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Effects of Isolated *Lactobacillus acidophilus* as a Probiotic on Chicken Vaccinated and Infected with *Salmonella typhimurium*

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Abstract: The efficacy of probiotics, prebiotics and synbiotic as alternatives to antibiotics in prevention of reduction of *Salmonella typhimurium (S. typhimurium)* infections in broiler chickens were studied, as well as the effect on shedding of *S. typhimurium* in both intestine content and internal organs in the experimental chickens. Obtained results proved that addition of probiotic *Lactobacillus acidophilus* (*L. acidophilus*) and/or prebiotic (Agrimos) to the ration of the chickens which were vaccinated with live attenuated *S. typhimurium* vaccine enhance the immune response and produced higher antibody titres than those vaccinated only. The use of probiotics, prebiotic as well as synbiotic may be an effective therapy against *S. typhimurium* shedding after vaccination with live attenuated vaccines or after exposure to the infection by *S. typhimurium* virulent organism. The treated non-vaccinated groups gave better results than vaccinated non-treated group in controlling of *S. typhimurium* organism shedding and this clarified the role of probiotic and/or prebiotic in declining the shedding of the organism. Incorporation of probiotic and/or prebiotic with vaccination with live attenuated *S. typhimurium* vaccine gave better results in decreasing the percentage of *S. typhimurium* reisolation from the internal organs of the chickens than in case of vaccination alone. As well as, in non-vaccinated groups, the using of probiotic and/or prebiotic reduced the percentage of reisolation if compared with non-treated group. Accordingly, it could be recommended using these friendly probiotic and prebiotic preparations beginning from the first day of chick life till marketing or end of production period as alternatives to antibiotics.

Key words: Probiotic · Prebiotic · *Lactobacillus acidophilus* · *S. typhimurium* · Immune Response

INTRODUCTION

Salmonella species is considered one of the major food borne pathogens that may colonize the gastrointestinal tract (GIT) of chickens. They can be associated with processed poultry and may cause sever illness and even death in humans [1].

Control of salmonella infections in poultry is posing itself as one of the difficult problems because of the fact that most of Salmonella serovars, which poultry harbor act as potential pathogens for man [2]. Many researchers, allover the world, have been trying to control and eradicate salmonellosis in poultry by vaccination. Live attenuated Salmonella vaccines may be hazardous because the residual virulence due to insufficient attenuation [3].

Probiotics have been reported to improve gastrointestinal tract balance through bacterial antagonisms, competitive exclusion and immune stimulation [4]. Prebiotics as non-digestible food ingredients stimulate not only growth but also activity of bifidobacteria and lactobacilli in host gut [5]. Synbiotic (probiotic plus prebiotic) may improve the survival rate of probiotics during their passage through the digestive tract, thus contributing to the stabilization and/or potentiation of the probiotic effects [6].

The present work was designed to investigate the effect of probiotic and/or prebiotic on the immunity of broilers. This will be achieved through 1. Isolation and identification of probiotic bacteria from chickens 2. Studying the effects of the isolated and identified probiotic *Lactobacillus acidophilus[L.acidophilus]* on

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broiler chickens vaccinated and challenged with *Salmonella typhimurium* 3. Evaluation of the effects of a prebiotic (Agrimos®) on broiler chickens vaccinated and challenged with *Salmonella typhimurium* 4. Using *Lactobacillus acidophilus* separately or in combination with Agrimos® on broiler chickens vaccinated and challenged with *Salmonella typhimurium* 5. Studying the comparative immune response of non-vaccinated chickens treated with *Lactobacillus acidophilus* and/or Agrimos®.

MATERIALS AND METHODS

Experimental Chickens: A total of 200, one day old, Specific Pathogen Free (SPF) chicks were obtained from SPF Farm at Koum Osheim, Fayoum Province, Egypt. They were divided into 8 groups, each group of 25 chicks as follows:

Group (1): Chickens administrated orally with prepared Lactobacillus (Probiotic) and vaccinated with live attenuated *Salmonella typhimurium* vaccine.

Group (2): Chickens administrated orally with prepared Lactobacillus, fed ration containing Agrimos (Synbiotic group) and vaccinated with live attenuated *Salmonella typhimurium* vaccine.

Group (3): Chickens fed ration containing Agrimos (Prebiotic) and vaccinated with live attenuated *Salmonella typhimurium* vaccine.

Group (4): Chickens vaccinated with live attenuated *Salmonella typhimurium* vaccine.

Group (5): Chickens administrated orally with Lactobacillus,

Group (6): Chickens administrated orally with Lactobacillus and fed ration containing Agrimos.

