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Efficacy and safety of peppermint extract (Mentha Piperita), probiotics, and the co-administration of peppermint plus probiotics in preventing ascites syndrome: A clinical animal study on male broilers

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ABSTRACT

Ascites syndrome is a serious and common condition in broilers. Therefore, we aimed to assess the preventive efficacy and safety of peppermint extract, probiotics, and their combination on ascitic broilers versus normal ones. In this clinical animal study, 168 male one-day-old broilers of the Ross 308 strain with similar weights were bred and vaccinated until day 20. On day 21, they were weighed and randomized into 8 groups of 21 broilers each: the negative control (no ascites and no treatments), peppermint extract administration, probiotics consumption, peppermint extract and probiotics co-administration, induced ascites (the positive control), induced ascites + peppermint extract, induced ascites + probiotics, and induced ascites + the co-administration of peppermint and probiotics. Until day 42, medications (levothyroxine, peppermint extract, or probiotics) were applied. On day 42, 10 broilers were randomly selected from each group (n=80), weighed, and blood-sampled. Data were analyzed at significant level 0.05 (α=0.05). Peppermint extract and probiotic administration alone were relatively safe. However, the combination of peppermint extract and probiotics might have deleterious effects in healthy broilers in terms of most of the parameters (AST/ALT/ALP/T3/T4/glucose/ cholesterol/triglycerides/total protein/RBC, p<0.05). In ascitic broilers, peppermint extract almost always had a positive therapeutic effect, either improving the parameters of the control levels

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or improving some levels between the control and diseased (all parameters except ALP and RBC). Probiotics had several such positive effects as well (in the case of AST, ALT, T3, T4, glucose, BUN, uric acid, total protein, albumin, globulin, hematocrit, weight gain), although mostly not as strong as those of peppermint. The co-administration of peppermint extract and probiotics caused slight improvements only for 2 parameters (uric acid and ALT) in ascitic broilers. Peppermint extract followed by probiotics (but not their combination) were safe and effective in preventing the ascites syndrome. The use of their combination should be avoided.

Keywords: *Peppermint Extract, Probiotics, Broilers, Ascites Syndrome, Herbal Medicine, Blood Parameters, Weight Gain.*

1 Introduction

Ascites syndrome is a pathological surge in non-inflammatory transudative liquid in the abdomen spaces of poultry (1). The buildup of this transudate usually occurs around the liver, particularly the abdominal portion of the hepatic-peritoneal space and the pericardial space (2-4). Ascites syndrome is a grave issue in the poultry industry worldwide, resulting in severe financial losses (5-7). This syndrome typically affects young and growing chickens and is considered a significant cause of death in chickens (5-7).

The most significant etiology for the progress of ascites syndrome is hypoxia (1, 4, 6). Some researchers suggest that meat breeding with low feed conversion ratios may reduce thyroid hormone activity and cause hypothyroidism, which in turn may reduce oxygen consumption and cause hypoxia (8-11).

Successful genetic selection in recent years has led to the upbringing of new broiler strains that are ready for the market much earlier than before. Such programs for genetic selection have performed very well in selecting superior strains of broilers. Nevertheless, they have yet to be equally fruitful with the growth rates of organs, especially those related to the cardiovascular system (12-15). Enhancing the growth rate requires increasing blood volume to transport nutrients to organs and excrete metabolites (8, 15, 16). As a result, this upbringing has enhanced the muscle growth of chickens without increasing their lungs' capacity; the lungs and heart of these chickens are not much improved. The resulting disproportion predisposes the animals to ascites (8, 12-16).

Due to the ban or restrictions on the use of antibiotics as food additives for animals and poultry, additives such as probiotics and probiotics as alternative substances have gained popularity with the livestock and especially the poultry industries (17-19). Furthermore, it might be possible to increase poultry production efficiency with the help of such additives (17-21).

Medicinal plants and their extracts are essential nutritional support. Besides antimicrobial properties, medicinal plants have other benefits, such as lowering serum lipids, boosting the immune system, and improving numerous blood factors (22-27). Herbal medicines are beneficial in terms of the simplicity of their use, the absence of adverse effects on livestock and poultry function, and the lack of harmful residues in products and the environment (22-26).

Phytogenic nutritional supplements are plant-based materials added to the food to improve its characteristics, increase livestock/poultry production, and improve quality (28-30). Probiotics are living microorganisms incorporated into livestock/poultry diets; they positively affect the function and health of poultry (23, 29, 31, 32). Probiotics have gained attention due to advantages like the lack of residues in the body and also benefiting poultry production (23, 29, 31, 32).

Research on the effects of medicinal plants and probiotics is quite active, introducing and testing many new materials (17-26, 29-33). Many results indicate a lack of harm and/or the presence of beneficial effects. For example, some studies found no substantial detrimental effects of probiotics on chickens' health or their hemoglobin levels, hematocrit percentages, leucocytes or erythrocytes, and erythrocyte index of plasma. In contrast, some others even favored probiotics (19, 27, 34-37). Moreover, it has been shown that phenolic substances of herbal medicines (including peppermint or Mentha Piperita) may boost the release of endogenous digestive enzymes and diminish the pathogenic bacteria or modify gut morphology because of their antioxidant and anti-inflammatory properties (21, 38-40).

Given the importance of ascites syndrome, it is crucial to find and test new, safe, economical, and effective medicinal

plants or probiotics that can prevent or treat it. In this regard, some methods are proposed to prevent ascites. One of these is probiotic consumption because, as stated above, probiotics may have favorable effects on various aspects of health, including gut development and ascites prevention (19, 27, 34-37). Nevertheless, studies on the efficacy of probiotics in preventing ascites are scarce and controversial. Therefore, more research is warranted.

Moreover, another potential therapeutic material in this regard can be peppermint and its extract because, as stated above, its phenolic compounds may have various beneficial effects that might reduce or prevent ascites syndrome (39, 40). However, the preventive effects of peppermint extract on ascites syndrome have not been assessed yet in broilers, other animals, or humans, except in a recent study (40).

Another potential therapeutic method deserving examination is the co-administration of peppermint products and probiotics. It is interesting to know if the combination of these two methods, each being famous for some potential therapeutic effects (at least in fields other than the ascites syndrome), can have synergistic effects. Nonetheless, to the best of our knowledge, no study to date has ever assessed such a topic on the ascites syndrome or any other disease. This is why we also included this third potentially therapeutic method in our interventions to examine its efficacy in preventing ascites syndrome. However, another crucial item to assess is the safety of potential treatments; the critical question is whether consuming these materials or their combination would be hazardous in healthy animals without ascites syndrome. This latter question sheds light on the practicality of these treatments as potential preventive medications to be consumed by broilers who have not necessarily developed ascites syndrome.

Due to the lack of any such studies on any animals (or humans, as detailed above) as well as the importance of the matter, the aim of this study was to assess, for the first time, the safety and potential preventive effects of peppermint extract in comparison with probiotics (Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium bifidum, Enterococcus faecium) and the combination of peppermint extract together with probiotics on broilers with experimental ascites induced by levothyroxine administration, in comparison with healthy broilers.

