essential oil of *R. officinalis* obtained by a hydro-distiller are of light-yellow color, with a yield of 1.2% (w/w), which is slightly higher than those (1.14%, w/w) obtained by Ladan (2015). Results showed that the EO was composed of monoterpenes and sesquiterpenes (Figure 1). A total of 15 active components of rosemary were identified. As shown in Table 2, 1,8-cineole (46.88%) was identified as the predominant active compound, followed by camphor (19.20%), α- pinene (9.56%), L.α.-terpineol (5.91%) and β-pinene (4,40%). Other compounds such as borneol (3.81%), camphene (3.12%), linalool (1.98%), terpinene-4-ol (1.08%), β-myrcene (1.22%), myrcenol (1.08%), β-caryophyllene (0,94%), bornyl acetate (0,45%) and caryophyllene oxide (0,41%) were detected at low concentrations (Table 2). These results are in agreement with the data of previous studies, especially those of Badreddine *et al*. (2015), who reported that 1,8-cineole was the major compound (50.44%) of the *R. officinalis* EO.

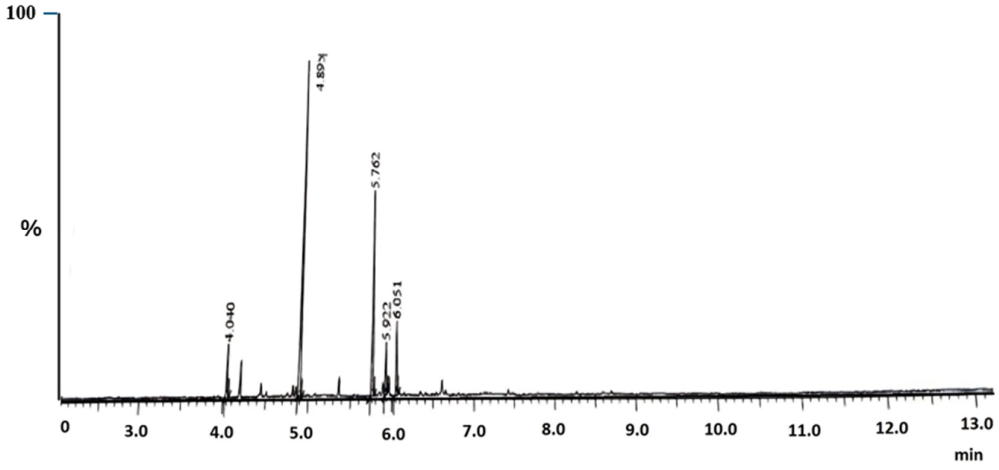

Figure 1. Chromatogram analysis (GC-MS) of the EO of *R. officinalis*

Table 2. Composition of the EO of *R. officinalis*

Name of compounds	RT	Area (%)
α-Pinene*	**4.134**	**9.50**
Camphene*	**4.291**	**3.12**
β-Pinene*	**4.526**	**4.40**
β-myrcene	4.590	0.78
1,8 cineol*	**4.983**	**46.88**
γ-Terpinene	5.155	0.53
Linalool	5.539	1.98
Camphor	5.882	19.2
Myrcenol	5.985	0.92
Borneol*	**6.020**	**3.81**
Terpinene-4-ol	6.053	1.08
L.α.-Terpineol*	**6.149**	**5.91**
Bornyl acetate	6.679	0.45
β-Caryophyllene	7.496	0.94
Caryophyllene oxide	8.364	0.41

*: Compounds in bold are the majority compounds.

Yield of extraction

The extraction of active ingredients from *R. officinalis* was performed subsequentially by solvents with different polarities [hexane, dichloromethane, ethanol and acetone/hexane mixture (1:1, v/v)]. From rosemary extracts, ethanolic extract (ER) has the highest yield followed by dichloromethane (DCMR), acetone/hexane (AR/HR) and, finally, hexane (HR) with yields of 22.86, 15.23, 12.86 and 6.09% respectively (Table 3).

