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Morphology and immunohistochemistry analysis of broiler intestine treated with immunostimulant agents

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Abstract

This study aims to examine the effects of *Saccharomyces cerevisiae* and a probiotic, mixing them on the intestine layer represented by (heights of the epithelium, depth of the crypts, width of villus and length of villi), and to observe the histopathological and immunohistochemical effects in broilers during 1-35 days. A total of sixty birds 1-day old broilers are randomly divided into four groups each with fifteen replicates, where G1 as a control group, G2 treated with 3 g/kg of *S. cerevisiae*, G3 treated with 200g/1000L of probiotics with water and G4 treated with a mixture of both probiotics and *S.C.* All broiler chickens are euthanized at days 7, 21 and 35 for histopathology and at days 7, 35 for immunohistochemistry. Results show a significant variation increase in the intestine layer of all groups in contrast to the control group, especially at G4 (the mixture of *S. cerevisiae* and probiotics). The administration of *S. cerevisiae* and probiotics significantly increases the permeability of the intestine via IL-6 proteins for cytoplasm and cell membrane and the gene expression is equal in intensity during the first and the fifth weeks of killing. From these results, we conclude that the materials used in G4 has given intense response of IL-6 in broiler chickens, which is a good indicator; therefore, they are proposed for stimulating innate and adaptive immunity.

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Introduction

The development, function and structure of the animal digestive system depend on the type of diet (1-4). Intestinal microfilaria formation is influenced by absorption of nutrition, metabolism and the development of tissue (5-7), resulting in alterations in the bowel bacterial fermentation of carbohydrates (8,9) and protein (10-12). Intestinal tract epithelium, which is involved in nutrients absorption also acts as a barrier between the internal and external milieu of the organism (13-15). Live yeast and probiotics are biologically active materials, which are used in poultry as a supplemented feeding (16-18). A probiotic is a group of microbes, which is added to meals to increase its nutritional value and preserve health of guts (19,20). The products containing both live yeast and probiotics are called symbiotics. Bioactive materials enhance the microstructure of the intestine and have favorable effects on the production and animal health through enhancing the short chain of fatty acid and the composition of microbiota (21-23). Due to the intensive breeding work, two advantage lines were obtained (broilers and lying hens), growth rates between these groups differed due to the development of the digestive system (24-26) morphological parameters of the intestine like width and heights of the villi, as well as crypts depth and absorption area size, could be related to broilers greater body weights in contrast to lying hens (27).

Nevertheless, to our knowledge, comprehensive results about the effects of diet supplemented with live yeast, probiotic and mixture of them in the response of chickens in terms of bowel inflammatory pattern (IL-6), bowel whole-transcriptome side, performance and morphology of the

intestine are still lacking. Thus, this study aimed to determine the effects of live yeast, probiotics and a mixture of them on intestine morphological, and to assess these effects by immunohistochemistry proteins (IL-6).

Materials and methods

Ethical approval

Ethical approval was obtained from the ethics committee at University of Mosul, under reference no. UM.VET.2022.021.

Saccharomyces cerevisiae isolate

Saccharomyces cerevisiae (bread yeast) was obtained from the market. The suitable concentration dose of this yeast is 3 g/kg of body weights (28), while the probiotic concentration dose, which was also obtained from the market is 200g/1000L according to the manufacturer's instruction.

Experimental design

The experiment is conducted on 60 birds in the animal house for 35 days at College of Veterinary Medicine, University of Mosul. Birds' weights range 180-200g. The birds (number=60) were divided into 4 groups: G1 as a control groups, G2 provided with *saccharomyces cerevisiae*, G3 provided with probiotic and G4 provided with a mixture of both. By days 7, 21 and 35, birds were sacrificed (five replicates for each group), their intestine, thymus and bursa of fabricius were preserved for histopathological and immunohistochemical examination.

Tissue histology

Intestine and immunity organ (bursa and thymus) specimens were taken from all groups at days 7, 21, and 35. After tissue sampling, they were put in 10% of formalin, then all tissue specimens were placed in alcohol with ascending level of 70%, 80% and 90%. After that they were dehydrated, cleaned with xylene and then incorporated into wax to prepare the blocks (29).