2 Materials and methods

This was an experimental animal study on male broilers. The animals were treated in accordance with the ethical guidelines of the Animal Care and Use Committee (ACUC). The study protocol was approved by the Research Council of Darab Branch, Islamic Azad University, Darab, Iran (thesis number: 16850102951001).

2.1 Peppermint identification and extraction

A total of 10 Kg of peppermint was collected from Darab, Iran (28°45′07″N 54°32′40″E at an elevation of 1180 meters above sea level) and evaluated at the botanical laboratory of the Agricultural Research and Natural Resources Center; the same center confirmed the plant as peppermint (L. Mentha piperita; common name: Black Mint, Lamb-Mint; plant family: Labiatae). These plants were collected from nature with the permission of the Agricultural Research and Natural Resources Center of Fars Province (a governmental institute) under local legislation.

The leaves were dried under standard conditions in the shadow, and the samples were used for extraction in the laboratory. The extraction steps were: 1. Grinding. 2. Pouring the plant powder into labeled glass containers. 3. Adding 96% methanol ten times the volume of the plant. 4. Stir the mixture with a spoon so the plant is well soaked in alcohol and immersed. 5. Storing the mixture for 24 hours at 20 °C to dissolve the active ingredients of the plant. 6. Sift the plant and transfer the extract to an Erlenmeyer flask. 7. Concentrating the extract by a rotary device. 8. The final concentration at laboratory temperature for 48 hours.

The concentration of the peppermint extract was adjusted to 150 ppm. The chemical analysis of peppermint extract was out of the scope of this *clinical* animal study, which was designed to assess the safety and efficacy of peppermint against ascites syndrome. However, previous studies have already examined and elaborated on the various compounds within peppermint powder and its alcoholic extracts (40-42). The materials available in 100 g peppermint powder are previously documented (41). Moreover, the chromatographic report of peppermint powder has been reported previously (40). The substances within peppermint extract were not within the scope of this clinical animal study on the efficacy and safety of peppermint and probiotics and, therefore, were not examined again.

2.2 Settings and the sample

This experiment was performed in a broiler farm with a capacity of 20,000 chickens, with exploitation license and health license, at a longitude of 33118, a latitude of 252371, and an altitude of 1580 meters above sea level, and with a

water hardness of 3000 EC. The intervention was done in a poultry house measuring 17 × 65 meters. There were 8 groups in this study; therefore, 8 rectangular compartments (with three replications, 24 compartments) with 1 × 1 mm2 dimensions were created using cement blocks and chicken nets. In order to get used to the breeding environment and also to have casualties in the first days, 21 male broilers were entered in each group (7 broilers per compartment, 3 compartments per group, 8 groups).

2.3 Breeding

At the beginning of the breeding period, in each of the 24 compartments, 7 male one-day-old broilers with similar weights were randomly selected from the central part of the poultry farm and released into the compartments. The birds were weighed. Overall, 168 male one-day-old broilers of Ross 308 strain with an average weight of 47.3 grams entered the study. The animals were obtained commercially from Behparvar company, Iran (license number: 9669/15). Each group had a 4-liter water bowl and a separate eating tray. No casualties were seen in any of the groups.

Room preparation and disinfection were done 10 days before the intervention (i.e., on the 11th day of chickens' life) in the following order: 1. Washing all food utensils, water utensils, and equipment with water, detergent, and brush outside the room. 2. Wash the floor and walls of the hall up to a height of 1.5 meters with water, detergent, and brush. 3. Sealing the hall's walls, ceiling, doors, and windows. 4. Transfer and installation of equipment back in the hall. 5. Throwing straw. 6. Formalex application.

All breeding stages (including feeding principles [amount and frequency], light regime, density, room temperature, and ventilation) were performed according to the breeding instructions of Ross broilers. The breeding unit maintained complete hygiene control During the breeding period. In front of the hall door, the Bactergent disinfectant solution was used. The vaccination schedule was performed. All vaccines were potable. When the broilers were 3, 7, 10, 14, 17, and 20 days old, they were given vaccines for bronchitis, Newcastle b1, Gumboro, bronchitis, Newcastle, and Gumboro (Lasota), respectively. For vaccination, the birds were kept thirsty for 2 hours before vaccination. After vaccination, a water-soluble multivitamin was used for 24 hours to prevent vaccine-induced stress. All health steps were performed under the supervision of the farm veterinarian. No broilers were lost during these 20 days of breeding.

2.4 Ascites induction and therapeutic Interventions

On the 21st day of broilers' growth, all of them were weighed using a digital scale after 7 hours of fasting (to empty their gastrointestinal tract).

2.4.1 Grouping

All of them were randomly assigned to one of the eight interventions, regardless of their previous fences and compartments; hence, the previous order of groupings was completely changed after the randomization. After the randomization, the compartments were re-filled with 21 randomly assigned broilers each. Each of these compartments (21 animals) underwent one of the 8 interventions, which were: (1) the negative control (no ascites and no treatments), (2) peppermint extract administration, (3) probiotics consumption, (4) peppermint extract and probiotics co-administration, (5) induced ascites but without any treatments - this group acted as the positive control, (6) induced ascites + peppermint extract consumption only, (7) induced ascites + probiotics consumption only, and (8) induced ascites + the co-administration of peppermint and probiotics. Each group included 21 broilers (n = 8 × 21 = 168).

2.4.2 Interventions

From the 21st day until the 42nd (and last) day of the project, the medications were given to the allocated broilers daily by dissolving them in drinking water. To dissolve the peppermint extract, first, the extract was dissolved in an equal volume of ethanol and then dissolved in the birds' drinking water. The concentration of the used peppermint extract was 150 ppm.

On the 21st day of the project onward, probiotics "Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium bifidum and Enterococcus faecium" for $1x10^8$ CFU/g within water-soluble dextrose (Lactofeed, Takgene Zist Co., Tehran, Iran) at a concentration of 5 g per 100 liters of drinking water were administered daily by mixing the probiotics in the water.

The extracts and probiotics were given daily until the last (42nd) day. On the same 21st day of the project, half the specimens (4 groups out of 8, as detailed above) were inducted with ascites with levothyroxine.

The dose of levothyroxine for the induction of ascites was 1.5 g/Kg feed daily from the 21st day until the 42nd day. This method of ascites induction by increasing the activity of the thyroid gland is an accepted standard method used in the

literature (43). The development of ascites syndrome after receiving levothyroxine was established in the positive control group by examining and confirming the enlargement of the heart and fluid accumulation in the peritoneum (44).

By the end of the study period (42 days), none of the groups had any casualties, and there was no dropout (the sample size remained 168 broilers).

The chickens in all groups except the negative control were euthanized because they could not be consumed as food. The euthanasia method was manual cervical dislocation, which was chosen because it is recommended for sudden death and minimal suffering (45). The consumable chickens were slaughtered as food.

2.5 Outcomes

2.5.1 Blood parameters

On the 42nd day of broilers' growth, 10 of them were randomly selected from each group of 21 chickens and tested (n = 80). Blood sampling was performed: Approximately 8 ml of blood was taken from each chicken and poured into two test tubes, one containing anticoagulant (2.5 ml) and the other a simple test tube (about 6 ml). The blood tubes were coded, centrifuged (3000 rounds/min), and then kept in a freezer (-20 °C) until blood analysis. Blood samples were analyzed using spectrophotometry (Technicon RA-1000, Bayer, Leverkusen, Germany).

