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Original Article

The Effects of Saccharomyces Cerevisiae and Citric Acid on Productive Performance, Egg Quality Parameters, Small Intestinal Morphology, and Immune-Related Gene Expression in Laying Japanese Quails

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■Keywords

S. cerevisiae, citric acid, productive performance, intestine morphology, AvBD 1 and 2 gene expression, Japanese quail.

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ABSTRACT

This experiment evaluated the effects of *Saccharomyces cerevisiae (S. cerevisiae)* and citric acid on production performance, egg quality, intestine histomorphology, and avian β-defensin 1 and 2 (AvBD 1 and 2) gene expressions in laying Japanese quails. A total of 400 48-day-old quails were randomly assigned to a 2×2×2 factorial arrangement of treatments with 5 replicates (each containing 10 quails) for 7 weeks. Variable factors consisted of *S. cerevisiae* (0 and 100 mg/kg diet), citric acid (0 and 5 g/kg diet), and Virginiamycin (0 and 50 mg/kg diet). At the completion of the trial, one bird per replicate was randomly killed, and jejunal tissue samples were removed to evaluate intestinal morphometric characteristics. Samples were taken from the midpoint of the jejunum to measure the gene expression of AvBD 1 and 2. Dietary inclusion of both *S. cerevisiae* and citric acid resulted in increased egg weight, egg mass, reduced feed intake, and improved FCR (p<0.05). The addition of *S. cerevisiae* to diets containing citric acid reduced feed intake, increased egg weight, and improved FCR (p<0.05). Shell weight and shell thickness were increased in birds fed each of *S. cerevisiae* and citric acid supplements (p<0.05). Dietary *S. cerevisiae* and citric acid similarly increased intestinal villus height, width, surface area, and the villus height to crypt depth ratio (p<0.0001). Results showed that AvBD 1 and 2 genes expression were up-regulated on quails fed *S. cerevisiae*-supplemented diets (p<0.0001). In conclusion, these results suggest that supplementation of *S. cerevisiae* and citric acid as functional feed additives either alone or in combination could be a potential alternative to antibiotics in the diet of Japanese laying quails.

INTRODUCTION

In commercial egg production, feed supplements have been used to increase the growth rate of chickens, and improve the productive performance, and health status (Upadhayay & Vishwa, 2014). Antibiotics have beneficial effects on the performance, growth, and health of animals. However, there is increasing pressure to reduce and even eliminate them in poultry feed due to the development of antibiotic resistance in consumers (Wegener, 2003). In a bid to enhance poultry performance, ensure the production of healthy products, and maintain a safer environment, scientists have developed an interest in the search for antibiotic replacements such as prebiotics, probiotics, aromatic oils, and organic acids (Chichlowski *et al.,* 2007). Yeast preparations such as *Saccharomyces cerevisiae* (*S. cerevisiae*) are used in animal diets mainly as probiotics (live yeast cultures) or as a source of high-quality protein, vitamins, and minerals, and also as an excellent prebiotic (dried yeast cultures) (De Lange *et al.,* 2010). It has been reported that *S. cerevisiae* maintains health and improves chicken production performance (Sugiharto, 2016). Improvement of nutrient utilization and feed

conversion ratio has been previously reported in hens (Hassanein & Soliman, 2010). It was observed that yeast inclusion in layer diets improved egg and shell weight and thickness (Swain *et al.,* 2011). It has been shown that feeding diets containing *S. cerevisiae* in Japanese quail improves intestinal development over a 7-week experimental period (Zamanizadeh *et al.,* 2021). Other alternatives to antibiotic growth promoters have been proposed, such as organic acids, herbs and herbal products, essential oils, and enzymes. Among these, the organic acids (citric acid, formic acid, acetic acid, propionic acid) are the most promising alternatives for poultry (Chowdhury *et al.,* 2009). Citric acid can be classified as an antioxidant, growth promoter, acidifier, bacterial inhibitor, and antitoxin. Citric acid exerts its antimicrobial effect in the animal's digestive tract as well as in food. Citric acid exerts its antimicrobial effect in both animal feed and the digestive tract (Islam, 2012). Its effects in animal diets may also suppress the activity and growth of pathogens and improve digestion, absorption, while also causing topical effects on the intestinal brush border, and promoting mucosal immunity (Mroz, 2005). Yesilbag & Colpan (2006) reported that a mixture of propionic and formic acids and their ammonium salts increased egg production by prolonging the laying period and accelerating laying capacity. Adding organic acids to feed lowers the pH in the gastrointestinal tract. Low pH accelerates the conversion of pepsinogen to pepsin, which may reflect in an improved internal and external egg quality (Al-Harthi & Attia, 2015; Park *et al.,* 2009). Avian β-defensins (AvBD) are antimicrobial peptides that have a wide range of antimicrobial activity against fungi, bacteria, and viruses. Treating chicks with probiotics affects the innate immune system by modulating the expression of Toll-like receptors (TLR), proinflammatory and anti-inflammatory cytokines, and AvBD (Terada *et al.,* 2020). Previous studies have indicated that β-defensins expression from sheep as well as broilers can be induced by various yeasts and their cell wall components *in vitro* and *in vivo* (Garcia Diaz *et al.,* 2018; Shao *et al.,* 2016). Yeast β-glucans have been shown to possess immunomodulatory activities involving receptor recognition, and be most effective in enhancing the immune response against infectious pathogens in piglets (Saleh *et al.,* 2015). It was reported earlier that butyrate, bile acids, and retinoic acid induce host defense peptides (HDP) expression in humans, animals, or poultry, while also enhancing disease resistance (Campbell *et al.,* 2012).Therefore, the objective of the present study was to investigate the effects of *S. cerevisiae* and citric acid as potential replacements of antibiotics, synergistically or individually, on productivity, egg qualitative traits, intestine histomorphology, and AvBD 1 and 2 gene expression in laying Japanese quails.