IL-6 Immunohistochemistry

IL-6 expression in the intestine and immunity organs was exposed by IHC staining with IL-6 (rabbit anti-IL-6 serum purchased from Abcam, Ab6672), blocks of paraffin were used, specimens with 6 µm thickness were placed on a glass slide, these slides were heated for one hour at 55-60ºC. All specimens were washed with dimethyl benzene, dehydrated in an ascending alcohol and washed by phosphate buffered saline for five minutes. After that they were microwaved for 20 minutes before being immersed in a solution of antigen retrieval. These slides underwent heat, then cooled at room temperature, rinse with PBS for five minutes, IL-6 was put and incubated overnights at 35-37 ºC for 16 minutes, then rinsed in phosphate-buffered saline, followed by adding a color generator to slides, incubating, rinsing and washing of these slides with distilled water, staining by Harris' hematoxylin. At last, all slides are dehydrated with alcohol and dimethyl benzene, then left to dry at room temperature for 18-20 minutes, treated with DPX and coverslip (30).

Scoring of Immunohistochemistry

To assess the immunoreaction of antibody, all slides' sections were examined by pathologist at high and low power magnification x200 and x50. Positive expression of IL-6 was evaluated and the staining intensity was determined as +, ++ and +++, brown color dots for IL-6 were recorded and considered as positive results. A score system pointed (31) were used for the assessment of IL-6 proteins.

Statistical analysis

The results were analyzed statistically by the analysis of variants in one-way mode at $P<0.05$ and $P<0.01$ using SPSS version 22.0.

Results

Birds treated with *Saccharomyces cerevisiae* and probiotic (G4) showed significant statistical increase in the heights of epithelium, depth of crypt, width of villus and its length when compared to the control group during the first, third and fifth killing, as well as G2 and G3 showed moderate variation in epithelium heights, crypt depth, villus width and length of the villi, respectively in contrast to the G1 (control group) during the 1st,2nd and 3rd killing of the animals (Tables 1-3).

Histopathological finding

The effects of *Saccharomyces cerevisiae* and probiotic in the morphology of the intestine is presented in figure 1. The heights of the villus in the ileum and jejunum were higher at the ($P<0.05$) in contrast to the G1, G2 and G3, furthermore, the depth of crypts in G4 showed high significant variation when compared to the control group (Figure 2).

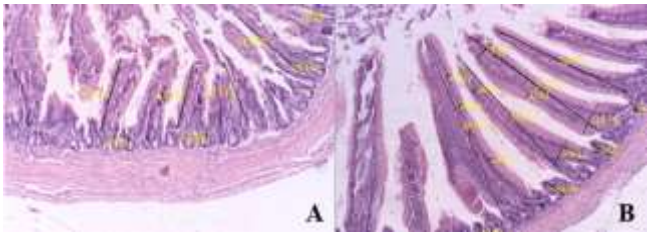

Figure 1: (A) Intestine micrograph showed the height of the villi and its depth in control group, while (B) showed the heights and depth of the villi in G4. H&E, x100.

Table 1: Effects of *S. cerevisiae* on epithelium heights, depth of crypt, width and length of villi

Parameter	Week	Groups	Mean (µM)	SD	T	Significant
Epithelium height	First	Control	23.80	1.82	2.94	0.026*
		S. cerevisiae	30.60	4.25		
	Third	Control	18.03	3.98	0.94	0.385^{NS}
		S. cerevisiae	20.15	2.17		
	Fifth	Control	19.58	1.71	1.13	0.300^{NS}
		S. cerevisiae	21.28	2.47		
Crypt depth	First	Control	64.80	2.84	1.24	0.260^{NS}
		S. cerevisiae	74.00	14.53		
	Third	Control	124.90	7.14	2.72	0.035*
		S. cerevisiae	136.25	4.33		
	Fifth	Control	139.25	40.03	1.01	0.353^{NS}
		S. cerevisiae	118.00	13.44		
Villus width	First	Control	239.05	40.51	8.98	0.000**
		S. cerevisiae	581.75	64.64		
	Third	Control	278.20	62.86	0.90	0.402^{NS}
		S. cerevisiae	445.78	366.70		
	Fifth	Control	567.25	82.75	0.59	0.574^{NS}
		S. cerevisiae	615.40	139.33		
Villi length	First	Control	70.95	18.30	1.05	0.336^{NS}
		S. cerevisiae	89.45	30.30		
	Third	Control	72.45	27.60	1.87	0.111^{NS}
		S. cerevisiae	115.58	37.03		
	Fifth	Control	103.50	59.52	0.90	0.401^{NS}
		S. cerevisiae	73.10	31.45		

* Significant variation at P<0.05. ** significant variation at P<0.01. NS means no significant differences.

