Dietary supplementation of *Bacillus subtilis, Saccharomyces cerevisiae* and their symbiotic effect on serum biochemical parameters in broilers challenged with *Clostridium perfringens*

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**ABSTRACT**

The study was conducted to evaluate the effect of an antimicrobial growth promoter (AGP), probiotic, prebiotic and their combination (symbiotic effect) on blood biochemistry of broiler chickens challenged with *Clostridium perfringens*. Two hundred and forty, 1-d-old Ross 308 broilers were distributed into six groups – the negative control (basal diet), positive control (*C. perfringens* infection), AGP + challenge, probiotic + challenge, prebiotic + challenge and symbiotic + challenge. The results showed significant differences in total protein, albumin, globulin and glucose in AGP with no significant difference between negative and positive control. Triglyceride and total cholesterol decreased significantly among all groups compared with positive control. Heterophils and heterophils-to-lymphocytes ratio (H/L) were significantly (*P* < .001) lower, and lymphocytes were significantly (*P* < .001) higher in all groups compared with positive control. It can be concluded from this study that the synergistic effect of *Bacillus subtilis* and Technomos was superior to that of the AGP in improving the blood biochemical profile of broiler challenged with *C. perfringens*.

**Introduction**

Necrotic enteritis is one of the deadliest diseases in broilers caused by *Clostridium perfringens*, which damages the intestinal mucosa, decreases digestion and absorption of nutrients (Abudabos et al., 2018) and leads to loss of performance (Abudabos and Yehia, 2013). The infection of *C. perfringens* is associated with poor feed efficiency, deteriorated gut morphology and severe alteration in blood biochemistry in the exposed broiler (Abudabos et al., 2018). Antibiotics are used several times at higher doses than a subtherapeutic level in animals to cure the infectious diseases (Khan, Nikousefat, et al., 2012; Khan, Naz, Nikousefat, et al., 2012). It has been suggested that antibiotics could be used at lower doses than the therapeutic standard in healthy birds to avoid any future challenge (Khan, Naz, Nikousefat, et al., 2012; Khan, Naz, Tufarelli, et al., 2012). Therefore, at the subtherapeutic level, antibiotics are used as antimicrobial growth promoters (AGPs) in poultry feed, which improve growth and prevent some specific intestinal diseases (Alzawqari et al., 2016; Abudabos et al., 2016, 2017; Khan et al., 2016). However, the use of AGPs has been linked with increasing concerns of bacterial resistance to antibiotics in both animals and human (Khan, Nikousefat, et al., 2012; Khan, Naz, Nikousefat, et al., 2012; Holmes et al., 2016). An intensive research is going to look for alternatives to AGPs to improve the performance in poultry (Khan, Naz, Tufarelli, et al., 2012; Khan, Naz, Javadani, et al., 2012). Among the alternative products, probiotics and prebiotics are being extensively used in poultry production (Chand et al., 2016).

It was reported that *B. subtilis* as a probiotic bacteria improved the microbial balance in the GIT of poultry by immune stimulation and competitive exclusion (Khan et al., 2016). *B. subtilis* which have a broad activity against *Clostridium* spp. was found to improve the immunity level, modulate intestinal microflora, inhibit pathogens and improve performance in broiler chickens (Khan and Naz, 2013; Abudabos et al., 2017).

Prebiotics such as mannan-oligosaccharide (MOS) and β-1,3-glucans, which are derived from the cell membrane of *S. cerevisiae*, was found to enhance the growth in chickens by improving the immune response and intestinal histomorphology under a stress condition (Chand et al., 2016). The probiotic mixed with the prebiotic is known as symbiotic. This mixture increases the existence of probiotics bacteria in the digestive tract of chicken, thus contributing to the increased stabilization of probiotic (Youssef et al., 2011).