MATERIALS AND METHODS

Animals and treatments

A total of 400 38-day-old laying Japanese quails (*Coturnix coturnix japonica*) with an average initial weight of 160.16 ±0.97 g were procured from a local market. They were fed with commercial diet over 10 days and then, on day 48, were randomly assigned to 8 experimental treatments with 5 replicates per treatment and 10 quails per replicate. The quails were raised in conventional wire cages (50× 30×50 cm^3) with individual feeders, nipple drinkers, and metallic trays for excreta collection. The 2×2×2 factorial arrangement was used in a completely randomized design. Variable factors were *S. cerevisiae* (0 and 100 mg/kg diet), citric acid (0 and 5 g/kg diet), and Virginiamycin (0 and 50 mg/kg diet). *S. cerevisiae* [Persian Type Culture Collection (PTCC)] were purchased from the Iranian Research Organization for Science and Technology, Tehran, Iran. The citric acid used was 99% pure and in the monohydrate form (Kimia Gharb Gostar, Kermanshah, Iran). The duration of the experiment was 7 weeks and birds were fed and watered ad libitum throughout the experiment. The quails were subjected to continuous light regimen of 16 L:8 D. The corn-soy bean meal based diets were formulated to be isonitrogenous and isocaloric according to National Research Council (NRC,1994) recommendations (Table 1). All animal protocols were approved by the Animal Ethics Committee of the Saravan Higher Education Complex.

Productive traits

Egg production (weight and number) was recorded daily throughout the experiment and calculated on hen-day basis. Feed intake per cage was recorded at the end of each week. At the end of the experiment, the feed conversion ratio (FCR) was calculated as the ratio of feed intake (g) divided by produced eggs (g). Damaged eggs (abnormal, shell-less, cracked, and broken eggs) were recorded as soon as they occurred.

Egg quality assessment

During the last 4-days of the experiment, 40 intact eggs were collected per replicate (1600 in total). Internal and external egg quality parameters were determined for randomly selected eggs from each replicate (3 eggs per replicate). Egg width and length (±1 μm) were then measured in mm using a digital micrometer.

Table 1 – Ingredients and chemical composition (%) of the basal diet.

Ingredient (%)	
Corn	56
Soybean meal	29
Corn gluten	3.51
Vegetable oil	3.24
CaCO3	5.3
Dicalcium phosphate	1.6
Sodium chloride	0.27
Sodium bicarbonate	0.11
DL-Methionin	0.15
L-Lysine	0.09
L-Threonine	0.08
Vitamin premix[1]	0.25
Mineral premix[2]	0.25
Washed sand	0.15
Calculated nutrients and energy	
AME, (kcal/kg)	2950
Crude protein (%)	20
L-Lysine (%)	1.03
DL-Methionin (%)	0.48
TSAA (%)	0.81
Calcium (%)	2.48
Nonphytate P (%)	0.45
Total P (%)	0.64
Analyzed values	
AME, (kcal/kg)	2946
Crude protein (%)	19.94
L-Lysine (%)	1.01
DL-Methionin (%)	0.45
TSAA (%)	0.78
Calcium (%)	2.46
Nonphytate P (%)	0.43
Total P (%)	0.62

[1]Vitamin mixture provided per kg of diet: 11000 IU of vitamin A, 1800 IU of vitamin D3,11 mg of vitamin E, 2 mg of vitamin K3, 4 mg of vitamin B1, 5.7 mg of vitamin B2, 2 mg of vitaminB6, 0.5 mg of folic acid, 2500 mg of choline chloride, 0.125 mg of antioxidants, 0.03mg of Biotin and 0.024 mg of vitamin B12.

[2]Mineral mixture provided per kg of diet: 500mg of FeSO4, 65 mg of CuSO4, 100mg of MnSO4, 0.5 mg of Iodine and 0.22gm of Selenium.