Group (7): Chickens fed ration containing Agrimos.

Group (8): Chickens were kept as non-treated control negative group.

***Salmonella typhimurium* Live Attenuated Vaccine:** Salmonella Vac T-Avipro (200 doses) produced by Lohmann Animal Health Company (LAH), Germany, Batch No. A026600. It was used in the vaccination of the experimental chicken. The vaccinal dose was 1×10^8 CFU.

Probiotic

Lactobacillus Acidophilus: It was a prepared probiotic used in a concentration of (10^7 CFU/bird) and administrated orally.

Prebiotic: Agrimos® (manno-oligisacchrides) was used in the feed by a concentration of 1kg/ton at one day old of the chickens. It was a product of Lallemand Co., France, distributed by Egavet Co., Egypt, Lot No. 005331E.

Samples

Fecal Swabs: Cloacal swabs were collected for bacteriological examination from all chicken groups for determination of *S. typhimurium* shedding at 3, 7, 14, 21 and 30 days post vaccination with *S. typhimurium* vaccine and at 1, 2, 3, 4, 5, 6, 7, 11, 14, 18, 21, 27 and 30 days post challenge with virulent *S. typhimurium* strain.

Blood Samples: Blood samples were taken from chickens vaccinated with *S. typhimurium* vaccine at 1, 2, 3 and 4 weeks post vaccination and at 3, 7, 14, 21 and 30 days post challenge with virulent *S. typhimurium* strain.

Organ Samples: At the end of the experiment, 4 weeks post challenge with *S. typhimurium* strain, all birds were sacrificed and liver, spleen and heart samples were collected and examined bacteriologically for presence of *S. typhimurium* (Clearance test).

Bacterial Strains

***Salmonella typhimurium* Strain:** Local field isolate of pathogenic strain of *Salmonella typhimurium* was kindly obtained Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo and used for challenge test with a concentration of 1.5×10^8 CFU/0.5ml.

Bacterial Culture Media

Media Used for Isolation and Identification of Lactobacillus

Pre-Enrichment Fluid Media: MRS (Man, Rogsa and Sharpe) broth: It was purchased from Laboratories CONDA, Spain.

Plating Solid Media: MRS-Agar: It was made from adding Agar- agar to the MRS broth.

Soft Agar-Agar Medium: It was purchased from Oxoid LTD. Basing stake, Hampshire, England.

Isolation and Identification of Lactobacillus Strains:
Isolation of Lactobacillus Strain: It was carried out according to Tharmaraj and Shah [7].

Identification of Lactobacillus Strain: According to Bergey's Manual [8]; Tharmaraj and Shah [7] and Messaouda *et al*. [9] (Table 1).

Evaluation of the Humoral Immune Response to *Salmonella typhimurium* Vaccine by Using ELISA: It was performed according to Voller *et al*. [10].

Intestinal Shedding: Cloacal swabs were collected from five birds / group at 3, 7, 14, 21 and 30 days post vaccination with *Salmonella typhimurium* vaccine and at 1, 2, 3, 4, 5, 6, 7, 11, 14, 18, 21, 27 and 30 days post challenge with virulent *Salmonella typhimurium* strain for exploring the frequency of *Salmonella typhimurium* fecal shedding [11].

Challenge Test Against *Salmonella typhimurium*: Challenge test was done using 0.5 ml of *Salmonella typhimurium* containing 1.5 X 10^8 CFU of *Salmonella typhimurium.* Chicks were challenged orally by dropper at four weeks after vaccination and observed for one month. The degree of protection was assessed according to the severity of the clinical signs, the mortality rate and the reisolation of the challenge organisms from post mortem materials [12].

RESULTS

Isolation and Identification of *Lactobacillus acidophilus*:

DISCUSSION

Salmonella is considered as one of the important causative agents which infect poultry farms specially that which apply the modern intensive system of rearing and management. Any contributions for elimination of salmonella in birds could have a major influence in reducing the populations of the organism under natural conditions [13].