When an infectious organism enters into an animal body, the blood biochemistry is expected to change. There are contradicting results regarding the effects of probiotics, prebiotics and symbiotic on the blood biochemical components in broilers. Kannan et al. (2005) reported that MOS decreased some biochemical blood components in broilers such as serum total cholesterol (TC) and total globulin. Recently, Abudabos et al. (2017) reported that TC decreased and blood protein and albumin increased significantly in response to *Bacillus subtilis* supplementation in broilers infected with *Salmonella typhimurium*. In addition, Abd El-Azeem (2002) reported that probiotic
supplementation increased the blood concentration of total protein (TP), albumin and globulin. Conversely, several studies showed that probiotics had no effect on serum TC, TG and low-density lipoprotein (LDL) cholesterol in broiler chickens (Chawla et al., 2013). Also, dietary supplementation with probiotic and prebiotic did not have any effect on TP, albumin and globulin in broiler chickens (Shahir et al., 2014). On the other hand, these supplements have a beneficial effect in lowering stress levels to resist infection and increase lymphocytes in the broiler (Paryad & Mahmoudi, 2008). Most of the contradictory results published have documented the effect of probiotics and prebiotics under the healthy condition of the birds. The objective of the present study was to compare the effects of AGP, probiotic, prebiotic and their combination on blood biochemistry and leukocytes dynamics in broiler chickens challenged with C. perfringens during the finisher stages.

Materials and methods
This study was approved by the committee on ethics and animal welfare, Department of Animal Production, King Saud University, Saudi Arabia.

**Birds husbandry and experimental design**
Two hundred and forty, 1-day-old Ross 308 broiler chicks (mixed sex) were randomly allotted to 60 experimental units (4 chicks per unit). The experiment was conducted in an environmentally controlled room from 1 to 35 days. The temperature was maintained at 22°C during the finisher stage. Feed and water were given ad libitum under a 24 h light schedule.

All birds were fed on corn and soybean diet including a starter (21.5% CP and ME 3000 kcal/kg feed from 1 to 13 days), and finisher (21% CP and ME 3100 kcal/kg feed from 14 to 35 days) diets formulated according to Ross Management Guide recommendations. On day 14, birds were randomly distributed to six groups as follows: Groups 1 and 2, negative and positive control, unsupplemented diets; Group 3, antibiotic, Neoxyval® (0.5 g/kg), contained 200 mg oxytetracycline and 200 mg neomycin (Kela, Belgium) dosed at the therapeutic level; Group 4, probiotic, GalliPro® (0.6 g/kg, Brioche, Lone, Germany), containing B. subtilis (1 × 10⁶ CFU); Group 5, probiotic as MOS (TechnoMos®, 0.75 g/kg, Brioche, Lone, Germany); Group 6, supplemented with the same mixture of probiotic (GalliPro®, 0.6 g/kg) and prebiotic (TechnoMos®, 0.75 g/kg) (synbiotic) under a challenged condition of C. perfringens. On day 14, Groups 2–6 were challenged with C. perfringens (4 × 10⁸ CFU/ml) according to the procedure described by Abudabos et al. (2017).

**Table 1.** Effect of treatments and age on TP, albumin, globulin and glucose in broilers.

| Treatment         | Challenge | TP (g/dl) | Albumin (g/dl) | Globulin (g/dl) | Glucose (mg/dl) |
|-------------------|-----------|-----------|----------------|----------------|-----------------|
| Negative control  | –         | 3.44ab    | 1.86           | 1.58b          | 205.5           |
| Positive control  | +         | 3.26cd    | 1.90bc         | 1.36b          | 204.0           |
| Antibiotic (AGP)  | +         | 3.16d     | 2.03a          | 1.13c          | 210.5           |
| Probiotic         | +         | 3.54ab    | 2.02a          | 1.52b          | 193.1           |
| Prebiotic         | +         | 3.42bc    | 1.99bc         | 1.42b          | 199.5           |
| Symbiotic         | +         | 3.63a     | 1.87bc         | 1.76a          | 200.7           |
| SEM               |           | 0.068     | 0.053          | 0.071          | 4.732           |
| P-value           | ***       | ***       | ***            | ns             |                 |
| Age (days)        | Challenge |           |                |                |                 |
| 14                |           | 3.78a     | 2.23a          | 1.55a          | 190.6           |
| 21                |           | 3.08d     | 1.77c          | 1.31b          | 186.6           |
| 28                |           | 3.52a     | 1.83c          | 1.69a          | 227.3           |
| 35                |           | 3.27a     | 2.00d          | 1.27b          | 204.5           |
| SEM               |           | 0.053     | 0.043          | 0.058          | 3.86            |
| P-value           | ***       | ***       | ***            | ns             | **              |
| Treatment*age     | P-value   |           |                |                |                 |
| ns                |           | ***       | **             | *              |                 |

*Note:* a–d Mean values within a column with different superscripts are significantly different; ns = non-significant; *P < .05; ** P < .01; *** P < .001.