The egg shape index was subsequently determined as performed in leghorn chickens (Anderson *et al.,* 2004):

Egg shape index = [egg width/egg length] ×100

Then, eggs were broken-out on a flat glass surface for internal quality analysis. The egg yolks were gently separated from the albumen and rolled over tissue papers to remove the residual albumen and weighed. The yolk index was calculated by dividing the height of the yolk by its width (Funk, 1958). Egg yolk color was determined by the Roche color fan (Hoffman-La Roche Ltd., Basel, Switzerland) (15 = dark orange and 1 = light pale). The shells were thoroughly washed with distilled water, left for 24 h at room temperature for drying, and then weighed to the nearest 0.01 g. Shell thickness was measured at three different points (sharp end, equator, and air cell) of each shell using a digital micrometer with a 0.01 µm accuracy. The mean value of three measurements was recorded as the egg shell thickness. The albumen weight was then calculated by subtracting yolk weight and dry shell weight from the initial whole egg weight. Haugh unit was calculated according to the following formula (Sari *et al.,* 2016):

Haugh unit= 100 log [albumen height – 1.7(egg weight)$^{0.37}$ + 7.57]

The relative weights of the albumen, yolk, and dry egg shell were expressed based on egg weight.

Serum biochemical parameters, sampling, and intestinal morphology

At the end of the experimental period, five birds per treatment (one bird/replicate) were randomly selected, deprived of feed for 12 h, weighed, stunned electrically, and then blood samples were taken from the jugular vein. The samples were centrifuged at 4 °C, and serum was stored at –20°C until further analysis. Serum measurements of triglyceride, cholesterol, HDL, LDL, AST, and ALT were performed using Automatic Biochemical Analyzer (A15 Autoanalyser, Biosystems SA, Spain). Afterward, the selected quails were sacrificed by cervical dislocation. To evaluate intestinal morphology, a 1.5-cm-long section of the jejunum was obtained from each bird. Tissue samples were flushed with cold saline (PBS, pH 7.2) and immediately placed in 10% buffered formalin (pH 7.0). Histologic samples were then gradually dehydrated in a graded series of alcohols, sectioned into small pieces (6 µm thickness), and embedded in paraffin. Paraffin blocks were cross-sectioned at 5 µm, placed on slides, and stained with hematoxylin-eosin. Morphometric indices, including villus width, villus height, and crypt depth were measured using a light microscope connected to Soft Imaging System (Olysia, Germany). Villus surface area was calculated according to Sakamoto *et al.* (2000) based on the formula (2π) × (villus width/2) × (villus height). To assess the gene expression of AvBD 1 and 2, tissues (150–200 mg) were freshly collected from the midpoint of the jejunum. The tissues were washed with ice-cold PBS, minced, and then stored at -80°C in RNAlater (Qiagen, Hilden, Germany) until RNA isolation.

Isolation of total RNA and reverse transcription

Total RNA was isolated from all samples of small intestine tissue using a RNX-plus Kit (SinaClon,Tehran). After that, the concentration and purity of total RNA

were measured by the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at a wavelength of 260–280 nm. All RNA samples were kept at –80 °C for the next experiments. Synthesis of cDNA was performed from 1 -10 micrograms of total RNA using a 2-steps RT-PCR kit (Cat. No. RTPL12, Vivantis Technologies) in 20 µl final volume.

Quantitative real-time PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out in a 48-well plate format of Real-Time PCR System (Applied Biosystems). Reactions of the real-time PCR were performed using the SYBR Green Master Mix, cDNA, as well as primers for either the housekeeping gene or the target genes. Each sample was run in triplicate, and no template control was included. The primers used in PCR reactions included AvBD 1, AvBD 2, and β-Actin as an endogenous control gene (Table 2). The primers were designed using NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast), and their qualities were checked by the Oligo Analyzer 3.1 program (http://sg.idtdna.com/calc/analyzer). The reaction conditions were as follows: 1 cycle of 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 59.5 °C for 30 s, 72 °C for 40 s, and finally one cycle of 72 °C for 10 min for extension; the melting curve was obtained over the range 59.5–95 °C. The relative expression of genes of interest was calculated based on the $2^{-\Delta\Delta Ct}$ method according to Livak & Schmittgen (2001).

Table 2 – Primer sequences used for the quantitative real-time PCR.

Gene[a]	Primer sequence[c] (5`-3`)	Annealing temp °C)	Amplicon (bp)	Accession no.
AvBD 1	F: GGTTCGTTCCTTCCTCTTGGC R: ACACTCGCCCTTCATCTTCCG	61 °C	158	XM_032443218.1
AvBD 2	F: TGGAGTTCCCATTCCTGAGTGAT R: TGAGGCGGTTCTACATCCAGG	61 °C	135	XM_015859640.2
β-actin	F: AACACCCACACCCCTGTGAT R: TGAGTCAAGCGCCAAAAGAA	60 °C	136	XM_015876619.1

[a] AvBD 1: avian β-defensin 1.

[b] AvBD 2: avian β-defensin 2.

[c] F: Forward primer; R: Reverse primer.

Statistical analysis

Data were analyzed statistically using the general linear model procedure of SAS (SAS Institute, 2003) as a three factorial arrangement, including dietary *S. cerevisiae* (0 and 100 mg/kg diet), citric acid (0 and 5 g/kg diet), and Virginiamycin (0 and 50 mg/kg diet). Differences among treatments were determined using Duncan's multiple-range test. The differences between treatments are considered statistically significant at $p \leq 0.05$.