The blood parameters analyzed included: triglycerides, cholesterol, hematocrit, hemoglobin, thyroid hormones T4 and T3, blood glucose, uric acid, blood urea nitrogen (BUN), serum albumin concentration, serum globulin concentration, and bilirubin. Also, liver function enzymes alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), and total protein were measured.

2.5.2 Weight gain

After blood sampling, 7 hours of fasting were considered to empty the broilers' gastrointestinal tract before weighing them. The birds were then weighed using a digital scale with an accuracy of ± 5 g. Weight gain was calculated for each chicken by subtracting the weight on the 21st day from the weight on the 42nd day.

2.6 Statistical analysis

Descriptive statistics (means and standard deviations [SDs]) were computed for each of the 16 variables within each of the 8 groups. The normality of data was checked and passed using the One-Sample Kolmogorov-Smirnov test. The groups were compared with each other using a factorial two-way analysis of variance (ANOVA) accounting for the presence or absence of the experimental ascites induction as well the 4 treatments (control, peppermint, probiotic, peppermint + probiotic), and the interaction of these two factors, a one-way ANOVA, and a Tukey Post Hoc test following the one-way ANOVA. The software used was SPSS 16 (SPSS, Chicago, IL, USA). The level of significance was set at 0.05.

3 Results

There was all the data. In all the following 16 variables, the one-way ANOVA became significant (detailed in Tables 1 to 4).

3.1 AST

The effects of ascites development ($p<0.001$), the difference across the 4 treatments ($p<0.001$), and the interaction of these variables ($p<0.001$, two-way ANOVA) were significant. The AST level was significantly higher in the positive control (ascites) compared to the negative control group (Tukey, $p<0.05$, Table 1). The post hoc test showed that groups 4, 5, 7, and 8 had significantly higher AST levels than the negative control (Tukey $p<0.05$). On the other hand, groups 2, 3, and 6 had AST levels similar to the negative control ($p>0.05$). Compared to group 5 (ascites, positive control), the peppermint extract (in group 6) had reduced AST back to the negative control levels; however, probiotics or the co-administration of probiotics and peppermint extract failed to do so (groups 7 and 8, Table 1, Figure 1). Still, probiotics alone improved ALT compared to the positive control, although not back to normal.

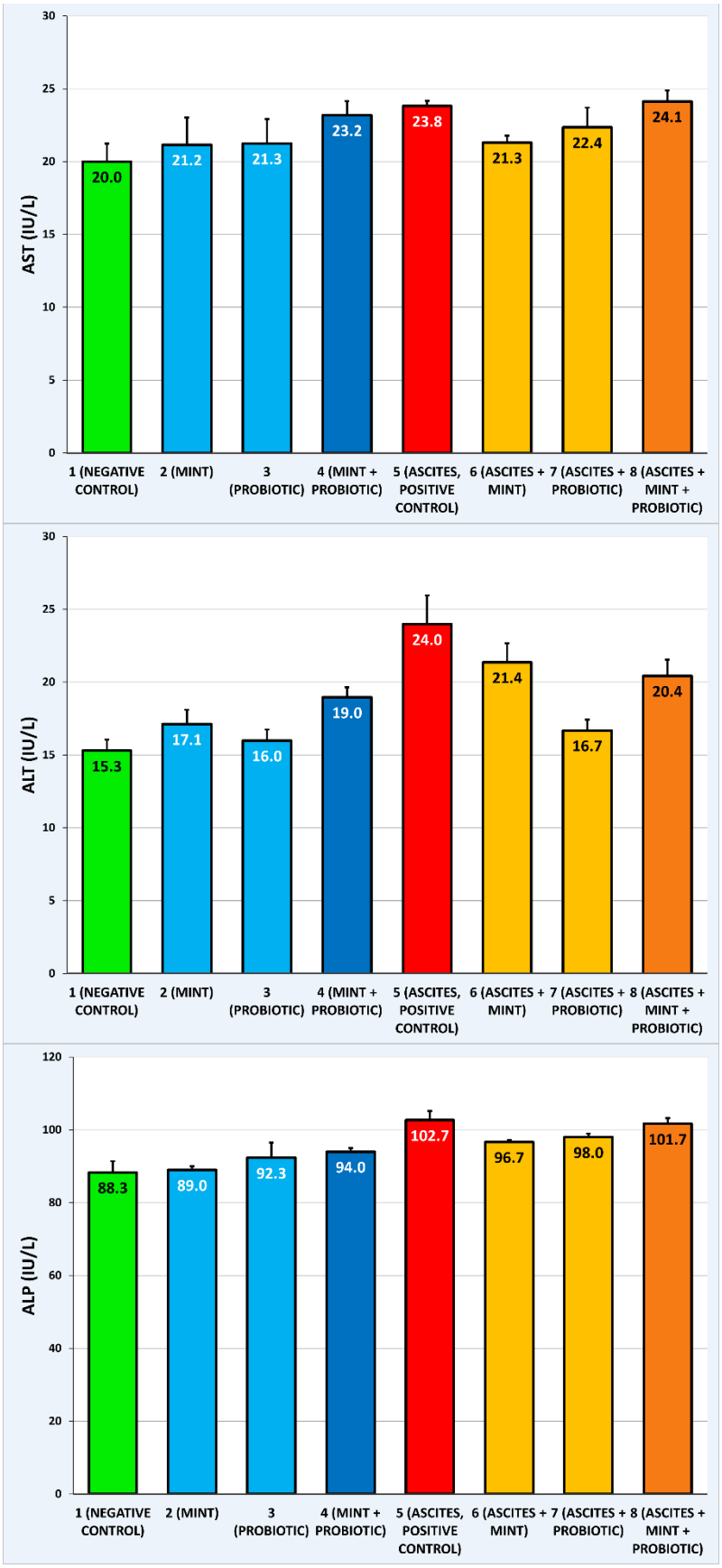
AST (IU/L)
30
25
20
15
10
5
0
20.0
21.2
21.3
23.2
23.8
21.3
22.4
24.1
1 (NEGATIVE CONTROL)
2 (MINT)
3 (PROBIOTIC)
4 (MINT + PROBIOTIC)
5 (ASCITES, POSITIVE CONTROL)
6 (ASCITES + MINT)
7 (ASCITES + PROBIOTIC)
8 (ASCITES + MINT + PROBIOTIC)
ALT (IU/L)
30
25
20
15
10
5
0
15.3
17.1
16.0
19.0
24.0
21.4
16.7
20.4
1 (NEGATIVE CONTROL)
2 (MINT)
3 (PROBIOTIC)
4 (MINT + PROBIOTIC)
5 (ASCITES, POSITIVE CONTROL)
6 (ASCITES + MINT)
7 (ASCITES + PROBIOTIC)
8 (ASCITES + MINT + PROBIOTIC)
ALP (IU/L)
120
100
80
60
40
20
0
88.3
89.0
92.3
94.0
102.7
96.7
98.0
101.7
1 (NEGATIVE CONTROL)
2 (MINT)
3 (PROBIOTIC)
4 (MINT + PROBIOTIC)
5 (ASCITES, POSITIVE CONTROL)
6 (ASCITES + MINT)
7 (ASCITES + PROBIOTIC)
8 (ASCITES + MINT + PROBIOTIC)

Figure 1. Mean (±SD) blood levels of liver enzymes (IU/liter) in different groups. The sample size for each group is 10 broilers.