Data effect of treatment are means of 40 replicate birds (n = 40) of dietary treatment each, data effect of age are means of 60 replicate birds (n = 60) and data interaction effect are means of 10 replicate birds (n = 10).

Confirmation of C. perfringens in infected birds
Upon arrival and before the introduction of infection, the chicks were confirmed for the absence of C. perfringens by the method described by Abudabos et al. (2018) (Figure 1).

Biochemical measurements of blood
At days 14, 21, 28 and 35, 5 ml of blood was collected from the wing vein from two birds per replicate. Blood samples were centrifuged at 3000 × g for 15 min. Serum was separated and stored at −80°C until measurements were performed. The analysis of TP, albumin, globulin, triglycerides (TG), TC and high-density lipoprotein (HDL) was conducted by using reagent kits (United Diagnostics Industry, KSA), with the help of a spectrophotometric analyzer (UDICHEM 310, KSA). To determine serum globulin, albumin concentration was subtracted from the TP level. The LDL cholesterol was calculated by using the formula, LDL cholesterol = TC − HDL cholesterol − (TG/5) as described by Panda et al. (2006) (Figure 7).

Heterophil (HET), lymphocyte (LYM) count and their ratio were determined by the method as described by Chand et al. (2017). Two birds were randomly selected from each replicate and blood samples (2 ml) were taken from the wing vein with the help of a sterile syringe without any anticoagulant. Hematological parameters were assessed by using one drop of blood and then smeared on a glass slide. The smears were fixed and stained using Giemsa. HET-to-LYM ratio was calculated by counting at least 100 leucocytes per bird per replicate of each group.

Statistical analysis
The experiment was conducted in a completely randomized design with 6 × 4 factorial design. Data obtained were subjected to statistical analysis using SAS (2008). Data were subjected to ANOVA by using JMP software version 11 (SAS, 2009). The statistical differences between means were analysed by Duncan’s multiple range tests (P ≤ .05). All values were expressed as statistical means ± standard error of the mean (SEM).
The following statistical model was used for the experiment:

\[ Y_{ijk} = \mu + T_i + A_j + TA_{ij} + B_k + e_{ijk}, \]

where \( T_i \) is the effect of treatment (\( i = 1, \ldots, 6 \)), \( A_j \) is the effect of age (\( j = 1, \ldots, 4 \)) and \( B_k \) is the effect of block (\( k = 1, \ldots, 10 \)). \( TA_{ij} \) is the interaction between treatment and age (\( 1, \ldots, 24 \)). \( \mu \) is the general mean and \( e_{ijk} \) is the random error associated with \( Y_{ijk} \) observation.

**Results**

Effects of all dietary treatments on TP, albumin, globulin and glucose are presented in Table 1. Birds received synbiotic showed the highest TP level, but it was not different from those which had received probiotic and negative control. A significant interaction between treatments and age for TP (\( P < .001 \)) was observed (Figure 1). Birds which had received probiotic and prebiotic had a higher TP at day 14 of age compared with all other treatments. At day 35, TP values were higher for probiotic and prebiotic (3.41 and 3.43 g/dl, respectively) as compared to the AGP (2.90 g/dl).

Birds received AGP showed a higher albumin level as compared to synbiotic and negative control, but it was not different from those who had received probiotic, prebiotic and positive control (\( P > .05 \)). Albumin concentration was the lowest when measured at 21 days, while it was the highest when measured at 14 days. The interaction between treatments and age did not show any significant changes in albumin concentration.

Birds which had received synbiotic showed the highest globulin concentration, while those which had received AGP had the lowest globulin (\( P < .001 \)). At the 14th day, birds which received probiotic and prebiotic had a higher globulin level as compared to positive and negative control. Birds received positive and negative control (1.40 and 1.75 g/dl, respectively) had a higher globulin level at day 21 as compared to probiotic and prebiotic. A significant interaction was found between age and treatments (Figure 2).