RESULTS

Productive performance

The results showed that *S. cerevisiae* has significant main effects on all production parameters (Table 3). Supplementation of *S. cerevisiae* at 100 mg/kg increased egg production, egg mass, and egg weight ($p<0.0001$). On the other hand, it reduced feed intake and improved FCR ($p<0.0001$). Citric acid supplementation also led to a significant increase in egg weight and egg mass ($p<0.0001$). It also significantly reduced feed intake and improved FCR ($p<0.0001$). However, a substantial trend toward significance was observed in egg production ($p=0.069$). The results showed that there was a significant interaction between *S. cerevisiae* and citric acid on egg weight, feed intake, and FCR ($p<0.05$). The two-way interaction on egg weight showed that regardless of the antibiotic level, the addition of *S. cerevisiae* when birds are fed a diet containing citric acid leads to a significant increase in egg weight (11.97 vs. 11.74 g; $p<0.05$). The two-way interaction on feed intake follows a similar trend. Adding *S. cerevisiae* to diets containing citric acid has significantly reduced feed intake (31.31 vs. 31.13 g; $p<0.0001$). This interaction with a similar trend has also affected the FCR. The addition of *S. cerevisiae* to diets containing citric acid has led to a significant reduction in FCR (2.59 vs. 2.67; $p<0.0001$).

Egg quality traits

The results showed that *S. cerevisiae* has significant main effects on shell thickness, shell weight, and shell percentage (0.189 vs. 0.175 mm, 1.20 vs. 1.12 g, and 10.12 vs. 9.55 %, respectively; $p<0.0001$) (Table 4). Supplementation of *S. cerevisiae* has also resulted in significant main effects on the yolk index ($p<0.05$). Birds that received diets containing *S. cerevisiae* had significantly higher yolk index (48.38 vs. 48.07;

Table 3 – Effect of *S. cerevisiae*, citric acid, and virginiamycin on the productive performance of laying Japanese quails.

Treatment			Parameter				
S. cerevisiae (mg/kg)	Citric acid (g/kg)	Virginiamycin (mg/kg)	Egg production (hen-day %)	Egg weight (g)	Feed intake (g/quail/day)	Feed conversion ratio (FCR, g feed/g egg)	Egg mass (g)
0	0	0	86.12	11.70^{c}	32.56^{a}	2.78^{a}	10.07
0	0	50	86.22	11.71^{c}	32.51^{a}	2.77^{a}	10.09
0	5	0	86.59	11.75^{c}	31.40^{b}	2.67^{b}	10.18
0	5	50	86.69	11.74^{c}	31.31^{b}	2.66^{b}	10.18
100	0	0	88.55	11.84^{b}	31.11^{c}	2.62^{c}	10.48
100	0	50	88.58	11.84^{b}	31.12^{c}	2.62^{c}	10.49
100	5	0	89.35	11.97^{a}	31.10^{c}	2.59^{d}	10.69
100	5	50	89.31	11.94^{a}	31.13^{c}	2.60^{cd}	10.66
SEM			0.463	0.023	0.041	0.007	0.073
Main effect							
S. cerevisiae		0	86.40^{b}	11.72^{b}	31.94^{a}	2.72^{a}	10.13^{b}
		100	88.94^{a}	11.90^{a}	31.11^{b}	2.61^{b}	10.58^{a}
Citric acid		0	87.36	11.77^{b}	31.83^{a}	2.70^{a}	10.28^{b}
		5	87.98	11.85^{a}	31.23^{b}	2.63^{b}	10.43^{a}
Virginiamycin		0	87.65	11.81	31.54	2.67	10.35
		50	87.70	11.81	31.52	2.67	10.36
					p		
S. cerevisiae			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Citric acid			0.069	<0.0001	<0.0001	<0.0001	0.010
Virginiamycin			0.882	0.703	0.462	0.926	0.984
S. cerevisiae × Citric acid			0.659	0.043	<0.0001	<0.0001	0.352
S. cerevisiae × Virginiamycin			0.877	0.703	0.121	0.315	0.848
Citric acid × Virginiamycin			0.954	0.397	0.866	0.646	0.774
S. cerevisiae × Citric acid × Virginiamycin			0.954	0.883	0.687	0.782	0.939

$^{a-d}$Means within each column with no common superscript differ significantly ($p<0.05$).

Table 4 – Effect of *S. cerevisiae*, citric acid, and virginiamycin on egg quality traits of laying Japanese quails.