Table 3. Yields (mean values ± standard deviations), expressed in percentage (w/w) of the extraction of PC in different organic solvent extracts of *R. officinalis*.

Extract	Yield (%)
Ethanol	22.86 ± 1.86
Acetone/hexane	12.86 ± 0.28
Hexane	6.09 ± 1.83
Dichloromethane	15.23 ± 1.40

Infrared spectroscopy

Analysis of rosemary extracts by Fourier-transform infrared spectroscopy in the range between 400 and 4000 cm^{-1} showed that bands between 2959 and 2848 cm^{-1} were present in each extract and characterize symmetric and asymmetric stretching of alkanes. The bands around 3402-3453 cm^{-1} are associated with alcohol function and their intensity varies depending on plant and solvent used. The most intense is ethanol extract. This is due to presence of considerable amounts of alcohol and carboxylic acid in these extracts and its intensity weakens by decreasing the polarity of solvents used (dichloromethane, acetone/hexane mixture and hexane extracts). In addition, this function is characterized by a band at 1367-1386 cm^{-1} and deformation of O-H carboxylic acid is reflected at 885-947 cm^{-1}. Moreover, remarkable bands at 3600 cm^{-1} and 1688 cm^{-1}, which reveals alcohol function of phenols as well as aromatic phenol cycle, confirm the high quantity of phenolic compounds at the ethanolic extract level of rosemary. On the other hand, these bands disappear in rosemary hexane extract. Bands of ester function appeared (1730-1734 cm^{-1}) in all rosemary extracts, while the ether function appeared in these FTIR spectrograms at 1244 cm^{-1} at ethanolic extract level.

Total phenolic compounds

The use of solvents with different polarities made a distinction in the content of phenolic compounds in the extracts. As depicted in Table 4, the most considerable quantity of phenolic compounds was found in the ethanolic extract, followed by those of acetone-hexane mixture, with dichloromethane and hexane extracts having lowest values. Among rosemary extracts, ethanolic extract had highest concentration of phenolic compounds representing 22.10 mg GAL/g of plant. This characterization is due probably to high polarity of solvent used, richness in phenolic compounds and high extraction yield (Table 4).

Table 4. Total phenolic compound (PC) content (mean values ± standard deviations), expressed as milligrams of garlic acid (GA) per gram of extract or plant sample, in different organic solvent extracts of *R. officinalis*

Extract	mg GA/ g extract	mg GA/ g plant
Ethanol (ER)	114.93	22.10 ± 0.67
Acetone/hexane (1:1, v/v) (AR/HR)	103.93	16.70 ± 0.04
Hexane (HR)	68.13	5.79 ± 0.59
Dichloromethane (DCMR)	72.70	8.30 ± 0.62

Chick growth assessment

To assess the economic impact of dietary formulations used in this work, we used the Consumption Index (CI) assessment method. This method gives indirect information on organism cellular maintenance which affects chick's weight yield.

The obtained results showed that the consumption index is 0.78 and 0.77, respectively, for the control batches (CBWS and CBSA), and 0.66 and 0.62, respectively, for the batches fed with a basal diet supplemented with formulation containing rosemary powder and chitin (TB1 and TB2). This difference is maintained on days 22 and 37 of rearing. Feed consumption index differs in treated batches, when compared to the control ones, because of the effect of dose administered (20 and 50 g of the formulation per Kg of basal diet). One also noticed that when animal weight increases, feed CI decreases. The effect of the formulation used (rosemary powder containing chitin at 1%, w/w, on growth performance, live weight, feed consumed and feed conversion ratio were summarized in Table 5.

