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Dietary Supplementation of *Bacillus subtilis* Probiotics Improves Growth Performance and Immune Response of Ducks-Experimentally Infected with Riemerella Anatipestifer

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Abstract

Riemerella anatipestifer (RA) is a major bacterial disease affecting the poultry industry worldwide, particularly causing high economic losses in duck farms in Egypt. The objective of this experimental study was firstly to investigate the effects of RA-experimental infection on the morbidity and mortality rates and growth performance in Pekin ducks. Secondly the effects of RA on the transcriptomic expression of the immune related genes (interleukins (IL)1β, 6, 8, 10 and 17A) in the infected ducks were also examined using real time quantitative PCR. Thirdly, the protective effects of *Bacillus subtilis* (*B. subtilis*) as a probiotic dietary supplementation against RA infection in ducks were also investigated. The achieved results indicated that the mortality rate in RA-experimentally infected group was 80% and this percentage was reduced to 20% when birds received probiotics in their diet before challenge with RA. Furthermore, RA infection reduced the average feed intake, average body gain with poor average feed conversion ratio, while dietary supplementation of probiotics to ducks' feed significantly improved growth performance parameters. RA modulated the gene expression of the immune related genes, however, probiotics significantly upregulated mRNA expressions of the examined cytokines as a mechanism for the protective roles of probiotics against RA infection in ducks. Therefore, it is highly recommended to use probiotics dietary supplementation in duck farms in order to improve the growth performance and reduce the disease burden.

Keywords: Riemerella anatipestifer; *Bacillus subtilis*; Probiotics; Immunity; Growth performance; Ducks

Introduction

Riemerella anatipestifer (RA) is a Gram's negative bacteria that cause respiratory disease in ducks, geese, turkey and chicken [1]. Several outbreaks of RA infection occurred worldwide, particularly in the duck [2,3]. The diseased birds suffer from respiratory and nervous symptoms and during necropsy, the dead birds had pericarditis, perihepatitis, and meningitis [4]. RA was responsible for many outbreaks in ducks and ducklings reared in Egypt causing severe economic losses [5].

Antimicrobials are extensively used in poultry and duck farms in Egypt and other African countries for the purposes of prevention and control of bacterial diseases and as feed additives to improve the bird's performance and feed-conversion ratio [6]. However, the abuse of these antimicrobials led to development of multidrug resistant pathogens. Multidrug-resistant RA was isolated from intensively reared ducks, water fowls and migratory birds in several reports [7-9].

Probiotics are beneficial microorganisms that can be added into the bird's ration in order to replace the gut harmful microbiota and can improve the overall performance and productivity of the birds mainly via a) activation of the immune system [10], b) maintain the gut microbial homeostasis [11], c) antimicrobial functions [12], d) improvement of the metabolic functions and detoxification of toxins and mutagens [13]. However, few reports had investigated the protective effects of probiotics supplementation on RA infection in ducks.

In sight of the previous facts, this study aimed at investigating the protective and beneficial effects of probiotics supplementation on RA experimental infection in ducks via studying the feed conversion ratio, feed intake and body gain in the experimental groups. Furthermore, the effects of probiotics on RA-down regulated immune related genes were investigated using quantitative real-time PCR (qPCR).

Materials and Methods

Birds and experimental groups

Eighty (one day old) Pekin ducklings (Anas platyrhynchos domesticus) were purchased from a commercial hatchery in Egypt. Birds were grouped separately into four experimental groups; the first group received normal starter duck ration (crude protein 22%, crude fat 3.59%, crude fiber 3.84% and energy 2900 kcal/kg, Alfa Feed International, Egypt) and considered as a control group, the second group received the same ration and challenged with RA serotype 1 via intramuscular injection on their 15th day and named here and after RA group, the third group received *Bacillus subtilis* (probiotic) - supplemented ration and challenged with an intramuscular injection of RA on their 15th day and named RA+probiotic group, the fourth group received *Bacillus subtilis*-supplemented ration with no microbial challenge and named probiotic group. *Bacillus subtilis* (1×10^7 CFU/g, Weifang, China) was added to the commercial duck starter ration at 250 g/ton). RA serotype 1, the challenging pathogenic strain, was

kindly gifted from Animal Research Institute, Cairo, Egypt and it was originally isolated from a commercial duck farm and prepared according to Fernandez et al. [14]. All birds received free access to antibiotic free-water and feed. Constant light was provided during the experimental duration (30 days). Survival percentages and growth performance were recorded. On the age of 30th day, five birds from each group were sacrificed by cervical dislocation and liver and spleen tissues were collected and snap frozen for total RNA isolation. All animal experiments were done according to the guidelines for the animal use adopted by Faculty of Veterinary Medicine, Zagazig University, Egypt.