Table 1: Summary of isolation and identification results of *Lactobacillus acidophilus*

Test	Result
* Growth on MRS agar	Small (0.5 mm) shiny yellowish brown colonies
* Gram Stain	Gram Positive Small Bacilli
* Motility Test	Non-Motile
* Catalase Test	Catalase Negative
* Glucose Fermentation Test	- Pink colour (Glucose fermentation) (Positive)
- No CO_2 formation (Negative)	
* Sorbitol Fermentation Test	- No Sorbitol fermentation (Negative)

The previous results proved that the suspected Lactobacillus isolate was *Lactobacillus acidophilus*

Table 2: ELISA antibody titre of chickens treated with prepared probiotic (Lactobacillus), patent prebiotic (Agrimos®) post vaccination with live attenuated *Salmonella typhimurium* vaccine and challenge with virulent *Salmonella typhimurium*

	Weeks Post Vaccination				Days Post Challenge				
Groups	1	2	3	4*	3	7	14	21	30
Group (1)	866.96	977.24	1002.31	1106.62	788.86	1339.68	2415.46	2760.58	4111.49
Group (2)	843.33	1088.93	1749.85	1849.27	2192.8	3006.08	3258.37	3334.26	4864.07
Group (3)	274.16	421.69	772.68	1073.98	1018.59	1749.85	2517.68	2594.18	3443.49
Group (4)	610.94	883.08	1672.94	2060.63	1315.22	1667.24	2041.73	2280.34	2488.86
Group (5)	170.03	201.11	199.10	250.25	255.89	1342.76	2666.86	2500.3	2238.7
Group (6)	165.50	180.55	245.43	190.04	321.07	2582.26	3221.07	3047.0	2606.15
Group (7)	295.74	159.20	303.10	248.25	342.77	501.2	1745.8	1625.55	331.1
Group (8)	188.79	369.83	102.56	358.09	687.07	827.94	2904.02	1577.61	1415.79

*Time of Challenge

Table 3: Faecal shedding of *Salmonella typhimurium* from chickens treated with prepared probiotic (Lactobacillus), patent probiotic (Bactocell®), patent prebiotic (Agrimos®) post vaccination with live attenuated *Salmonella typhimurium* vaccine

		Days Post Vaccination						
Groups	No. of samples	3	7	14	21	30	No. of +ve / Total No.	% of +ve
Group (1)	25	3.5	0.5	0.5	0.5	0.5	3.25	12%
Group (2)	25	1.5	3.5	0.5	0.5	0.5	4.25	16%
Group (3)	25	2.5	5.5	0.5	0.5	0.5	7.25	28%
Group (4)	25	1.5	1.5	0.5	0.5	0.5	2.25	8%

Table 4: Faecal shedding of *Salmonella typhimurium* from chickens treated with prepared probiotic (Lactobacillus), patent prebiotic (Agrimos®) post vaccination with live attenuated *Salmonella typhimurium* vaccine and challenged with virulent *Salmonella typhimurium* strain

		Days Post Challenge														
Groups	No. of samples	1	2	3	4	5	6	7	11	14	18	21	27	30	No. of +ve / Total No.	% of +ve
Group (1)	65	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.65	0%
Group (2)	65	0.5	0.5	1.5	1.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	2.65	3.8%
Group (3)	65	1.5	1.5	1.5	1.5	1.5	1.5	2.5	3.5	5.5	5.5	4.5	1.5	0.5	26.65	40%
Group (4)	65	0.5	1.5	4.5	3.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	0.5	16.65	24.6%
Group (5)	65	3.5	2.5	3.5	1.5	2.5	1.5	1.5	1.5	1.5	1.5	0.5	0.5	0.5	16.65	24.6%
Group (6)	65	5.5	4.5	3.5	2.5	1.5	1.5	2.5	1.5	0.5	0.5	0.5	0.5	0.5	19.65	29.2%
Group (7)	65	3.5	4.5	2.5	5.5	1.5	2.5	2.5	2.5	1.5	2.5	2.5	2.5	2.5	30.65	46.2%
Group (8)	65	4.5	4.5	3.5	2.5	1.5	1.5	2.5	2.5	3.5	4.5	4.5	3.5	3.5	36.65	55.4%

Table 5: Isolation of *Salmonella typhimurium* (Clearance test) from Liver, Spleen and Heart of chickens treated with prepared probiotic (Lactobacillus), patent prebiotic (Agrimos®) post vaccination with live attenuated *Salmonella typhimurium* vaccine and challenged with virulent *Salmonella typhimurium* (4 weeks post challenge)

Groups	No. of samples	Liver	Spleen	Heart	Total +ve / Total No.	% of +ve
Group (1)	24	0.8	0.8	0.8	0.24	0%
Group (2)	24	0.8	0.8	0.8	0.24	0%
Group (3)	24	0.8	1.8	0.8	1.24	4.2%
Group (4)	24	0.8	1.8	0.8	1.24	4.2%
Group (5)	24	2.8	0.8	4.8	6.24	25%
Group (6)	24	0.8	0.8	4.8	4.24	16.7%
Group (7)	24	4.8	3.8	4.8	11.24	45.8%
Group (8)	18*	6.6	5.6	4.6	15.18	83.3%

* On the day 14 post challenge, 3 out of 10 chickens were died.