Treatment			Parameter									
S. cerevisiae (mg/kg)	Citric acid (g/kg)	Virginiamycin (mg/kg)	Shell thickness (mm)	Shell weight (g)	Shell (%)	Yolk weight (g)	Yolk (%)	Yolk index	Yolk color	Albumen (%)	Egg shape index	Haugh unit
0	0	0	0.174	1.10	9.42	4.01	34.31	48.03	5.19	56.26	77.46	87.34
0	0	50	0.172	1.11	9.54	3.95	33.77	48.12	5.20	56.68	77.74	87.35
0	5	0	0.176	1.12	9.52	4.06	34.57	48.01	5.21	55.89	77.74	87.32
0	5	50	0.178	1.14	9.70	4.01	34.15	48.13	5.19	56.14	77.94	87.31
100	0	0	0.188	1.19	10.06	4.02	34.95	48.21	5.23	55.97	77.91	87.30
100	0	50	0.188	1.20	10.14	4.04	34.12	48.27	5.21	55.73	77.54	87.36
100	5	0	0.192	1.22	10.20	4.13	34.52	48.46	5.21	55.27	77.94	87.32
100	5	50	0.190	1.20	10.10	4.13	34.60	48.60	5.20	55.29	77.83	87.38
SEM			0.001	0.009	0.086	0.114	0.979	0.171	0.015	0.969	0.227	0.064
Main effect												
S. cerevisiae		0	0.175^{b}	1.12^{b}	9.55^{b}	4.01	34.20	48.07^{b}	5.20	56.24	77.71	87.33
		100	0.189^{a}	1.20^{a}	10.12^{a}	4.08	34.30	48.38^{a}	5.21	55.56	77.80	87.34
Citric acid		0	0.181^{b}	1.15^{b}	9.79	4.00	34.04	48.16	5.21	56.16	77.66	87.33
		5	0.184^{a}	1.17^{a}	9.88	4.00	34.46	48.30	5.20	55.65	77.86	87.33
Virginiamycin		0	0.182	1.15	9.80	4.05	34.34	48.18	5.21	55.85	77.76	87.32
		50	0.182	1.16	9.87	4.03	34.16	48.28	5.20	55.96	77.75	87.35
								p				
S. cerevisiae			<0.0001	<0.0001	<0.0001	0.386	0.887	0.015	0.254	0.330	0.613	0.812
Citric acid			0.008	0.010	0.143	0.354	0.548	0.249	0.679	0.461	0.228	0.948
Virginiamycin			0.628	0.280	0.256	0.777	0.799	0.400	0.384	0.874	0.977	0.491
S. cerevisiae × Citric acid			0.567	0.827	0.470	0.768	0.883	0.233	0.293	0.934	0.818	0.575
S. cerevisiae × Virginiamycin			0.894	0.133	0.190	0.685	0.665	0.951	0.818	0.752	0.148	0.504
Citric acid × Virginiamycin			0.757	0.426	0.586	0.960	0.990	0.816	0.747	0.971	0.781	0.948
S. cerevisiae × Citric acid × Virginiamycin			0.152	0.280	0.320	0.941	0.943	0.912	0.384	0.873	0.597	0.862

$^{a-b}$Means within each column with no common superscript differ significantly ($p<0.05$).

p<0.05). It was observed that the supplementation of citric acid significantly increased the thickness and weight of egg shells (0.184 vs. 0.181 and 1.17 vs. 1.15, respectively; p<0.05).

Intestinal morphology

Results showed that both *S. cerevisiae* and citric acid had similar significant main effects on increasing the villus height (547.70 vs. 436.95 µm and 513.35 vs. 471.30 µm, respectively; p<0.0001), villus width (143.90 vs. 119.60 µm and 137.30 vs. 126.20 µm, respectively; p<0.0001), villus surface area (247518 vs. 165215 µm^2 and 222413 vs. 190320 µm^2, respectively; p<0.0001), and villi height to crypt depth ratio (8.146 vs. 6.603 µm/µm and 7.728 vs. 7.022 µm/µm, respectively, p<0.0001) (Table 5). Also, a two-way interaction was

Table 5 – Effect of *S. cerevisiae*, citric acid, and virginiamycin on jejunum morphology of laying Japanese quails.

Treatment			Parameter				
S. cerevisiae (mg/kg)	Citric acid (g/kg)	Virginiamycin (mg/kg)	Villus height (µm)	Villus width (µm)	Crypt depth (µm)	Villus surface area (µm^2)	Villus height:crypt depthratio (µm /µm)
0	0	0	403.00^{c}	109.80^{c}	66.19	138963^{d}	6.12
0	0	50	400.00^{c}	110.20^{c}	67.19	138384^{d}	5.98
0	5	0	471.60^{b}	128.40^{b}	66.30	190186^{c}	7.12
0	5	50	473.20^{b}	130.00^{b}	66.00	193326^{c}	7.18
100	0	0	540.00^{a}	142.00^{a}	67.60	240833^{b}	8.01
100	0	50	542.20^{a}	142.80^{a}	68.12	243097^{ab}	7.96
100	5	0	553.60^{a}	146.00^{a}	67.06	253800^{a}	8.27
100	5	50	555.00^{a}	144.80^{a}	66.69	252340^{ab}	8.33
SEM			4.854	1.729	1.899	3048	0.196
Main effect							
S. cerevisiae		0	436.95^{b}	119.60^{b}	66.42	165215^{b}	6.603^{b}
		100	547.70^{a}	143.90^{a}	67.32	247518^{a}	8.146^{a}
Citric acid		0	471.30^{b}	126.20^{b}	67.23	190320^{b}	7.022^{b}
		5	513.35^{a}	137.30^{a}	66.51	222413^{a}	7.728^{a}
Virginiamycin		0	492.05	131.55	66.78	205946	7.383
		50	492.60	131.95	66.94	206787	7.367
					p		
S. cerevisiae			<0.0001	<0.0001	0.491	<0.0001	<0.0001
Citric acid			<0.0001	<0.0001	0.579	<0.0001	<0.0001
Virginiamycin			0.873	0.745	0.076	0.759	0.911
S. cerevisiae × Citric acid			<0.0001	<0.0001	0.871	<0.0001	0.007
S. cerevisiae × Virginiamycin			0.718	0.627	0.921	0.872	0.873
Citric acid × Virginiamycin			0.783	0.871	0.690	0.999	0.601
S. cerevisiae × Citric acid × Virginiamycin			0.696	0.517	0.941	0.499	0.862