Bacterial re-isolation

Re-isolation of RA from the liver and spleen of the dead birds or from collected tissue at the end of the experiment according to Fernandez et al. [14] with slight modifications. Briefly, one g of liver and spleen was aseptically collected and homogenized separately in 500 ml of tryptic soy broth using tissue homogenizers. A loopful from each homogenized sample was streaked on 5% sheep blood agar plates, and incubated at 37°C. The bacterial isolates were gram-negative coccobacilli and identification was confirmed biochemically and serologically.

Growth performance

Growth performance parameters including average feed intake (AFI), average body gain (ABG) and average feed conversion ratio (AFCR) were recorded at five-day intervals according to Abudabos et al. [15].

RNA isolation

Total RNA was extracted from liver and spleen of the sacrificed ducks according to the method published before [16] using TRI reagent (Sigma, Sigma, St. Louis, USA) combined with homogenization (Bead Blaster 24 Tissue Homogenizer, Sayreville, NJ 08872, USA). The clear lysate was strongly mixed with chloroform for 2 min and followed by centrifugation at 13000 rpm for 20 min at 4°C. RNA was precipitated using isopropanol and centrifugation at the same conditions followed by washing with 70% ethanol and RNA was dissolved in RNAse free water. RNA concentrations and qualities were determined using a Nanodrop ND-1000 spectrophotometer (DYMO, Stamford, Conn., USA).

cDNA synthesis

For cDNA synthesis, Rever Tra Ace® qPCR RT Master Mix with gDNA remover (Toyobo Co. Ltd., Osaka, Japan) was used as described in the manufacturer's instructions. cDNA samples were stored at -20°C for further analysis.

Quantitative real time PCR (qPCR) for immune response genes

The mRNA expression levels of the immune response genes including interleukins (IL-) 1β, 6,8,10 and 17A were determined using qPCR, in Step One Plus Real-Time PCR system (Applied Biosystems, Foster, CA). The PCR mixture contained 600 ng of cDNA, Fast SYBR® Master Mix, 5 µM of each primer, with RNase-free water added to a final volume of 10 µL. The reaction cycle started with initial incubation for 20 s at 95°C, followed by 40 cycles of 3 s at 95°C (denaturation), 30 s at 60°C (annealing) and 15 s extension at 95°C. Single amplicon amplification was confirmed using melting curve analysis. The absence of primer dimers and genomic DNA amplification were confirmed by agarose gel electrophoresis. GAPDH was used for normalization by the comparative ΔΔCt method. Primer sets used were prepared according to published reports [17,18] and were displayed in Table 1.

Target	Nucleotide sequence	Product size (bp)	Accession number
IL-1β F	5'-TCATCTTCTACCGCCTGGAC-3'	149	DQ393268
IL-1β R	5'-GTAGGTGGCGATGTTGACCT-3'		
IL-8 F	5'-AAGTTCATCCACCCTAAATC-3'	182	DQ393274
IL-8 R	5'-GCATCAGAATTGAGCTGAGC-3'		
IL-6 F	5'-TTCGACGAGGAGAAATGCTT-3'	150	AB191038
IL-6 R	5'-CCTTATCGTCGTTGCCAGAT-3'		
IL-10 F	5'-CTGACCTCCTACCAGCGAAG-3'	179	NM001310368
IL-10 R	5'-CTCCATGTAGAACCGCATCA-3'		
IL-17A F	5'-ATGTCTCCAACCCTTCGT-3'	185	EU366165
IL-17A R	5'-CCGTATCACCTTCCCGTA-3'		
GAPDH F	5'-ATGTTCGTGATGGGTGTGAA-3'	176	AY436595
GAPDH R	5'-CTGTCTTCGTGTGTGGCTGT-3'		

Table 1: Primer sequences used in the present study.

Statistical analysis

Statistical significances among experimental groups were analyzed using Tukey's Kramer HSD test (JMP program, SAS Institute, Cary, NC, USA) with $P<0.05$ considered as significant.

Results and Discussion

Ducks experimentally infected with RA in the present study suffered from loss of appetite, poor growth nervous manifestations, respiratory symptoms, and diarrhea. The morbidity rate in RA-experimentally infected group was 100%. While this percentage was reduced to 70% in the RA-experimentally infected group that received probiotics-supplemented ration (Table 2). Similar symptoms were reported in ducks naturally infected with RA [5]. The mortality rate in RA-experimentally infected group was 80% and this percentage was reduced to 20% when birds received probiotics in their diet before challenge with RA. Likely, Subramaniam et al. [19] demonstrated that RA causes mortality rates as high as 75% in ducks. The re-isolation percentages were 100% and 70% in both RA and RA +probiotics group (Table 2).