Table 1. Descriptive statistics for the blood concentrations of liver enzymes (all in IU/Liter) in different groups. The n (sample size) of each group is 10 broilers (total n = 8 groups × 10).

Group	AST		ALT		ALP	
	Mean	SD	Mean	SD	Mean	SD
1 (negative control)	20.00^{c}	1.23	15.29^{d}	0.76	88.33^{e}	3.06
2 (mint)	21.16^{c}	1.87	17.12^{c}	0.98	89.00^{de}	1.00
3 (probiotics)	21.25^{c}	1.67	15.99^{d}	0.76	92.33^{cde}	4.16
4 (mint + probiotics)	23.19^{b}	0.96	18.96^{bc}	0.68	94.00^{bc}	1.00
5 (ascites, positive control)	23.82^{a}	0.35	23.98^{a}	1.97	102.67^{a}	2.52
6 (ascites + peppermint)	21.31^{c}	0.47	21.36^{b}	1.31	96.67^{ab}	0.58
7 (ascites + probiotics)	22.35^{b}	1.35	16.69^{d}	0.74	98.00^{abc}	1.00
8 (ascites + peppermint + probiotics)	24.13^{a}	0.76	20.43^{b}	1.12	101.67^{abc}	1.53
One-way ANOVA's F	6.451		21.187		17.970	
One-way ANOVA's *P*	<0.001		<0.001		<0.001	

The superscript alphabets mark the non-significant pairwise comparisons (Tukey, $p>0.05$). SD: standard deviation.

3.2 ALT

The variables ascites induction ($p<0.001$), intervention ($p<0.001$), and their interaction ($p<0.001$, two-way ANOVA) became significant. The ALT level was significantly higher in the positive control (ascites) compared to the negative control group (Tukey, $p<0.05$, Table 1). Mint and probiotics did not significantly increase ALT compared to the negative control, but their combination did (Table 1). Compared to group 5 (ascites, positive control), probiotics (in group 7) had reduced ALT back to the negative control levels (healthy broilers); however, peppermint alone (group 6) or the co-administration of probiotics + peppermint extract (group 8) failed to improve ALT back to the negative control level (groups 7 and 8, Table 1, Figure 1). Still, in these groups 6 and 8, ALT slightly improved compared to the positive control ($p<0.05$).

3.3 ALP

The roles of ascites stimulation ($p<0.001$), interventions ($p<0.001$), and their interaction ($p<0.001$, two-way ANOVA) were significant. The ALP level was significantly higher in the positive control (ascites) compared to the negative control group (Tukey, $p<0.05$, Table 1). Mint and probiotics did not significantly increase ALP compared to the negative control, but their combination did (Table 1). None of the treatments could significantly reduce ALP levels compared to group 5 (ascites); still, the best one among the three was the addition of peppermint, and the worst ALP was seen in the mint + probiotics group (group 8, Table 1, Figure 1).

3.4 T3

Ascites induction ($p=0.006$), treatments ($p=0.001$), and their interaction ($p=0.004$, two-way ANOVA) significantly affected T3. The post hoc test showed that the ascites and peppermint + probiotics (5 and 4) groups significantly increased T3 compared to the negative control. Mint consumption did not significantly alter T3. In ascitic broilers, each of the treatments 'meant or probiotics' reduced T3 significantly (but only to above-normal levels) to similar extents. However, their combination failed to reduce T3 (Table 2, Figure 2).

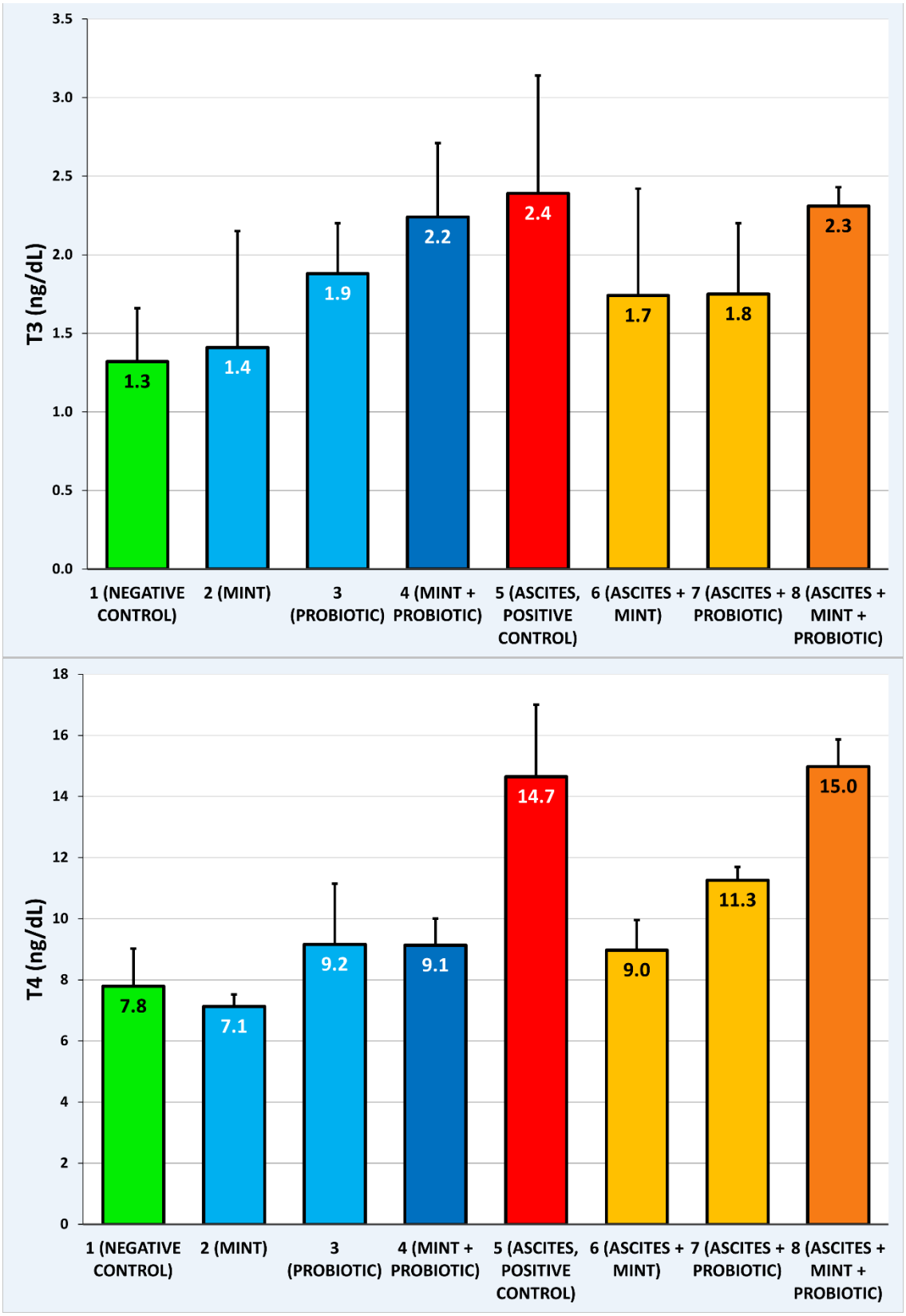

Figure 2. Mean (±SD) blood levels of thyroid enzymes (ng/dL) in different groups (total n = 8 × 10 broilers).