$^{a-d}$Means within each column with no common superscript differ significantly (p<0.05).

observed between *S. cerevisiae* and citric acid on villus height, villus width, and villus surface area (p<0.0001). This interaction clearly showed that the addition of *S. cerevisiae* to diets containing citric acid increased villus height, width, and surface area (555 vs. 471.60 µm, 144.80 vs. 128.40 µm and 253800 vs. 190186 µm/µm, respectively; p<0.0001). The interaction also showed that the addition of citric acid to bird diets containing *S. cerevisiae* increased the villus surface area (253800 vs. 240233 µm/µm; p<0.0001).

Serum biochemical parameters

As shown in Table 6, except for triglycerides, serum biochemical parameters were not affected by experimental treatments (p<0.05). Regarding triglycerides, the birds fed diets supplemented with *S. cerevisiae* had significantly lower serum triglyceride levels (1803 vs. 1856 mg/dL; p<0.05).

Gene expression of AvBD 1 and 2

As shown in Table 7, the expression of AvBD 1 and 2 genes were significantly increased by the supplementation of *S. cerevisiae* (p<0.0001).

DISCUSSION

In this study, we found that *S. cerevisiae* has beneficial effects on the productive performance of laying Japanese quails. The findings of this experiment regarding the effect of *S. cerevisiae* on production performance parameters are in accordance with the findings of Zamanizadeh *et al.* (2021), who showed that the addition of *S. cerevisiae* to diets of laying

Table 6 – Effect of *S. cerevisiae*, citric acid, and virginiamycin on blood serum parameters of laying Japanese quails.

Treatment			Parameter					
S. cerevisiae (mg/kg)	Citric acid (g/kg)	Virginiamycin (mg/kg)	Triglyceride (mg/dL)	LDL (mg/dL)	HDL (mg/dL)	Cholesterol (mg/dL)	AST (U/L)	ALT (U/L)
0	0	0	1852	27.15	66.40	184.00	167.80	12.58
0	0	50	1872	27.34	65.46	182.20	169.60	12.14
0	5	0	1853	26.97	69.41	175.80	172.40	12.24
0	5	50	1847	27.82	68.20	181.00	168.80	11.94
100	0	0	1779	26.92	69.35	179.40	171.40	12.16
100	0	50	1826	28.50	69.00	178.00	173.60	12.77
100	5	0	1787	27.60	69.30	181.80	171.80	11.86
100	5	50	1822	28.79	67.98	184.20	169.40	12.21
SEM			36.10	1.467	3.139	6.765	4.897	0.381
Main effect								
S. cerevisiae		0	1856[a]	27.32	67.36	180.75	169.65	12.22
		100	1803[b]	27.95	68.90	180.85	171.55	12.25
Citric acid		0	1832	27.48	67.55	180.90	170.60	12.41
		5	1827	27.79	68.72	180.70	170.60	12.06
Virginiamycin		0	1817	27.16	68.61	180.25	170.85	12.21
		50	1842	28.11	67.66	181.35	170.35	12.26
					p			
S. cerevisiae			0.048	0.545	0.492	0.983	0.587	0.923
Citric acid			0.849	0.763	0.601	0.966	1.000	0.204
Virginiamycin			0.350	0.365	0.670	0.819	0.886	0.851
S. cerevisiae × Citric acid			0.794	0.871	0.448	0.354	0.587	0.768
S. cerevisiae × Virginiamycin			0.510	0.676	0.956	0.901	0.908	0.123
Citric acid × Virginiamycin			0.709	0.946	0.890	0.576	0.475	0.903
S. cerevisiae × Citric acid × Virginiamycin			0.901	0.802	0.938	0.868	0.954	0.710

[a-b]Means within each column with no common superscript differ significantly ($p<0.05$).