Rosemary containing 1% (w/w) chitin significantly decreased feed intake and improved body weight gain in chickens. A significant reduction in feed intake was observed in the chickens fed with a mixture of rosemary and chitin. The consumption indices of studied batches (CBWS, CBSA, TB1 and TB2) were respectively around 1.05, 1.04, 1.00 and 1.03 on the 37th day. These values are lower than those of the Hubbard F37 chicken dashboard (Broiler Guide, Hubbard, 2015). Thus, it can be concluded that the used formulations have a positive effect on parameters measurement of live weight of individuals (279.63, 278.32, 281.88 and 283.75 g for CBWS, CBSA, TB1 and TB2, respectively) for 10 days, with a quantity of food consumed, and consumption index.

It should be noted that antibiotic elimination in broiler diets may lead to some disease's emergence. However, the obtained results in this research revealed that the addition of formulated products at concentrations of 20 g/kg and 50 g/kg in livestock feed has a beneficial effect on the growth performances of broiler chickens.

This study is limited to the first two rearing phases (starting and growth proper) because these are the two critical phases, where poultry farmers come up against many dysfunctions, such as contamination and weight loss. Our results agree with those of (Khaouchene *et al*., 2016), who worked only on growth phase nutritional side. Subsequently, in this work we focused mainly on the start phase, where there is a maximum risk of chick mortality. It should be underlined that chick mortality was around 8.3% (Table 5), and which was limited on the two control batches (CBWS and CBSA). Conversely, the TB1 and TB2 treated batches (receiving 20 and 50 g/kg of the formulation, respectively) showed no mortality.

Table 5. Zootechnical performances of studied batches during starter feed growth feed phases

Trait	Batches	Control		Treated	
	Parameters	**CBWS[a]**	**CBSA[b]**	**TB1[c]**	**TB2[d]**
	Weight J0 (g)	40.1	40.05	40.08	40,04
Start D10	Live weight J10 (g)	279.63	278.32	281.88	283.75
	Feed consumed (g)	219	218	186	177
	CI (10)	0.78	0.77	0.66	0.62
	Mortality (%)	0	0	0	0
Growth D21	D21 live weight	965.57	965	955.86	919.71
	Weight gain D10/D21	686	684	674	636
	Feed consumed (g)	879	888	766	754
	CI (21)	0.91	0.9	0.80	0.82
	Mortality (%)	0	0	0	0
Growth D37	Live weight D37	2372.83	2366	2465.16	2088.71
	Weight gain D21/D37	1407	1401	1509	1169
	Feed consumed (g)	2470	2477	2471	2167
	CI (37)	1.04	1.05	1	1.03
	Mortality (%)	8.3	8.3	0	0

[a] Control batch without supplementation; [b] Control batch supplemented with antibiotic; [c] Batch treated with 20 g/kg of formulated powder and Batch treated with 50 g/kg of formulated powder. **Trait**: Treatment; **Weight J0 (g)**: D0 live weight at day 0 ; **Live weight J10 (g)**: D10 live weight at day 10

Results illustrated in Table 5 are the means of three tests and the difference in the means obtained were the subject of an analysis of variance (ANOVA). Results of this test showed that the difference is significant, with a very low probability, because the F value found is much higher than the F' value read on the Fisher-Snedecor table.

Physiological parameters

Serum protein assay

Serological analysis provides information on livestock status health. Determination of certain metabolites and certain enzymes provide information for veterinarians to inquire about the health status of animals. Indeed, serum proteins reflect the body's responses to its environment and its diet. Serum proteins levels in plasma of the chicks belonging to different batches are summarized in Figure 2.