Table 2. Descriptive statistics for the level of thyroid enzymes (both in ng/dL) in different groups. The n of each row is 10 broilers (total n = 8 × 10). The P value is calculated using the one-way ANOVA.

Group	T4		T3	
	Mean	SD	Mean	SD
1 (negative control)	7.79^{d}	1.23	1.32^{c}	0.34
2 (mint)	7.13^{d}	0.39	1.41^{c}	0.74
3 (probiotics)	9.16^{c}	1.98	1.88^{b}	0.32
4 (mint + probiotics)	9.13^{c}	0.87	2.24^{ab}	0.47
5 (ascites, positive control)	14.65^{a}	2.35	2.39^{a}	0.75
6 (ascites + peppermint)	8.97^{bc}	0.98	1.74^{b}	0.68
7 (ascites + probiotics)	11.26^{b}	0.43	1.75^{b}	0.45
8 (ascites + peppermint + probiotics)	14.98^{a}	0.89	2.31^{a}	0.12
One-way ANOVA's F	29.124		6.322	
One-way ANOVA's *P*	<0.001		0.012	

The superscript alphabets mark the non-significant pairwise comparisons (Tukey, $p>0.05$). SD: standard deviation.

3.5 T4

Blood T4 was altered by ascites development ($p<0.001$), treatments ($p<0.001$), and their interaction ($p<0.001$, two-way ANOVA). Mint alone did not increase T4 compared to the control. However, probiotics and the co-administration of peppermint + probiotics increase T4 significantly (Tukey, Table 2, Figure 2). The induction of ascites also significantly increased T4 compared to the negative control (Table 2, Figure 2). In the cases with ascites, peppermint significantly reduced the level of T4 to almost normal levels though above normal; probiotics also reduced T4 but not as much as mint; the combination of mint + probiotics failed to reduce T4 (Table 2, Figure 2).

3.6 Glucose

Blood glucose was significantly under the influence of ascites generation ($p<0.001$), intervention ($p<0.001$), and their interaction ($p<0.001$, two-way ANOVA). Compared to the negative control, groups 4 and 5 increased their blood glucose levels (Tukey, Table 3, Figure 3). However, peppermint or probiotics alone did not increase blood glucose significantly. Compared to the positive control group, mint reduced the glucose level significantly (although not to the normal levels). Probiotic administration reduced blood glucose levels, not as much, but still statistically significantly. Their combination, however, failed to reduce the elevated blood glucose (Table 3, Figure 3).

3.7 Cholesterol

The role of ascites induction ($p<0.001$), treatments ($p<0.001$), and their interaction ($p=0.007$, two-way ANOVA) was significant. Compared to group 1, groups 4 and 5 they increased their blood cholesterol levels (Tukey, Table 3, Figure 3). However, peppermint or probiotics alone did not increase blood cholesterol significantly. Compared to group 5 (positive control), none of the groups significantly changed cholesterol levels; however, the decrease in group 6 (ascites + peppermint) was such that the cholesterol level reached normal levels (while still being insignificantly different from group 5). In groups 7 and 8, mean cholesterol levels had increased.

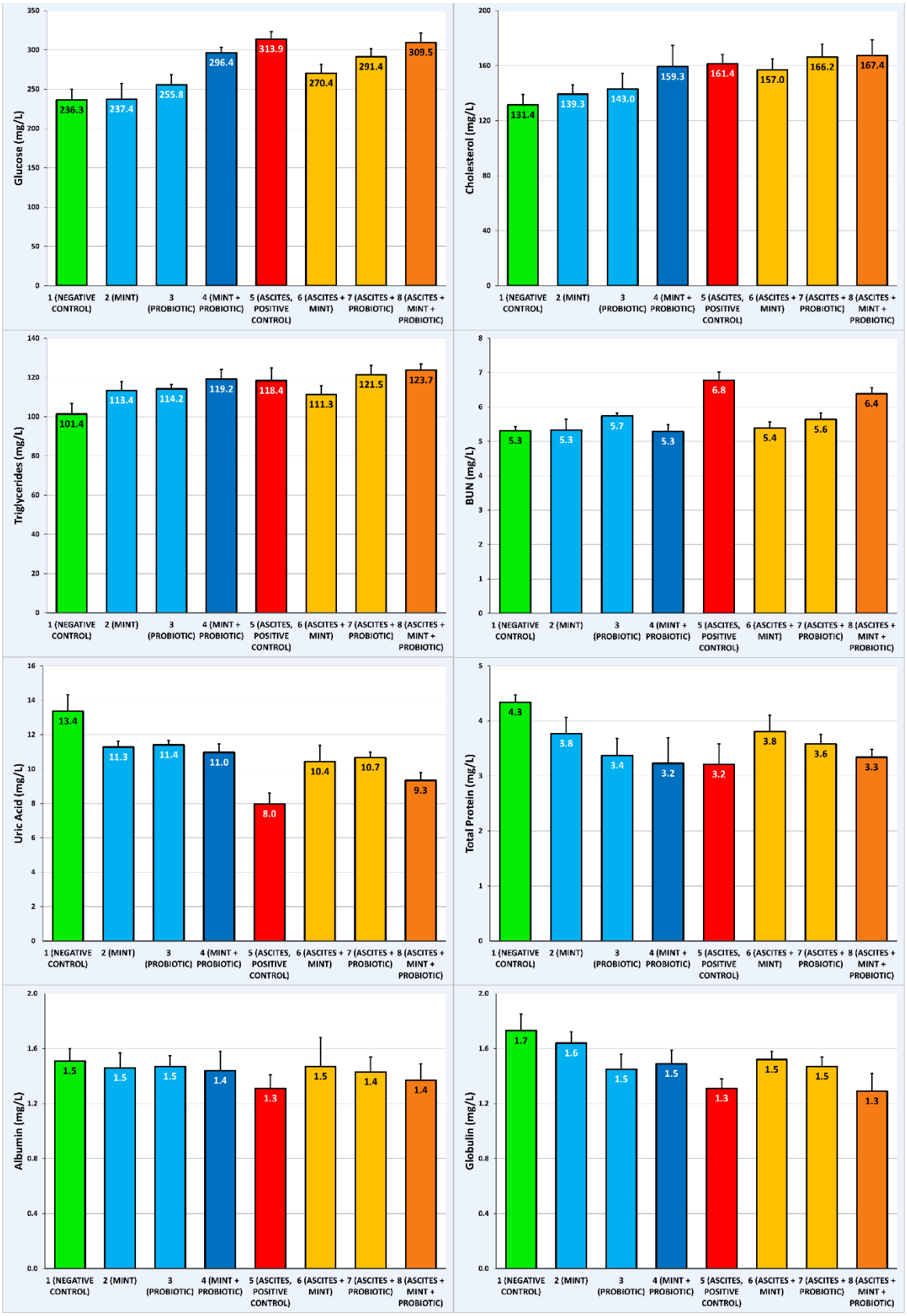

Figure 3. Mean (±SD) blood parameters (mg/dL) in different groups (total n = 8 × 10 broilers).