At necropsy, the most prominent findings were fibrinous perihepatitis, pericarditis, and airsacculitis in RA group which agrees with Sandhu [4]. Relatively higher mortality rate (16%) in RA-naturally infected ducks was reported in Japan during 2014. The diseased birds were 15-21 days old, suffering from ataxia, leg paddling, and dorsal decumbency; while at postmortem inspection, diseased birds showed meningitis, air sacculitis, pericarditis and perihepatitis [20]. Furthermore, high prevalence rate (65.6%) for RA in naturally infected waterfowls was reported in Taiwan [21]. Concerning the growth performance of the ducks experimentally infected with RA, the achieved data were reported in Table 3.

Group	Morbidity	Mortality	Re-isolation
Control	0	0	0
RA	100%	80%	100%
RA+Probiotic	70%	20%	70%
Probiotic	0	0	0

Table 2: Effects of probiotic supplementation on the survival rates of ducks experimentally infected with RA.

Group	AFI (g/day)		ABG (g/day)		AFCR	
	D1-15	D16-30	D1-15	D16-30	D1-15	D16-30
Control	50.22 ± 5.15a	105.22 ± 9.15a	27.14 ± 4.22a	45.22 ± 3.32a	1.85	2.33
RA	55.17 ± 4.12a	72.17 ± 6.12b	29.22 ± 5.25a	21.22 ± 2.11b	1.88	3.4
RA+Probiotic	81.11 ± 7.14b	100.11 ± 5.14a	53.61 ± 8.15b	43.51 ± 5.17a	1.51	2.3
Probiotic	85.14 ± 7.21b	152.14 ± 9.21c	55.18 ± 5.17b	80.28 ± 9.77c	1.54	1.89

Table 3: Effect of probiotic supplementation on the growth performance of ducks experimentally-infected with Riemerella anatipestifer. RA: Riemerella anatipestifer; AFI: average feed intake; ABG: average body gain; AFCR: average feed conversion ratio. Values in the same column carrying different super script letter are significantly different with each other at $P<0.05$.

It was clear that AFI (g/day) was significantly reduced in RA infected group (72.17 ± 6.12) compared with the control (105.22 ± 9.15) and RA+Probiotic (100.11 ± 5.14). Similarly, the ABG (g/day) was the lowest in RA group (21.22 ± 2.11) in a comparison with the control group (45.22 ± 3.32) and RA+Probiotic (43.51 ± 5.17). When birds received only probiotics-supplemented ration, their AFI (152.14 ± 9.21 g/day) and ABG (80.28 ± 9.77 g/day) were the highest among the tested group. Likely, AFCR value was the best in ducks receiving probiotics in their diet, while RA raised AFCR (3.4) reflecting the poor growth performance in the diseased birds, which much improved in the ducks infected with RA while receiving probiotics (2.3) in their diet (Table 3). Similar poor growth performance was reported in RA-naturally infected dusks in Egypt [5], Japan [20] and Taiwan [21].

Interleukins have emerged as critical molecules in host protective immunity and inflammatory diseases [22,23]. Therefore, screening of the transcriptomic changes of immune related interleukins expressed in both liver and spleen was investigated in order to explain the possible reasons for the high morbidity and mortality rates in RA-experimentally infected group. This screening was conducted using qPCR analysis as clear in Figures 1 and 2. RA mainly targets both liver and spleen tissues as reported before [14]. The achieved results in Figure 1 indicated significant down regulation (fold relative to control) of IL-8 (0.33 ± 0.04) and IL-10 (0.39 ± 0.09) in the liver tissue of RA-experimentally infected ducks. In the spleen, the expression levels of these interleukins were in the same trend as the liver as clear in Figure 2. The reduction (fold relative to control) levels were as follows: IL-8 (0.44 ± 0.04) and IL-10 (0.47 ± 0.15). Interestingly, IL-1β (1.35 ± 0.03 and 1.83 ± 0.15), IL-6 (2.61 ± 0.05 and 1.54 ± 0.02) and IL-17A (2.11 ± 0.07 and 3.35 ± 0.11) were drastically up regulated as a response for RA infection in the liver and spleen, respectively. Supplementation of probiotics to ducks significantly up regulated the examined cytokines in the challenged group with RA as clear in Figures 1 and 2. This might explain the protective roles of the probiotics against RA infection and the significant reduction in the morbidity and mortality rates.