quails at 200 mg/kg reduced feed intake, increased egg production and egg mass, and improved FCR. Previous research showed supplementation of animal diets with *S. cerevisiae* increases the activity of trypsin, chymotrypsin, and α-amylase enzymes in the duodenal chyme, leading to better nutrient utilization and higher protein digestibility (Ahiwe *et al.,* 2020). Moreover, manganese oligosaccharides in the yeast cell wall reduce the harmful bacteria load in the intestine, therefore efficiently diverting the nutrients in the diet toward production and leading to improved egg production in layers and breeders (Shashidhara & Devegowda, 2003). Regarding the effect of citric acid on production performance, our results agree with those of Boling *et al.* (2000), which reported that the inclusion of citric acid in the diet of laying hens at the level of 4% reduced feed intake and improved FCR. A possible reason for reduced feed intake may be related to high levels of supplemented citric acid, which in turn has negative effects on birds' appetite and diet palatability. The increase in egg weight due to supplementation of citric acid could be partly explained by the increase in shell weight. Citric acid capacity to maintain the balance of pH and microorganisms in the digestive tract might be one of the causes of the feed digestion process becoming more optimal and FCR being improved (Youssef *et al.,* 2013). According to the results of the present experiment, combined supplementation of citric acid (5 g/kg) *S.* and *cerevisiae* (100 mg/kg) showed synergistic effects in reducing feed intake, as well as improving egg weight and FCR throughout the experiment. A possible explanation could be that *S. cerevisiae* + citric acid diet improved protein, mineral, and energy utilization, which may account for the additive effects of *S. cerevisiae* and citric acid in promoting the productive performance of quails in the present study. The findings of this experiment in terms of beneficial effects of dietary *S. cerevisiae* on egg shell properties are in accordance with the findings of Zamanizadeh *et al.* (2021), who showed that supplementation of yeast at 100 and 200 mg/kg in Japanese quails significantly improved shell thickness and percentage. In regards to shell weight, these results are similar to those reported by Ayanwale *et al.* (2006) in laying hens fed diets supplemented with *S. cerevisiae* at 0.75 %. It has been suggested that the improvement of shell thickness in birds receiving diets containing yeast may be related to the

Table 7 – Effect of S. cerevisiae, citric acid, and virginiamycin on the relative mRNA expression of avian β-defensins in the intestine of laying Japanese quails.

Treatment				
S. cerevisiae (mg/kg)	Citric acid (g/kg)	Virginiamycin (mg/kg)	AvBD 1	AvBD 2
0	0	0	0.774	0.844
0	0	50	0.796	0.830
0	5	0	0.874	0.946
0	5	50	0.844	0.874
100	0	0	1.510	1.574
100	0	50	1.488	1.524
100	5	0	1.404	1.564
100	5	50	1.518	1.548
SEM			0.030	0.058
Main effect				
S. cerevisiae		0	0.822[b]	0.873[b]
		100	1.500[a]	1.552[a]
Citric acid		0	1.142	1.193
		5	1.180	1.233
Fish oil		0	1.160	1.232
		2	1.161	1.194
			p	
S. cerevisiae			<0.0001	<0.0001
Citric acid			0.086	0.342
Virginiamycin			0.963	0.366
S. cerevisiae × Citric acid			0.104	0.432
S. cerevisiae × Virginiamycin			0.817	0.904
Citric acid × Virginiamycin			0.963	0.885
S. cerevisiae ×Citric acid× Virginiamycin			0.218	0.583

[a-b]Means within each column with no common superscript differ significantly ($p<0.05$).

enhancement of calcium bioavailability (Elkaiaty *et al.,* 2019).On the other hand, diets containing probiotics have been reported to develop an acidic environment in the gastrointestinal tract, facilitating ionization and the subsequent absorption of minerals by developing lactic acid bacteria (Haddadin *et al.,* 1996). Regarding the effects of citric acid on the improvement of shell quality, our results are consistent with the findings of Youssef *et al.* (2013), who stated that the supplementation of organic acids (Galliacid) in diets of laying hens at 0.06% leads to an increase in shell thickness. Similarly, Al-Harthi & Attia (2015) found that dietary citric acid at 0.2% improved the weight and quality of the egg shell in laying hens. Improving the thickness of the shell may be the consequence of increasing the absorption of minerals and proteins, which in turn is reflected in the deposition of calcium and protein in the shell, which contributes to the improvement of shell quality (Youssef *et al.,* 2013). Organic acids act in such a way that, by lowering the pH of the gastrointestinal tract, they accelerate the conversion of pepsinogen to pepsin and increase the rate of absorption of proteins and minerals. In this regard, it has been reported that citric acid can prevent the formation of the calcium phytate complex, making phytate phosphorus available and subsequently releasing minerals bound to the phytate molecule (Islam, 2012). Our findings concerning the effects of dietary *S. cerevisiae* on yolk index are in line with those of Yoruk *et al.* (2004), who found that the addition of probiotics to the diets of laying hens leads to an improved yolk index. The egg yolk index, defined as the ratio of yolk height over yolk diameter, provides an indication of egg freshness. As egg deterioration progresses, the yolk index score becomes lower (Khan *et al.,* 2011). It seems that the improvement of the yolk index can be associated with the localization of yellow pigments in the yolk membrane between lipid membranes, which may be associated with higher consumption of corn xanthophylls. Moreover, yeast can also utilize the starch and fiber of the carbon source materials to produce energy required for the growth of beneficial microorganisms, thereby affecting the egg yolk index (Gnanadesigan *et al.,* 2014). Concerning the effects of *S. cerevisiae* on jejunum morphology, similar results were found in a study performed on laying Japanese quails, whereby birds' villus height, width, and absorptive surface area were significantly affected by dietary *S. cerevisiae* supplementation at the level of 1.5% (Tomaszewska *et al.,* 2018). Yeast increases short-chain fatty acids (SCFAs) synthesis, which in turn stimulates intestinal epithelial cells proliferation and eventually leads to wider and longer villi, and a larger absorptive surface area (Tomaszewska *et al.,* 2018). Furthermore, it has been shown that Lactic acid bacteria can increase the villus length by stimulating the digestion of carbohydrates and the production of SCFAs (Shah *et al.,* 2019). As previously mentioned, citric acid supplementation also had similar beneficial effects on jejunum morphology. Similar effects were seen by Nourmohammadi & Afzali (2013), who showed that inclusion of citric acid at 3 and 6% in the diets of broilers increased villus height, villus width, and villus height:crypt depth ratio. It has been demonstrated that the inclusion of organic acids in poultry diets reduces gram-negative bacteria, while also increasing *Lactobacillus spp.* in the gastrointestinal tract (Wang *et al.,* 2010). On the other hand, as *Lactobacillus spp.* increases, intestinal integrity improves, and its positive effects on intestinal health become apparent. This leads to lower inflammatory damage to the intestinal mucosa, which increases villus height and secretion functions, leading to increased nutrient digestion and