The currently available vaccines against salmonellosis can be divided into three major classes: bacterins, attenuated and subunit vaccines. Protection induced by bacterins in poultry is generally mild; killed vaccines elicit good antibody responses but induce poor cell mediated immunity. Live attenuated vaccines have multiple advantages because of their ease of administration, ability to carry heterologous antigens and capacity to induce cellular and humoral immune responses [14].

These mentioned facts attracted attention of poultry men to be back to the nature by using environmentally friendly products such as probiotics, prebiotics and synbiotic as a substitute to antibiotics to avoid their bad effect, high cost and bacterial contaminated table eggs. The "WHO" is urging the meat producing countries around the world to use “Environmentally Friendly” alternative methods of controlling infectious diseases. Probiotics, prebiotics, synbiotics and organic acids have been suggested to be these alternatives.

For all aforementioned facts, the aim of this study was designed to study the effect of using of prebiotics, probiotic and synbiotics on the broiler chickens either vaccinated with *S. typhimurium* vaccine, infected with salmonella or control (without vaccination or infection). In this study, a probiotic was used, *L. acidophilus* which was isolated from gastrointestinal tract of chickens (whereas it is found normally in the gut) and a prebiotic that was called Agrimos®. So, it was very important to isolate and identify the used *L. acidophilus*.

The result of isolation showed that the colonies grown on MRS agar (selective media for lactobacillus) were small, shiny yellowish brown in colour (typical characters of lactobacillus), as described by Tharmaraj and Shah [7]. Because of presence of several species of lactobacillus (*L. acidophilus, L. casi spp. casi*, *L. casi spp. rhamnosus* and *L. fermentum*) gave the same colony characters, identification of the isolated colonies was carried out to determine the lactobacillus species. Gram staining, microscopical examination, motility test and biochemical tests revealed that the isolate was Gram positive bacilli, non-motile and catalase negative. The four species of lactobacillus gave the same results. On performing the sugar fermentation tests, it was found that the isolate did not give CO_2 production from glucose (-ve). So, *L. fermentum* was excluded. On the other side, the isolate gave negative result of acid production (in case of sorbitol) and positive result (in case of glucose). Depending on this result, *L. casi spp. casi*, *L. casi spp. rhamnosus* were also excluded, where they gave positive results with both sugars (sorbitol and glucose). From all previous results of isolation and identification tests, it was confirmed that the isolated species was *Lactobacillus acidophilus*. These results were in agreement with that of Bergey's Manual [8] and Tharmaraj and Shah [7].

Regarding the antibody titres against *S. typhimurium* that was measured using ELISA, it can be noted from the data illustrated in Table 2 that the antibody titres of G1, G2, G3 and G4, began with (866.96, 843.33, 274.16 and 610.94) in the first week post vaccination, respectively, then increased gradually till reached (1106.62, 1849.27, 1073.98 and 2060.63) in the fourth week post vaccination for G1, G2, G3 and G4, respectively, while the non vaccine groups G5, G6, G7 and G8 began with (170.03, 165.5, 295.74 and 188.79) and ended after 4 weeks with (250.25, 190.04, 248.25 and 358.09).

After challenge with virulent *S. typhimurium*, the titres of the G1, G3 and G4 showed slight decrease (788.86, 1018.59 and 1315.22, respectively) on the 3rd day post challenge, contrarily G2 showed slight increase in the same day (2192.8). From the 7th day post challenge, the previous groups (G1, G2, G3 and G4) continued increasing gradually till reached the maximum levels in 30th day post challenge (G1 "4111.49", G2 "4864.07", G3 "3443.49" and G4 "2488.86").

On the other hand, in non-vaccinated groups (G5, G6, G7 and G8), on infection with virulent *S. typhimurium*, the antibody titres began with (255.89, 321.07, 342.77 and 687.07), respectively on the 3rd day post infection, then increased gradually till reached the peak on the 14th day post infection (2666.86 "G5", 3221.07 "G6", 1745.8 "G7" and 2904.02 "G8"). The antibody titres of these groups began to decline again till reached 2238.7, 2606.15, 331.1 and 1415.79 for G5, G6, G7 and G8, respectively.