A significant interaction between treatments and age in glucose (\( P < .01 \)) was found (Figure 3). Birds received probiotic, prebiotic and synbiotic had lower glucose levels at day 14 (161.83, 171.78 and 174.73 mg/dl, respectively) as compared to positive and negative control (206.38 and 207.93 mg/dl, respectively). At day 21, glucose was lower for probiotic and synbiotic as compared to all other treatments (170.20 and 174.53 mg/dl, respectively) of age.

The effect of all dietary treatments on TG, TC, HDL and LDL are presented in Table 2. Birds received synbiotic and prebiotic showed the lowest TG level when compared to all other treatments. In addition, TG was influenced by time of collection (age) (\( P < .001 \)). TG concentration was decreased (\( P < .001 \)) linearly from days 14 to 35.

A significant interaction between treatment and age for TG (\( P < .001 \)) was observed (Figure 4). Birds received prebiotic
had lower TG at days 21 and 28 of age as compared to all other treatments (127.21 and 104.75 mg/dl, respectively), while it was higher than synbiotic at day 35 (98.38 and 90.12 mg/dl, respectively). The result of this trial showed that TC was affected by treatments ($P < .001$), age ($P < .001$) and their interaction ($P < .001$) as shown in Figure 5. Birds received synbiotic had the lowest TC level, but it was not different from those received probiotic or AGP. On the other hand, negative and positive control had the highest TC concentration. Moreover, TC was influenced by time of collection (age) ($P < .001$). Serum TC was the highest when measured after the challenge at day 21 of age, and the lowest at day 35 (Table 2).

Similarly, TC values were affected by the interaction between treatments and age ($P < .001$). Birds received probiotic, prebiotic and synbiotic had lower TC concentration at days 14 and 35 as compared to positive control. On day 21, TC concentration was higher for probiotic, prebiotic and synbiotic as compared to the positive control. Conversely, birds received positive control had lower TC at day 21 of age as compared to the negative control, while the positive control was higher than the negative control at day 35.

The result showed that HDL was affected by treatments ($P < .001$) (Table 2). Birds received AGP showed the lowest HDL level, but it was not different from those received synbiotic (96.2 mg/dl). Birds received negative or positive control or probiotic had the highest HDL. HDL concentration was influenced by time of collection (age) ($P < .001$). HDL concentration was the highest when measured at day 35.

A significant interaction between treatment and age for HDL ($P < .01$) was observed (Figure 6). Birds received probiotic had a higher HDL as compared to AGP at day 14 of age (104.9 and 84.94 mg/dl, respectively), while prebiotic was lower than AGP at day 21 (89.3 and 95.4 mg/dl, respectively). On day 35, HDL values were the lowest for AGP and synbiotic as compared to all other treatments (96.7 and 96.4 mg/dl, respectively).

The result showed that LDL was not affected by treatments but it was affected by age and interaction between age and treatment ($P > .05$, $P < .001$ and $P < .001$, respectively) as shown in Figure 7. LDL was the highest when measured after the challenge at 21 days (Table 2). Birds which had received probiotic and prebiotic had lower LDL values as compared to the positive control on day 14, while they
were higher than the positive control on day 21. At day 35, LDL values were the lowest for probiotic and synbiotic as compared to the AGP and positive control.

The effect of all dietary treatments on LYM, HET and H/L ratio are presented in Table 3. The results showed that LYM, HET and H/L ratio were affected by treatments ($P < .001$), week of collection ($P < .001$) and their interaction ($P < .001$). Birds that received synbiotic had the highest LYM (77.7%). The H/L ratio was the highest for positive control and AGP. This observation could be due to the fact that infected birds fed with probiotic, prebiotic and synbiotic diet had a better capacity in decreasing the stress level compared to the positive control. The positive control group had the lowest LYM, the highest HET and H/L ratio as compared to the negative control which could be due to the infection by $C.\ perfringens$.

HET, LYM and H/L ratio were influenced by time of collection (age) ($P < .001$). HET was the highest, whereas LYM and H/L ratio were the lowest when measured after the challenge on day 28.

A significant interaction between treatments and age for the percentage of HET on days 21, 28 and 35 ($P < .001$) was observed (Figure 8). Birds received positive control had a higher HET percentage on 21 and 28 days of age as compared to all other treatments. At day 35, HET percentage was the higher for positive control and AGP as compared to all other treatments. The interaction of age and treatment for LYM is shown in Figure 9.