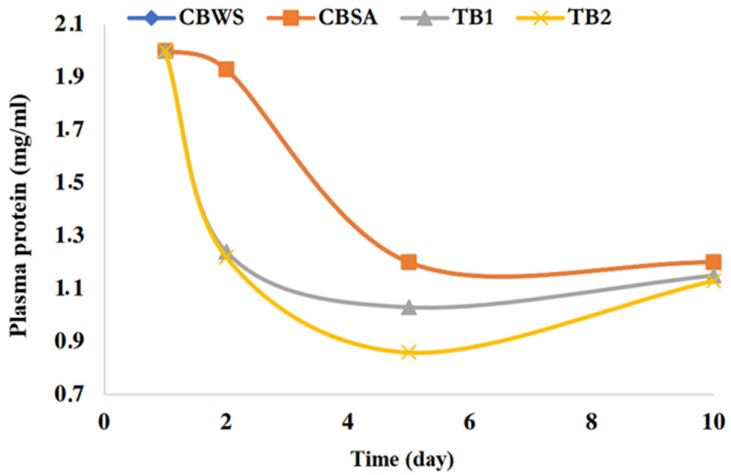

Figure 2. Level of serum proteins in chicks of different batches as a function of time

Note: CBWS: Control batch without supplementation; CBSA: Control batch supplemented with antibiotic; TB1: Batch treated with 20 g/kg of formulated powder; and TB2: Batch treated with 50 g/kg of formulated powder.

Results in Figure 2 unfolded that there is a progressive decrease in plasma proteins in control batches (CBSA and CBWS). This reduction becomes greater in batches fed with basal diet supplemented with rosemary powder containing 1% (w/w) chitin (TB1 and TB2) at 20 and 50 g of the formulation. Protein contents were of the order of 1.2, 1.2, 1.15 and 1.13 mg/mL for the studied batches CBWS, CBSA, TB1 and TB2, respectively.

Catalase assay

Catalase activity was introduced in this study to investigate the state of oxidative stress in organisms of treated and untreated chicks. This metabolic indicator may give essential information on potential toxicity of extracts or virtues that rosemary powder combined with chitin can play. The obtained results (Table 6) show that catalase activity of the studied batches increases as a function of time. Such an effect is much higher in the chicks having received TB2 feed. whose catalase activity has a value of 4.06 µmoL, followed by batch of the chicks having consumed the formula TB1 feed (2.79). On the other hand, the control batches (CBWS and CBSA) have a catalase activity of 0.97 and 0.97 µmoL, respectively (Table 6). These results allowed us to emphasize that formulation incorporation at doses of 20 and 50 g/kg in chicks leads to an increase in catalase activity when compared to the controls.

Table 6. Plasma catalase activity (mean values ± standard deviations) of the studied chicks

Day	Cat CBWS	Cat CBSA	Cat TB1	Cat TB2
2	0.08 ± 0.01	0.09 ± 0.01	1.49 ± 0.02	3.85 ± 0.03
5	1.56 ± 0.02	1.57 ± 0.02	2.92 ± 0.01	3.97 ± 0.02
6	1.26 ± 0.03	1.25 ± 0.06	3.97 ± 0.11	4.34 ± 0.05

Note: Statistical significance: significant at $p<0.05$; Cat CBWS: Catalase activity of control group fed without supplementation; Cat CBSA: Catalase activity of the control batch supplemented with antibiotic; Cat TB1: Catalase activity of batch fed with basal diel containing 20 g/kg of the formulation; and Cat TB2: Catalase activity of batch treated with 50 g/kg of the formulated powder preparation.

Discussion

According to Lemos *et al*. (2015), the contents of phenolic compounds varies considerably depending on rosemary harvest period. However, its maceration in ethanol for seven days results in a quantity of phenolic compounds comprising between 75.2 and 99 mg GAL/g of dry extract. These values are lower than those obtained with a content of 114.93 mg GAL/g dry-extract and is closer to Uruguayan rosemary which contains 52.9 mg GAL/g dry-extract of phenolic compounds in the hexane extract and 123.9 mg/g dry-extract in the 75% ethanol dry-extract (Vieitez *et al*., 2018).