Table 2: Effects of probiotic on epithelium heights, depth of crypt, width and length of villi

Parameter	Week	Groups	Mean (µM)	SD	T	Significant
Epithelium height	First	Control	23.80	1.82	2.42	0.052^{NS}
		Probiotic	26.53	1.33		
	Third	Control	18.03	3.98	4.57	0.004**
		Probiotic	31.05	4.08		
	Fifth	Control	19.58	1.71	4.93	0.003**
		Probiotic	25.15	1.48		
Crypt depth	First	Control	64.80	2.84	3.55	0.012*
		Probiotic	95.28	16.94		
	Third	Control	124.90	7.14	13.77	0.000**
		Probiotic	235.13	14.33		
	Fifth	Control	139.25	40.03	2.79	0.032*
		Probiotic	195.25	3.59		
Villus width	First	Control	239.05	40.51	4.79	0.003**
		Probiotic	414.85	61.28		
	Third	Control	278.20	62.86	9.26	0.000**
		Probiotic	594.98	26.95		
	Fifth	Control	567.25	82.75	1.94	0.100^{NS}
		Probiotic	647.98	8.17		
Villi length	First	Control	70.95	18.30	0.70	0.509^{NS}
		Probiotic	60.38	23.97		
	Third	Control	72.45	27.60	1.95	0.099^{NS}
		Probiotic	123.03	43.99		
	Fifth	Control	103.50	59.52	0.13	0.904^{NS}
		Probiotic	107.25	4.65		

* Significant variation at P<0.05. ** significant variation at P<0.01. NS means no significant differences.

Table 3: Effects of mixing of *S. cerevisiae* and probiotic on epithelium heights, depth of crypt, width and length of villi

Parameter	Week	Groups	Mean (µM)	SD	T	Significant
Epithelium height	First	Control	23.80	1.82	2.78	0.032*
		S. cerevisiae + Probiotic	32.43	5.93		
	Third	Control	18.03	3.98	3.44	0.014*
		S. cerevisiae + Probiotic	25.05	0.91		
	Fifth	Control	19.58	1.71	4.56	0.004**
		S. cerevisiae + Probiotic	25.03	1.67		
Crypt depth	First	Control	64.80	2.84	10.45	0.000**
		S. cerevisiae + Probiotic	162.18	18.41		
	Third	Control	124.90	7.14	2.23	0.067^{NS}
		S. cerevisiae + Probiotic	137.50	8.74		
	Fifth	Control	139.25	40.03	4.27	0.005**
		S. cerevisiae + Probiotic	265.75	43.64		
Villus width	First	Control	239.05	40.51	19.05	0.000**
		S. cerevisiae + Probiotic	731.80	32.18		
	Third	Control	278.20	62.86	13.46	0.000**
		S. cerevisiae + Probiotic	993.75	85.76		
	Fifth	Control	567.25	82.75	8.25	0.000**
		S. cerevisiae + Probiotic	1146.25	113.41		
Villi length	First	Control	70.95	18.30	10.01	0.000**
		S. cerevisiae + Probiotic	176.05	10.32		
	Third	Control	72.45	27.60	0.20	0.852^{NS}
		S. cerevisiae + Probiotic	68.68	27.05		
	Fifth	Control	103.50	59.52	0.27	0.796^{NS}
		S. cerevisiae + Probiotic	92.75	52.93		

* Significant variation at P<0.05. ** significant variation at P<0.01. NS means no significant differences.

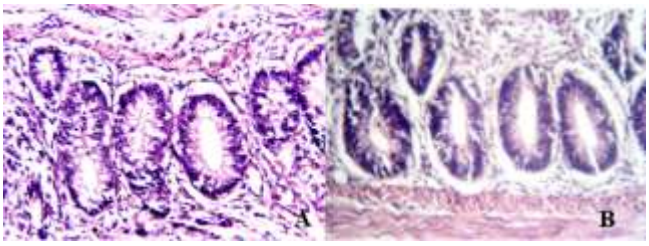

Figure 2: (A) intestine micrograph showed the depth of the crypts in the control group, while (B) showed the depth of the crypts in G4. H&E, x100.