Percentages of HET were affected by the interaction between treatment and age ($P < .001$). Birds received positive control had lower HET percentage on days 21 and 28 of age as compared to all other treatments. On day 35, HET percentage was lower for positive control and AGP as compared to all other treatments. A significant interaction between treatments and age for the H/L ratio ($P < .001$) was observed (Figure 10). Birds received positive control had a higher H/L ratio on days 21 and 28 of age as compared to all other treatments. On day 35, the H/L ratio was higher for positive control and AGP as compared to all other treatments.

The result of this study shows that alternatives used for AGP in this trial had a clear impact in increasing TP and globulin while decreasing TG and cholesterol, which appeared to reduce the damaging effect of $C.\ perfringens$ infection.

**Discussion**

In the present study, a lower content of protein in the positive control could be due to the effect of $C.\ perfringens$ on the intestinal wall. This result is in agreement with Kaldhusdal et al. (2001) who reported that intestinal mucosal damage by $C.\ perfringens$ led to the poor digestion and absorption of nutrients. Dietary supplementation with prebiotic increased TP in serum compared with AGP, which indicates that prebiotic can improve body protein anabolism in broilers (Hassanein and Soliman, 2010). Hassanein and Soliman (2010) showed that TP of birds fed on 1.6% probiotic of $S.\ cerevisiae$ was higher than the positive control in layers. In contrast, Wang et al. (2015)
reported that probiotic supplementation of *B. subtilis* and *B. licheniformis* decreased TP as compared to the control group. Variations in the efficacy of probiotics can be explained by the difference in microorganism strains used.
The result obtained in this trial, regarding the albumin concentration, is in agreement with Djouvinov et al. (2005) who showed that probiotic supplementation had no effect on albumin in broilers. Similarly, dietary supplementation with probiotic (S. cerevisiae) and prebiotic (MOS) did not have any effect on albumin (Shahir et al., 2014). The results of globulin in the
The present study are in agreement with those obtained by Abd El-Azeem (2002) who reported that birds which had received symbiotic showed the highest globulin concentration due to the improvement in the absorption and utilization of nutrients. No differences in globulin concentration were found between positive and negative control, probably due to the shift from the acute phase of injury to the chronic phase or could be the result of a decrease in the activity of *C. perfringens* (Klasing and Austic, 1984). The result of the present study showed that glucose was not affected by treatments (*P > 0.05*). This is in agreement with the results of Najafi and Taherpour (2014). Other studies showed that serum glucose was the highest when probiotic was used (Shareef and Al-Dabbagh, 2009). The glucose level was not affected on days 21, 28, and 35 in response to all dietary treatments (*P > 0.05*). These results are in agreement with the findings of Chelladurai et al. (2013). The present values of TC and TG concentration are consistent with the findings of Mahdavi et al. (2005) in hens, and Najafi and Taherpour (2014) in broilers. Ashayerizadeh et al. (2009) reported that the use of probiotic decreased TC and TG in serum when compared with AGP and control. Similarly, in the current study, dietary supplementation with probiotic decreased TC concentration on day 21 as compared to the control, probiotic and AGP. The reduction of TC and TG as a result of probiotic supplementation is consistent with the findings of Kananan et al. (2005) who reported that the use of MOS reduced TC and TG in broilers. However, these findings were in disagreement with Mohebbifar et al. (2013) who reported no significant differences in serum TC, HDL, LDL cholesterol and TG as a result of probiotic and probiotic supplementation. Decreasing TC in this trial may be due to *B. subtilis*, which can be effective in reducing the activity of acetyl coenzyme A carboxylase, a key enzyme involved in the synthesis of fatty acids (Santoso et al., 1995).

The results of blood cells and their indices are in agreement with those obtained elsewhere (Paryad and Mahmoudi, 2008). Whereas the H/L ratio was lower in the probiotic group than prebiotic and control in broilers (Klasing and Austic, 1984). Similar results were indicated by improved immunity by supplementation of probiotic (Tollba et al., 2007).

**Conclusion**

The results of the present study indicated that the combined effect of *Bacillus subtilis* and MOS was superior to that of anti-biotic in broilers challenged with *C. perfringens*.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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