Because of the antibiotic's restriction in poultry feeds, scientists are exploring new feed additives to improve the poultry growth and create innovations for producers. Natural extracts of plants show immense potential as natural alternatives instead of chemical additives (Valenzuela-Grijalva *et al*., 2017). Rosemary is a medicinal plant that has been widely used in traditional medicine, perfumery and food industries (Yao *et al*., 2023). Its extract is mainly composed of flavonoids, di-, triterpenoids, monoterpenes, sesquiterpenes, alcohols, among others (Ribeiro-Santos *et al*., 2015). The present research effort demonstrates that feeding a diet enriched with rosemary and chitin increased live weight and weight gain, while, simultaneously, decreasing feed conversion. These results could be attributed to the growth stimulating effect of certain ingredients in the proposed mixture. This hypothesis agrees with previous studies (Saleh *et al*., 2018), which reported an increase in body weight, weight gain and organ weight in birds fed a diet containing a mixture of grasses (Abbas *et al*., 2021).

Puvača *et al*. (2022) showed that adding rosemary at 0.5, 1.0 and 2.0% (w/w) to broiler chicken diets increased weight gain – which indicated that rosemary had the potential to promote growth in poultry as a feed additive. Yesilbag *et al.* (2011) noted that zootechnical performance of chicks was not affected by dietary supplementation with EO obtained from rosemary. Additionally, Yildirim *et al*. (2018) demonstrated inconsistent results showing that adding 100 and 200 mg/kg of rosemary ethanol extract to the diet decreased the body weight and feed intake in broiler chickens from 1 to 42 days old. This could be due to differences in rosemary processing methodologies.

The qualitative and quantitative analyses of the bioactive compounds extracted from rosemary are deposited on the choice of the appropriate treatment. Factors such as plant conditions, plant portions, extraction methods and doses of actives can affect the final results (Azmir *et al*., 2013). Ethanol extract and rosemary EO did not receive enough bioactive phytochemicals compared to rosemary powder for growth effects. The present study shows results similar to the previous one, namely that feeding with rosemary-chitin has a positive effect on the growth performance when compared to feeding with antibiotics (*viz*., Flavomycin). The reason for this difference may be related to the presence of metals (Ca, K, Na, P, As, Cd, Cr, Mg) in the investigated samples. A treatment with 50 g/kg of formulation is more favorable for the start growth phase of chicken rearing. However, a treatment with 20 g/kg of the formulated product is more suitable for restarting the growth phase of rearing. This clearly shows that the use of the formulated products enhanced chicks' immunity of treated batches (TB1 and TB2).

The feed conversion index shows the positive effect of the developed formulation for improvement of poultry feed quality. These results in a better assimilation of all poultry feed components widely cited by certain authors (Yesilbag *et al*., 2011). Salehi *et al*. (2018) showed that rosemary EO and phenolic compounds can improve food digestibility in digestive tract. Additionally, Tariq *et al.* (2019) pointed out that two compounds of rosemary EO (1,8-cineole and α-pinene) limit pathogenic bacteria development in chicken gut.

Nevertheless, other studies have reported that live weight, average daily gain, amount of food ingested and feed conversion were not improved following the addition of supplements (Gadde *et al*., 2017). It should be emphasized that the disadvantage of using pure EO in the diet is that they cause a disturbance of microbiological composition or even a loss of a large part of the non-pathogenic bacteria of intestinal microbiota. Hence, the interest of the present formulation, which undeniably constitutes a natural reservoir which releases active ingredients (EO and phenolic compounds) at a low rate in poultry feed matrix.

Thus, the incorporation of 20 and 50 g/kg of rosemary-chitin in poultry feed leads to a decrease in serum protein levels due to infections and a reduction in toxicity (Abd El-Hack *et al*., 2022). The integration of chitin in poultry feed formulation reduces EO toxicity. Indeed, the use of the rosemary-chitin formula in broiler diets causes an increase in catalase activity when compared to controls. This incorporation seems to have an antioxidant effect in the treated batches. This advantageous metabolic activity is particularly important in individuals treated with a dose of 50 g/kg of the formulation compared to those treated with 20 g/kg.