absorption by the mucosa (Baurhoo *et al.,* 2007). In this study, the morphological characteristics were improved with dietary *S. cerevisiae* and citric acid supplementation. This may be due to an increased rate of nutrient absorption, as well as the migration of enterocytes from the crypt to the villus. The ratio of villus height:crypt depth is an important indicator of digestive capacity in the small intestine. An increase in this ratio is associated with improved nutrient digestion and absorption (Montagne *et al.,* 2003). As previously mentioned, the synergistic interaction effects between yeast and citric acid improved the morphological characteristics of the intestine. Significant changes in intestinal morphological traits occurred with the addition of *S. cerevisiae* to diets containing citric acid. In regards to the effects of *S. cerevisiae* on the reduction of serum triglycerides, similar effects were recorded by Yalçın *et al.* (2012), who reported that supplementary *S. cerevisiae* at 2 g/kg in the diet of laying hens reduced serum triglycerides. Decreased serum triglycerides may be associated with an increase in the population of *Lactobacillus spp.* and lactic acid-producing *Bifidobacteria* in the digestive gut, which in turn consume yeast cell wall mannan-oligosaccharides. These bacteria increase lactic acid fermentation in the gut, which in turn reduces serum triglycerides (Konca *et al.,* 2009). Our results have shown that *S. cerevisiae* can induce the expression of AvBD 1 and 2 in laying Japanese quails. Our observations are in accordance with those of Shao *et al.* (2016), who concluded that β-glucan, a yeast cell wall component, increases the expression of BD in chickens. AvBDs were differentially expressed in the chicken gastrointestinal tract. Antimicrobial activities of AvBDs have been demonstrated against a wide range of Gram-negative and Gram-positive pathogens. It has been shown that chickens possess a Dectin-1-like β -glucan receptor and a collection of mannan-binding lectins that activates the complement cascade (Nerren & Kogut, 2009). The expression of vertebrates defensin genes has been previously investigated, the first being pattern recognition receptors (PRRs), such as TLRs, Cytosolic DNA sensor Nucleotide oligomerisation domain (NOD)-like receptors (NLR), and C-type lectin receptor (CLR) detect pathogen-related molecular patterns (PAMPs) (Takaoka *et al.,* 2007). PAMPs are specific protected molecules within a class of microorganisms such as β-glucans, peptidoglycans, bacterial DNA (unmethylated CpG DNA), and flagellin, lipopolysaccharide (LPS). TLRs transduce the signal through adaptors, such as Toll/interleukin-1 receptor (TIR) domain-containing adaptor protein (TIRAP), myeloid differentiation factor 88 (MyD88), Toll-receptor-associated activator of interferon (TRIF), and Toll-receptor associate molecule (TRAM) (Akira & Takeda, 2004). These adaptors activate NF-κB proteins through signal propagation. NF-kB belongs to a family of dimeric transcription factors that perform functions such as expression of genes involved in the inflammatory process, development, control of apoptosis, and immune response (Gilmore & Wolenski, 2012).

CONCLUSION

In conclusion, this study demonstrated that *S. cerevisiae* and citric acid as feed additives (instead of the antibiotic growth promoter Virginiamycin), either alone or with synergistic effects, improved the productive performance of laying Japanese quails. They also improved egg quality by increasing shell weight and shell thickness. These supplements exerted positive effects on the morphological characteristics of intestinal villi, and improved intestinal digestive and absorption capacity. The results suggest that dietary supplementation of *S. cerevisiae* exerts beneficial effects on AvBD 1 and 2gene expression. Further experiments will be necessary for understanding the molecular mechanisms regulated by dietary factors.

CONFLICT OF INTEREST

There is no conflict of interest regarding this article

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