In table 4 the scoring system result were shown, the estimation of IL-6 levels in the intestine of birds at the 1st and 5th killing. Birds' intestine sections were stained with anti-IL-6 marker in the cytoplasm and cell membrane. As shown in figures 3 and 4, which represented negative control, mild, moderate and severe positive reactive patterns of IL-6 in G4 during the first sacrificing, while high level of IL-6 staining intensity showed in figures 5 and 6 in contrast to the control group.

Table 4: Scoring of IL-6 proteins in tissue sections

IL-6	Control	1st week	5th week
Intestine	-	++	+++
adventitia	-	+	+++
Muscularis layer	-	+	+++
Crypts	-	++	+++
Villi	-	++	+++
Lamina propria	-	+	+++

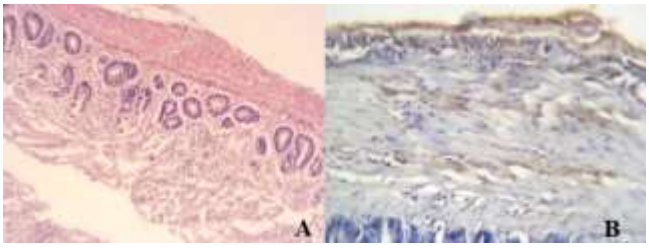

Figure 3: Intestine micrograph at 7 days. G4, (A) negative section. (B) showed mild dotes with brown colour of IL-6. Gene expression, x200.

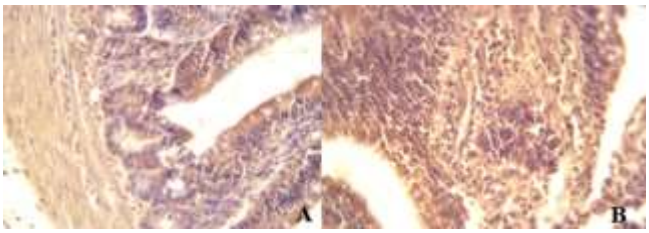

Figure 4: Intestine micrograph at 7 days, G4. (A) Showed moderate dotes with brown color of IL-6 gene expression, while (B) showed sever expression of IL-6. IHC-IL-6, x200.

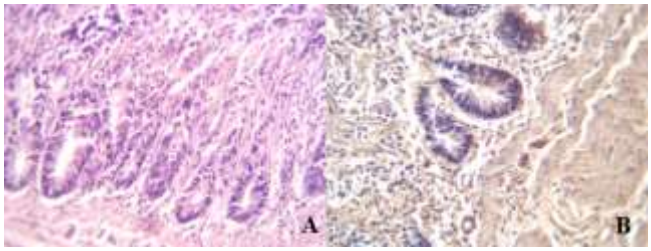

Figure 5: Intestine micrograph at 35 days, G4. (A) Negative section. (B) Mild dotes with brown color of IL-6 gene expression. IHC-IL-6, x200.

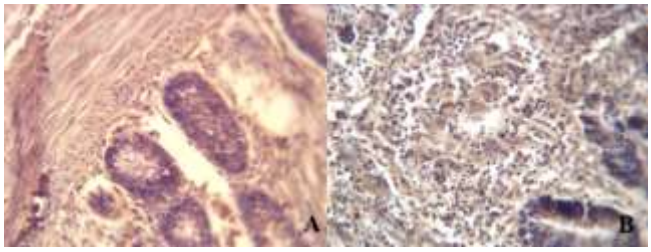

Figure 6: Intestine micrograph at 35 days, G4. (A) Moderate dotes with brown color of IL-6 gene expression, while (B) sever expression of IL-6. IHC-IL-6, x200.