Induction of catalase activity by the formulation improves meat quality. This is explained by the plant used rich in phenolic compounds (flavonoids) which inhibit the formation of free radicals, as it was pointed out by previous studies (Yesilbag *et al*., 2011). The antioxidant effect observed is based on its ability to inactivate the free radicals produced during auto-oxidation reaction (Babovic et al., 2010). Moreover, the antioxidant activity was also an important indicator reflecting bird's status health. The appearance of oxidative stress in birds reared in an intensive system disturbs cellular redox balance. This triggers an adaptive physiological response to redistribute metabolic energy that impacts the growth (Lauridsen, 2019).

The proposed feed improves chicken's weight yield by reducing feed conversion index and increasing cellular antioxidant activity during the two growth stages of broiler rearing cycle (start and of growth phase).

In the present study EO levels were of the order of 238 mg/kg of food in the first treatment (TBE1), and 594 mg/kg of food in the second treatment (TBE2). Di *et al*. (2022) noted that dietary supplementation of 1,8-cineole at 10 to 40 mg/kg improves the growth performance of broilers by strengthening antioxidant

capacity, immunity and intestinal morphology. It should be highlighted that 1,8-cineole, a major compound in rosemary, exhibits a range of biological properties, including anti-inflammatory, antioxidant, antimicrobial, bronchodilatory, analgesic and pro-apoptotic (Hoch *et al*., 2023). The authors have highlighted the benefits of this molecule for human health, as demonstrated in clinical trials involving patients suffering from respiratory disorders, including chronic obstructive pulmonary disease, asthma, bronchitis, and rhinosinusitis. A comprehensive understanding of the pharmacodynamics and safety aspects of 1,8-cineole, as well as the development of effective formulations, could help exploit its therapeutic value. This work opens avenues for future research into the various human and animal health benefits and potential uses of 1,8-cineole in combating therapeutic conditions. In recent decades, the poultry industry has leaned toward the use of plant-based feed supplements and their derivatives to improve their health and productivity. These benefits on the health of the host are mainly attributed to the secondary metabolites of plants, namely polyphenols, which are known for their antioxidant, immunomodulatory, antimutagenic and anti-inflammatory properties (Abdel-Moneim *et al*., 2020).

Conclusions

Overall, it can be concluded from this study that aromatic and medicinal plants are of great interest in broiler breeding. The formulation used in the present work showed a beneficial effect on the growth of chicks (in both growth and growth initiation phases). The proposed formulations (rosemary and chitin) seem to be a proper substitute for antibiotics widely used in poultry farming and which are responsible for the global emergence of infectious diseases due to bacterial multidrug resistance. Results of the present research study showed that the incorporation of rosemary powder and chitin in the diet of broiler chickens can be used as a natural alternative solution to avoid antibiotics use and as promoters or animal growth.

Since the use of antibiotics in poultry is known for its negative effects such as antimicrobial resistance, destruction of beneficial bacteria in the gut and imbalance of the microbiota, alternative solutions are urgently needed and are highly demanded. Indeed, aromatic, and medicinal plants are known to be a good source of natural preservatives that can act for the activation of the immune system of animals and elimination of pathogens. This study therefore opens avenues through an innovative approach for the valorisation of agri-food by-products and natural resources, and sustainable management in chick farming.

Authors' Contributions

Conceptualization: F.E. and N.C.; Methodology: L.E.G. and S.M.R.; Validation: N.C. and A.Z.; Investigation: F.S.K. and S.M.R.; Resources: F.S.K. and N.C.; Writing—original draft preparation: F.S.K.; Writing—review and editing: A.Z. and J.M.R.; Supervision: F.E. and S.M.R. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

The animal study protocol was approved by the Institutional Review Board (or Ethics Committee) of the Fes Hospital-University Ethics Committee (CEHUF) protocol (code 001, approval date 2020) for studies involving animals.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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