The achieved results indicate different roles for each interleukin. In agreement with the achieved results, Zhoe et al. [24] reported that RA infection in ducks modulated the transcriptomic expression of several genes in the affected ducks. For instances, immune response genes such as beta-defensins were downregulated, however IL-6 was upregulated as it plays an important role in the inflammatory response of the birds. In addition, Fernandez et al. [14] had reported significant decreases in the gene expression of IL-2 and IL-4 and increase in the expression of IL-1β, IL-6 and IL17A as a response to RA infection.

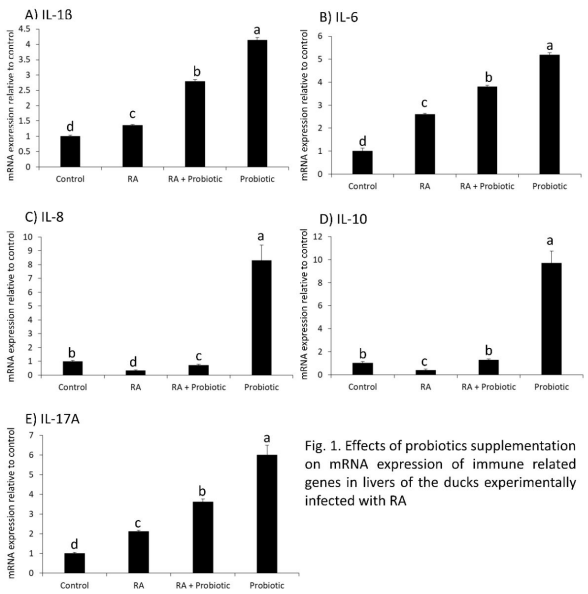

Fig. 1. Effects of probiotics supplementation on mRNA expression of immune related genes in livers of the ducks experimentally infected with RA

Figure 1: Effects of probiotics supplementation on mRNA expression of immune related genes in livers of the ducks experimentally infected with RA.

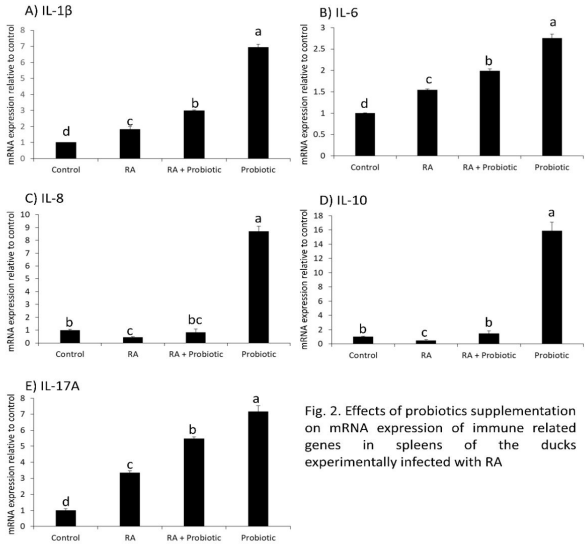

Fig. 2. Effects of probiotics supplementation on mRNA expression of immune related genes in spleens of the ducks experimentally infected with RA

Figure 2: Effects of probiotics supplementation on mRNA expression of immune related genes in spleens of the ducks experimentally infected with RA.

It notes worthy to mention that the supplementation of the duck ration with *B. subtilis* probiotics had led to a significant elevation in the immune response. For instances, the expression levels (fold relative to control) of IL-1β (4.13 ± 0.09), IL-6 (3.18 ± 0.09), IL-8 (8.32 ± 1.08), IL-10 (9.70 ± 1.07) and IL-17A (5.99 ± 0.49) in the liver. Similarly, the expression levels (fold relative to control) of the tested interleukins were significantly up regulated, IL-1β (6.92 ± 0.19), IL-6 (2.75 ± 0.09), IL-8 (8.71 ± 0.39), IL-10 (15.88 ± 1.21) and IL-17A (7.17 ± 0.35). The protective effects of *B. subtilis* probiotics were reported in ducks challenged with *E. coli* and novel duck reovirus, as *B. subtilis* improved innate immune response, growth performance and resistance to pathogens [25]. The possible mechanisms for the protective effects of the dietary probiotics were suggested to include improving gut barrier function, antimicrobial activity, improving the resistance against pathogens, competition for nutrients, degradation of toxins and secretion of inhibitory substances [26].

Conclusion

This experimental study demonstrated the protective roles of *B. subtilis* as a probiotic supplemented to duck ration against RA experimental infection. This probiotic improved the growth performance survival rates and reduced the morbidity of the disease. The protection mechanism is suggested to involve up regulation of the immune and inflammation related genes in the liver and spleen of ducks. Therefore, it is highly recommended to use this probiotic type in duck farms as a preventive measure against RA infection.

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Conflict of Interest

None.

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