Discussion

The digestive system is made up of the gastrointestinal tract (large and small intestines). Small intestine acts as a barrier, separating the external and internal environment of the body. It is also a highly specialized organ for the absorption and digestion of the nutrients. In chickens, the small intestine is rather short, and the surface area of the intestinal villi determines the majority of the absorption area (32-34). Small intestine structures provide information on the health condition of the digestive system. Because of the mucosal surface proximity and the intestinal tract content, the pathogenic bacteria and toxic dietary substances may impact the intestinal tract mucosa condition and their impact may be seen in alteration to the intestinal villi structure and crypts depth of the morphological and morphometric parameters of the digestive system, especially the small intestinal tract, which is significantly affected by the diet composition and the supplement utilized, including *Saccharomyces cerevisiae* and the probiotic (35,36). In this research, a significant increase in the width of the villi in G4 resulted in the heights and the largest of the intestinal tract villi area. This suggests that G4 has a positive effect in the intestinal tract absorption surfaces. *Saccharomyces cerevisiae and* the probiotic enhance the morphological characteristics of the intestinal tract mucosa and the effects in the absorption surfaces area (37,38). Interestingly, there is an increase in the size of the goblet cell in birds fed in G4 in contrast to animals fed in the G1. It is probable that the

bigger size of the cell in G1 was due to the storage and accumulations of Muncie, whereas in G4, numerous cells were continuously making and releasing mucus (39). Live yeast could organize immune response by increasing expression of the anti-inflammatory cytokine, the alternative material to antibiotics like yeast, probiotic and prebiotic could organize immune response for the body through the increase in the expression of the anti-inflammatory cytokine as IL-6 (40). Live yeast could boost the host immune response by the specific interaction with different immunological-competent, live yeast structural elements like mannan oligosaccharides, B-1, 3, B1-6 Glucans and their cell walls help birds to produce more immunoglobulin, which in turn improves the immunological response (41,42). Furthermore, the presence of B-1, 3, B1-6 Glucans in *Saccharomyces cerevisiae* has also been identified as an immune-stimulating substances in mankind and animals.

Conclusion

From the findings, we concluded that *Saccharomyces cerevisiae* at 3 g/kg (BW) and the probiotics at 200 g/ 1000 L improved intestinal histological performances through using the routine stain. These materials increased the gene expression of IL-6 in the cytoplasm and cell membrane of the intestine and increased the immune responses.

Conflict of interest

The authors declare none.

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التحليل الشكلي القياسي والكيمياء المناعية النسجية لأمعاء فروج اللحم المعاملة بمحفز مناعي

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الخلاصة

تهدف هذه الدراسة الى تقييم تأثير خميرة الخبز والمعزز الحيوي ومزجهما معاً على طبقات الأمعاء والمتمثلة ب (ارتفاع الظهارة، عمق الخبايا، عرض الز غابات وطولها) وتحديد التأثيرات النسيجية وتقنية الكيمياء المناعية على الدجاج اللاحم خلال فترة ٣٥ يوماً، استخدم في التجربة ستون طائراً وقسمت عشوائيا الى أربع مجاميع بواقع خمسة عشر طائر مكرر في كل مجموعة بعمر يوم واحد. كانت المجموعة الأولى مجموعة السيطرة، وعوملت المجموعة الثانية بخميرة الخبز بجرعة ٣ غم/ كغم في العلف، والمجموعة الثالثة أعطيت المعزز الحيوي بجرعة ٢٠٠غم/ ١٠٠٠لتر في ماء الشرب والمجموعة الرابعة عوملت بمزيج من المادتين. تم التضحية بالدجاج اللاحم خلال ٧ و ٢١ و ٣٥ يوماً لأخذ عينات للفحص النسجي المرضي وخلال ٧ و ٣٥ يوماً للكمياء المناعية النسجية. أظهرت النتائج وجود فرق معنوي واضح في ظهارة الأمعاء وارتفاعها وعمق الخبايا إضافة الى عرض الز غابات وارتفاعها بين المجاميع المعاملة مقارنة بمجموعة السيطرة وكانت أعلاها في المجموعة الرابعة. أما التعبير الجيني للانترلوكين السادس في سايتو هيولي وغشاء الخلية فقد كان موجبا ومتساوي الشدة ما بين القتلة الأولى والخامسة، نستنتج من نتائج الدراسة أن الاستجابة الشديدة للانترلوكين السادس في الدجاج اللاحم يقترح كسبب لتحفيز الاستجابة المناعية الفطرية والمكتسبة الناتجة من استخدام هذه المواد وخصوصا المزيج منها في المجموعة الرابعة.