Table 3. Descriptive statistics for the level of blood parameters (all in mg/dL) in different groups. N of each row is 10 broilers (total n = 8 × 10). The P value is calculated using the one-way ANOVA.

G	Glucose		Cholesterol		Triglycerides		BUN		Uric Acid		Total Protein		Albumin		Globulin	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	236.29^{d}	13.67	131.42^{b}	7.54	101.38^{b}	5.45	5.31^{b}	0.12	13.36^{a}	0.98	4.34^{a}	0.13	1.51^{a}	0.09	1.73^{a}	0.12
2	237.42^{d}	19.75	139.33^{ab}	6.75	113.43^{ab}	4.54	5.33^{b}	0.31	11.28^{ab}	0.34	3.77^{ab}	0.29	1.46^{ab}	0.11	1.64^{a}	0.08
3	255.78^{cd}	12.43	142.97^{ab}	11.27	114.21^{ab}	2.18	5.74^{b}	0.09	11.41^{ab}	0.26	3.37^{ab}	0.31	1.47^{ab}	0.08	1.45^{ab}	0.11
4	296.41^{b}	6.74	159.28^{a}	15.35	119.17^{a}	4.87	5.29^{b}	0.19	10.97^{ab}	0.48	3.23^{b}	0.46	1.44^{ab}	0.14	1.49^{ab}	0.10
5	313.87^{a}	9.75	161.37^{a}	6.74	118.42^{a}	6.48	6.78^{a}	0.23	7.97^{b}	0.65	3.21^{b}	0.37	1.31^{b}	0.10	1.31^{b}	0.07
6	270.36^{c}	11.16	156.97^{ab}	7.87	111.33^{ab}	4.56	5.39^{b}	0.17	10.43^{ab}	0.96	3.81^{ab}	0.29	1.47^{ab}	0.21	1.52^{ab}	0.06
7	291.38^{b}	9.97	166.24^{a}	9.35	121.47^{a}	4.74	5.64^{b}	0.19	10.67^{ab}	0.32	3.58^{ab}	0.17	1.43^{ab}	0.11	1.47^{ab}	0.07
8	309.49^{a}	12.24	167.42^{a}	11.34	123.71^{a}	3.21	6.39^{a}	0.18	9.34^{ab}	0.45	3.34^{b}	0.14	1.37^{b}	0.12	1.29^{b}	0.13
F	18.545		12.321		13.121		9.741		3.124		4.134		3.565		3.121	
P	<0.001		<0.001		<0.001		<0.001		0.021		0.003		0.016		0.019	

The superscript alphabets mark the non-significant pairwise comparisons (Tukey, $p>0.05$). SD: standard deviation. G: groups.

3.8 Triglycerides

Ascites induction ($p<0.001$), intervention ($p<0.001$), and their interaction ($p<0.001$, two-way ANOVA) significantly affected triglycerides. Compared to group 1, groups 4 and 5 increased triglyceride levels (Table 3, Figure 3). However, groups 2 or 3 did not increase them significantly. Compared to group 5 (positive control), none of the groups significantly changed triglycerides levels; however, the decrease in group 6 (ascites + peppermint) was so much that the triglycerides level reached normal levels (while still being insignificantly different from group 5). In groups 7 and 8, mean triglyceride levels had increased.

3.9 BUN

BUN was significantly altered by ascites ($p<0.001$), treatments ($p<0.001$), and their interaction ($p<0.001$, two-way ANOVA). The following Post Hoc observations were made: None of the 3 treatments (groups 2 to 4) increased BUN significantly higher than normal (Table 3, Figure 3). Ascites induction significantly increased BUN. Compared to this group (#5), each of the treatments, mint or probiotics, was able to lower BUN levels back to normal. However, their combination was not (Tukey, Table 3, Figure 3).

3.10 Uric Acid

The impacts of ascites stimulation ($p<0.001$), interventions ($p<0.001$), and their interaction ($p<0.001$, two-way ANOVA) were significant. Compared to the control, groups 2 to 4 slightly reduced uric acid (Tukey, Table 3, Figure 3). The only group significantly reducing it was the positive control. All 3 treatments were able to increase uric acid levels somehow close to normal (while still being close to the ascites group, i.e., between normal and diseased levels, Table 3, Figure 3).

3.11 Total protein

The variables ascites (p=0.004), interventions (p<0.001), and their interaction (p<0.001, two-way ANOVA) became significant. The post hoc test indicated that the 3 treatments could lower the total protein compared to normal broilers; this became significant only in the case of the combination of peppermint and probiotics. The induction of ascites could significantly reduce total protein. The consumption of peppermint extract or probiotics could slightly improve it to levels between normal and diseased. However, mint + probiotics could not improve it (Tukey, Table 3, Figure 3).

3.12 Albumin

Albumin was significantly affected by ascites induction (p=0.010) but not interventions (p=0.353) or their interaction (p=0.064, two-way ANOVA). The treatments lowered the albumin concentrations, but none of those reductions became statistically significant (Tukey). Only in the ascites group was albumin significantly reduced. Consuming peppermint extract or probiotics improves albumin concentration and levels between normal and diseased. However, the combination of peppermint and probiotics could not improve it (Table 3, Figure 3).

3.13 Globulin

Ascites (p<0.001), interventions (p<0.001), and their interaction (p<0.001, two-way ANOVA) had significant effects. The Tukey test showed that peppermint did not significantly reduce globulin levels, but probiotics and the co-administration of mint and probiotics reduced it to levels between the negative and positive controls. The positive control had a significantly reduced globulin level compared to normal broilers. In ascitic birds, peppermint extract or probiotics could slightly improve globulin levels to something between normal and diseased levels. However, mint + probiotics could not improve it (Table 3, Figure 3).

3.14 RBC

RBC levels were affected by ascites production (p<0.001), treatments (p<0.001), and their interaction (p<0.001, two-way ANOVA). According to the Tukey test, probiotics and combining peppermint + probiotics significantly increase RBC compared to normal broilers. However, mint did not significantly alter RBC (Table 4, Figure 4). The maximum RBC was seen in the ascites (positive control) group. All the 3 treatments were able to reduce RBC to a level between normal broilers and the ascitic ones (mint being the most successful in this regard).

3.15 Hematocrit

The role of ascites (p<0.001), interventions (p<0.001), and their interaction (p<0.001, two-way ANOVA) became significant. Hematocrit was the highest in normal broilers. It was significantly reduced in the ascites group (Tukey). In groups 2 to 4, hematocrit reduced but not significantly. In ascitic broilers, peppermint or probiotics similarly increased hematocrit to something between the negative and positive control levels. However, their co-administration failed to do so (Table 4, Figure 4).