It can be concluded from the previous observations, that the addition of probiotic (Lactobacillus) and/or prebiotic (Agrimos) to the ration of the chicken which were vaccinated with live attenuated *S. typhimurium* vaccine enhance the immune response and produced higher antibody titres than those vaccinated only [15-17] In comparing between non-vaccinated groups but treated with Lactobacillus, Bactocell and/or Agrimos (G5, G6 and G7) and the non-vaccinated non-treated group (G8), after infection with *S.* typhimurium, it was clear that G6 (Lactobacillus plus Agrimos) gave the highest antibody titres, when compared with that of G8 and even with G4 (vaccinated only), i.e. using of synbiotic (probiotic + prebiotic) in chicken feeding induced high immune response [18].

The results in Tables (3and4 illustrated that the shedding of *S. typhimurium* from chickens, it was noticed that the *S. typhimurium* microorganism could be isolated from the faecal swabs of vaccinated groups and treated with probiotic and/or prebiotic (G1, G2, G3, G4) only during the first 7 days post vaccination in all groups with somewhat differences in the percentage of positive samples between groups (Table 2); the lowest percent was found in G1 (Lactobacillus plus Vaccine) (12%) and then G2 (Lactobacillus plus Agrimos + Vaccine) (16%). When these previous groups were challenged with *S. typhimurium*, it was found that *S. typhimurium* microorganism could be isolated from the faecal swabs till 4th day post challenge (G2) (3.8%), while no detection was found after challenge in G1. On the other hand, the detection of shedding persisted till the 27th day post challenge in groups (3 and 4).

It could be concluded from the previous results that incorporation of probiotic and prebiotics with *S. typhimurium* vaccination decrease the possibility of organism shedding, where it was found that the best results were in group [1] whereas the vaccinated chickens were treated with *lactobacillus acidophilus* (probiotic).

On the other hand, the results in Table 4 clarified the faecal shedding of *S. typhimurium* and focusing on the non-vaccinated groups (G5, G6, G7) but treated with probiotic and/or prebiotic and infected with *S. typhimurium*, it was found that *S. typhimurium* organism could be isolated from the faecal swabs in

the first 11 days post infection in group [6] (Lactobacillus plus Agrimos), while in groups (5 "Lactobacillus") persisted till the 18th day post infection and finally in group [7](Agrimos) and group [8] (control negative) lasted till the end of the experiment (30th day post infection).

From the previous results, it was clear that the groups which were treated with synbiotic before infection namely; group [6] (Lactobacillus plus Agrimos) gave the best results where they helped in prevention of shedding of the *S. typhimurium* organism in the first 14 days post infection if they were compared with non-treated group [8] which showed shedding of the organism till the end of the experiment. Besides, on comparing vaccinated and non-treated group (G4) with non-vaccinated treated groups (G5 and G6), it was found that treated non-vaccinated groups gave better results than vaccinated non-treated group in controlling of *S. typhimurium* organism shedding and this clarified the role of probiotic and/or prebiotic in declining the shedding of the organism.

From results of this experiment, it can be concluded that the use of probiotics, prebiotic as well as synbiotic may be an effective therapy against *S. typhimurium* shedding after vaccination with live attenuated vaccines or after exposure to the infection by *S. typhimurium* virulent organism [19-22].

Concerning with the results of *S. typhimurium* isolation (clearance test) from liver, spleen and heart are shown in Table 5 It was found that the reisolations of *S. typhimurium* organism from liver, spleen and heart after 4 weeks post challenge were (0%) in group (1) (Lactobacillus plus Vaccine) and group (2) (Lactobacillus + Agrimos plus Vaccine), while the percentages of reisolation showed somewhat higher (4.2%) in group (3) (Agrimos plus Vaccine) and group (4)Vaccine only). On the other hand, in non-vaccinated and probiotic / prebiotic treated groups (G5, G6 and G7) gave (25%, 16.7% and 45.8%), respectively, while in non-vaccinated, non-treated and infected group (G8) the reisolation percentage was (83.3%).

It could be concluded that incorporation of probiotic and/or prebiotic with vaccination with live attenuated *S. typhimurium* vaccine (G1, G2) gave better results than in case of vaccination alone. As well as, in non-vaccinated groups, the using of probiotic and/or prebiotic reduced the percentage of reisolation if compared with non-treated group (G8) [23- 25].

In conclusion, probiotic (Lactobacillus) and/or prebiotic (Agrimos) enhanced the immune response, reduced *S. typhimurium* shedding and decreased the percentage of *S. typhimurium* reisolation from the internal organs. So, it could be recommended using these friendly probiotic and prebiotic preparations beginning from the first day of chick life till marketing or end of production period as alternatives to antibiotics.

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