3.16 Weight gain

Ascites (p<0.001), treatments (p<0.001), and their interaction (p<0.001, two-way ANOVA) influenced weight gain. None of the 3 treatments significantly reduced the extent of weight gain (Tukey, Table 4, Figure 4). However, weight gain in ascites broilers was much lower than normal ones. Broilers with ascites, peppermint, and probiotics improved weight gain to something between the normal and diseased broilers. However, their combination failed to do so (Table 4, Figure 4).

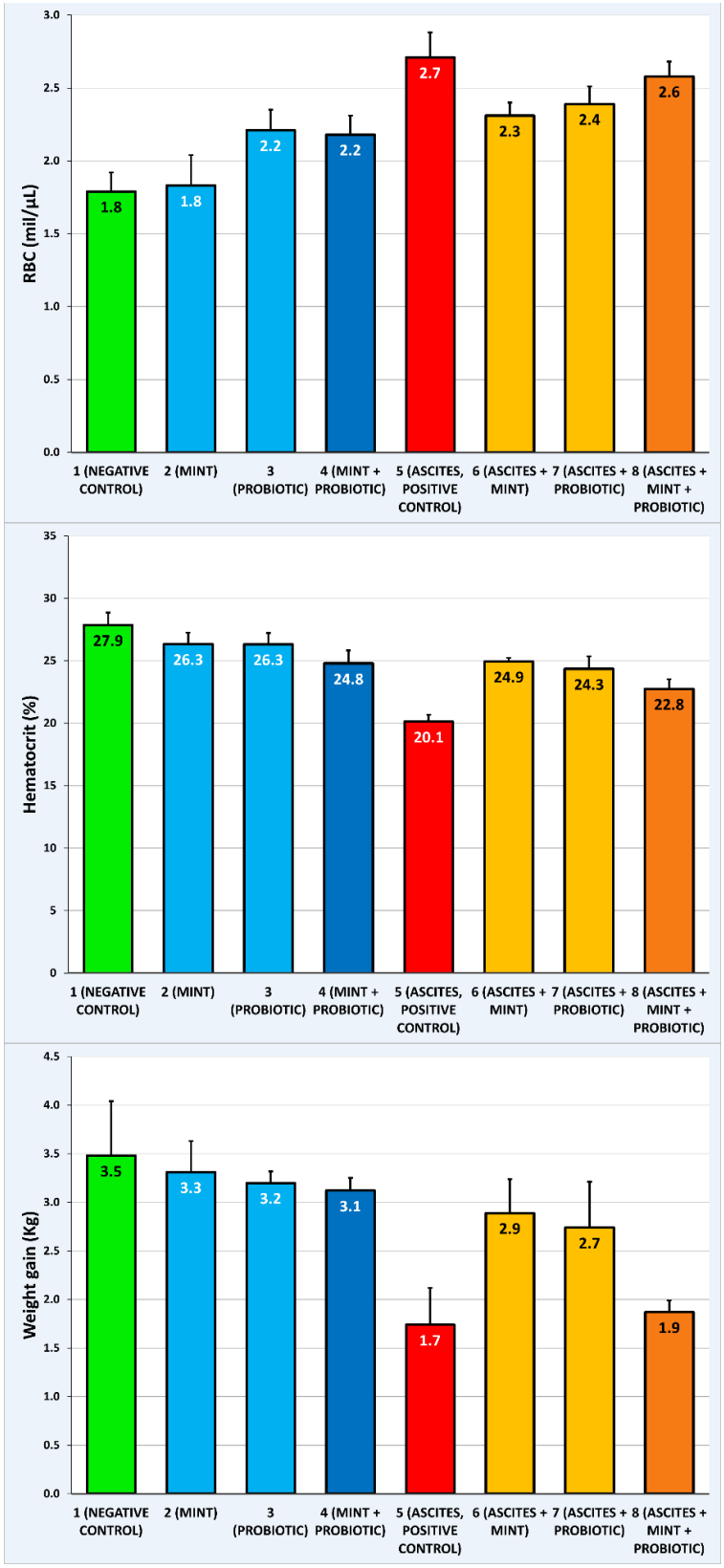

Figure 4. Mean (±SD) RBC (mil/µL), hematocrit (%), and weight gain (Kg) in different groups. The sample size for each group was 10 broilers (total n = 8 × 10 chickens).

Table 4. Descriptive statistics for the level of RBC (mil/µL), hematocrit (HTC, %), and weight gain (Kg) in different groups. The sample size of each group is 10 birds (total n = 8 × 10 broilers). The P value is calculated using the one-way ANOVA.

Group	RBC (mil/µL)		HTC (%)		Weight gain (Kg)	
	Mean	SD	Mean	SD	Mean	SD
1 (negative control)	1.79^{c}	0.13	27.86^{a}	0.99	3.48^{a}	0.56
2 (mint)	1.83^{c}	0.21	26.34^{ab}	0.91	3.31^{a}	0.32
3 (probiotics)	2.21^{b}	0.14	26.32^{ab}	0.92	3.20^{a}	0.12
4 (mint + probiotics)	2.18^{b}	0.13	24.78^{ab}	1.06	3.12^{a}	0.13
5 (ascites, positive control)	2.71^{a}	0.17	20.13^{c}	0.55	1.74^{b}	0.38
6 (ascites + peppermint)	2.31^{ab}	0.09	24.94^{abc}	0.29	2.89^{ab}	0.35
7 (ascites + probiotics)	2.39^{ab}	0.12	24.34^{abc}	1.01	2.74^{ab}	0.47
8 (ascites + peppermint + probiotics)	2.58^{ab}	0.10	22.75^{bc}	0.78	1.87^{b}	0.12
One-way ANOVA's F	9.754		20.746		16.354	
One-way ANOVA's *P*	<0.001		<0.001		<0.001	

The superscript alphabets mark the non-significant pairwise comparisons (Tukey, $p>0.05$).

4 Discussion

This study showed both the safety of peppermint extract and, to a slightly lesser degree, probiotics; it also indicated their efficacy in treating ascites. Only one 2022 abstract was available regarding peppermint's effect (without probiotics) on the reversal of ascites signs (40). In that study (40), 1% Mentha Piperita powder could improve ascites and vitamins C and E. This result was in line with our findings. Nevertheless, there was no study on combining peppermint with probiotics in treating or preventing ascites.

Regarding the efficacy of probiotics, results are controversial, with some studies failing to find proper effects (46) and some others succeeding to observe partially or fully beneficial effects (47), possibly due to different bacteria strains, forms, and dosages in use with different methods (47, 48). The therapeutic effects of probiotics may be explained through their favorable effects on the digestive system. As stated above, hypoxia is one of the major causes of ascites. By increasing the efficiency of the digestive system, probiotics reduce the consumption of oxygen by the digestive system and thereby reduce ascites. The digestive system is a metabolically active organ that needs a lot of nutrients and oxygen (49). Whereas the oxygen demand of poultry's digestive tract has not been estimated, it has been assessed in monogastric animals like pigs. In pigs, the digestive system is merely 5% of the animal's body weight, but it uses 25% of the whole uptaken oxygen (49). The digestive and cardiopulmonary systems are interdependent; however, their relationship can reverse in ascites due to factors such as inflammation, pathogens, the environment, and the high metabolism that occurs because of ascites (50). High gastrointestinal oxygen demand from the lungs and heart may explain why dietary restriction can lessen the occurrence of ascites in broilers (51).

The combination of peppermint extract and probiotics failed to produce proper therapeutic effects in ascitic broilers; also, it was not safe for healthy broilers either. Due to the lack of any similar studies in this regard, it is not possible at this stage to know why this happened. These interventions affect a broad range of multiple biological systems, many overlapped. Therefore, although each is beneficial, their interactions might not result in favorable results. No study in this regard allowed us to make comparisons and discuss more. Still, this particular type of probiotics should not be used in conjunction with peppermint extract. Future studies are warranted to identify such interactions and the mechanisms underlying them.

Levothyroxine can induce ascites through different routes: it is the artificial form of thyroxine or T4, a thyroid gland hormone (52). Therefore, it can increase metabolism, which can induce hypoxia (43, 53), resulting in ascites (5-8, 16). The second path is its potential to increase free radicals, increasing the ascites risk (54, 55). The development of ascites can damage the liver and kidney, deteriorate numerous physiological processes, and alter levels of multiple parameters such as ALT, AST, ALP, or uric acid (16, 56-61). Uric acid is one of the most important antioxidants in the body (62, 63). Plasma uric acid levels can be influenced by oxidative stress and free radicals (64-67), something prevalent in ascites (56, 60, 61). Therefore, it can be deduced that the

decrease in plasma uric acid levels in ascitic broilers could be caused by free radicals destroying xanthine oxidoreductase.

On the other hand, the leaves of medicinal plants such as mint are a rich source of phenolic compounds that are biologically active and have antioxidant, anti-inflammatory, and free radical scavenging potentials, and thus can prevent the reduction of plasma uric acid (68) as was seen in this study. Antioxidant substances of essential oils are phenolic terpenes like carvacrol, thymol, and eugenol (69, 70). Also, menthol and ketones like iso menthone, pulegone, carvone, piperine, and dihydro carvone may play a role in the effects of peppermint extract (41, 71-73). Among these, thymol, carvacrol, menthol, and paracymene may be the most effective materials (74).

The decrease in serum total protein of the current sample can be attributed to the secretion of high amounts of protein into ascites fluid and its decrease in the serum (75). In ascites as a disease with free radicals (56, 60, 61, 76), most of the membrane lipids of capillary walls are oxidized by free radicals produced by the oxidative stress in ascites and, therefore, increase capillary permeability in proportion to amounts of blood oxidants (76-79). Thus, some plasma proteins, such as albumin, will leak into the ventricular cavity through the capillary apertures (75, 76, 80-82). The antioxidant and phenolic compounds of peppermint leaves may also reduce this by inhibiting the oxidation of capillary membranes. Another possible reason might be the food intake: In the ascites group of this study, compared to the mint and probiotics groups, chickens may consume less feed, and as a result, they may receive less protein resulting in lower plasma levels of protein; however, more studies on this regard are needed before being able to link food intake to lowered plasma protein certainly.

The globulin reduction observed in our experimental ascites group can be attributed to the presence of high levels of thyroid hormones T4 and T3, which are given for ascites induction and/or caused by it (83, 84). Thyroid hormones and globulin form thyroxine-bound globulin, which reduces free blood globulin (83, 85).

Despite a decrease in their hematocrit, we noted increases in the red blood cells of ascitic broilers. Because the destruction of old red blood cells and the production of new ones both require metabolic processes, the more active the thyroid is, the higher the metabolism and the oxygen demand of red blood cells (86). Due to hypoxia, ascitic birds cannot provide adequate oxygen, causing them to increase red blood cell production and hematocrit as a compensatory mechanism (56, 87-90). Moreover, another factor contributing to the increased red blood cells and hematocrit might be the elevated levels of corticosterone (87). Factors that affect red blood cells might also affect hematocrit: Chickens raised at low temperatures may have higher hematocrit. With increasing metabolic activity in poultry, the percentages of hematocrit and neutrophils increase (56, 87-90). A negative relationship exists between hematocrit and blood oxygen saturation and ascites (56, 87-89).

Nevertheless, studies have reported controversial results regarding the link between hematocrit and ascites; some have reported a positive genetic correlation between ascites and hematocrit (91). Some have yet to observe notable associations between the two (10, 44, 88, 89, 92). Even some believe a negative relationship exists between the hematocrit level and blood oxygen saturation (93). In other studies, RBC and packed cell volume (9) or hematocrit and RBC (94) were decreased in chickens with ascites. In our study, although hematocrit decreased in ascitic broilers, RBC increased. Interpreting this finding requires more studies; the most probable reasons, however, might be either a decrease in the size of the RBCs contributing to the decline of hematocrit or an increase in the plasma volume. The latter might happen in ascitic animals (95).

Elevated blood glucose levels in ascites have been attributed to high plasma amounts of glucocorticoids and corticosteroids induced by ascites (56), as well as increased gluconeogenesis due to the release of amino acids from liver proteins in ascites (96).

In our study, we induced ascites using levothyroxine, associated with weight loss (97, 98). Nevertheless, weight loss is not limited to levothyroxine-induced ascites, as other forms, such as cold-induced ascites, also show weight loss (56). This indicates that ascites itself may cause reduced growth. This study showed for the first time that peppermint extract and probiotics could partially reverse these alterations caused by ascites. Since there was no such study, we could not compare our results. More data in this regard are necessary.

5 Conclusions

Peppermint extract is safe for healthy broilers based on all the evaluated parameters. Probiotic administration alone could deteriorate merely a few parameters in normal broilers (T3, T4, and RBC). However, the co-administration of peppermint extract and probiotics can have deleterious effects in healthy broilers (in terms of most parameters: AST, ALT, ALP, T3, T4, glucose, cholesterol, triglycerides, total protein, and RBC). The induction of ascites considerably worsened all

the assessed parameters. In ascitic broilers, peppermint extract almost always had a positive therapeutic effect (all parameters except ALP and RBC). Probiotics had such positive effects as well (in the case of AST, ALT, T3, T4, glucose, BUN, uric acid, total protein, albumin, globulin, hematocrit, and weight gain), although mostly not as strong as that of mint. The co-administration of mint extract and probiotics, however, failed to cause any improvements in almost all the parameters of ascitic broilers (except uric acid and ALT).

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Conflict of Interest

The authors have no conflict of interest to declare.

Author Contributions

M, H-J. searched the literature, conceived and designed the study, supervised the thesis, interpreted the findings, and contributed to the draft. E. T, searched the literature, designed the study, interpreted the findings, supervised the thesis, and interpreted the findings. M, N. searched the literature, collected the sample, performed the experiments, analyzed the findings, and wrote the thesis. V, R. searched the literature, contributed to the statistics, interpreted the findings, prepared the Figures and tables, and drafted the article. All authors reviewed the final version and agreed to submit the paper to this journal.

Data Availability Statement

The data is not shareable.

Ethical Considerations

All experiments were conducted by ethical guidelines of the Animal Care and Use Committees (ACUC).

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Abbreviations

Animal Care and Use Committee (ACUC)
Analysis of Variance (ANOVA)
Blood Urea Nitrogen (BUN)
Alanine Aminotransferase (ALT)
Alkaline Phosphatase (ALP)
Aspartate Aminotransferase (AST)

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