Probiotic Effect of Limosilactobacillus fermentum on Growth Performance and Competitive Exclusion of Salmonella Gallinarum in Poultry

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ABSTRACT
Fowl typhoid, an acute or chronic systemic infection of poultry is caused by Salmonella Gallinarum. It causes considerable economic losses in poultry in different countries including Pakistan. The aim of this study was to evaluate the effect of two previously characterized indigenous Limosilactobacillus fermentum strains (PC-10 and PC-76) on competitive exclusion of Salmonella Gallinarum in poultry gut. Day old chicks (n=90) were equally divided into six groups. Group 1 was kept as negative control and group 2 was positive control. Birds in group 3, 4 and 5 were administered with Limosilactobacillus fermentum strains PC-10, PC-76 and commercial probiotic, respectively from day 1-35. Group 6 was treated with antibiotic (florfenicol 30mg/kg) following the post infection of S. Gallinarum. All experimental groups except the negative control were challenged with S. Gallinarum (10^6CFU/bird) on day 21. The effect of probiotics on gut microbiota; Lactobacillus, total coliform and Salmonella were enumerated before and after the administration of challenge organism. Weight gain, feed conversion ratio and immune response to Newcastle disease and infectious bursal disease vaccine were determined. The results showed that Limosilactobacillus fermentum PC-10 and PC-76 significantly (P<0.05) decreased the growth of S. Gallinarum (3.92±0.37 vs 3.99±0.22 log10 CFU/g) in comparison to positive control group (6.88±0.22 log10 CFU/g) on day 35. The administration of these probiotics led to a significant increase in Lactobacillus count (>2log10) and reduction in coliforms count (1-2log10) in broilers. The probiotic fed groups exhibited less lesion scores and mortality rates compared to positive control group. Moreover, the broiler bird fed with Limosilactobacillus fermentum PC-10 and PC-76 group exhibited higher weight and immune response. Based on these findings, it was concluded that Limosilactobacillus fermentum PC-10 and PC-76 may be used as potential probiotics for targeted mitigation of S. Gallinarum in broilers.

INTRODUCTION
Fowl typhoid a septicemic infection in poultry, is caused by Salmonella enterica subspecies enterica serovar Gallinarum. It causes significant economic losses in poultry sector due to high morbidity and mortality (Spickler, 2019). However, the global burden of this disease still remains unknown (Zhou et al., 2022). In developed countries including United States and Canada, fowl typhoid has been eradicated from commercial poultry through the implementation of biosecurity measures, maintaining the hygienic practices, and screening and removal of infected flocks (Desin et al., 2013). However, it remains endemic in many developing countries including South America and Asia where inadequate control measures and favorable climatic conditions facilitate the spread of Salmonella Gallinarum in environment (Barrow and Neto, 2011).

The Salmonella Gallinarum is host restricted pathogen of poultry that has less zoonotic importance (Andino and Hanning, 2015). It primarily caused infection in chicken while seldom cause infection in other hosts such as pheasants, quails and guinea fowl (Shivaprasad, 2000). It is highly prevalent in commercial chicken...
particularly in brown layers and breeders (Penha et al., 2018). The infection in poultry cause anemia, depression, anorexia, difficulty in breathing, drop in egg production and diarrhea causing adherence of feces to the vent (Anonymous, 2018).

Antibiotics are commonly employed as preventive and therapeutic measures to control bacterial infections such as fowl typhoid. However, the excessive use of antibiotics has led to the increasing development of antibiotic resistant strains including the multidrug resistance Salmonella Gallinarum (Yasmin et al., 2019; Zhou et al., 2020). To overcome this issue, various alternatives to antibiotics are available such as probiotics, prebiotics, symbiotics, bacteriophages, organic acids, essential oils, cinnamaldehyde, chitosan, vaccines and nanoparticles. These alternatives can be used for prevention or control of fowl typhoid in poultry (Al-Razem et al., 2022; El-Saadony et al., 2022). Among these options, probiotics have gained significant attention as safe and efficient alternative to antibiotics (Chen et al., 2020).

Probiotics are live microorganisms when administered in sufficient amount provide health advantage to host (FAO/WHO, 2002). Probiotics are utilized as feed additives in poultry feed to promote the growth of beneficial bacteria, enhance the growth performance of birds and reduce the level of enteric pathogens in broiler (de Souza et al., 2022; El Jeni et al., 2021). The microbial genera include Lactobacillus, Lactococcus, Bifidobacterium, Streptococcus and Bacillus are commonly used as probiotics in poultry (Gadde et al., 2017; Rashid et al., 2023). Among these, Lactobacillus has the advantage of being Generally Regarded as Safe and are naturally occurring in intestinal tract (Robyn et al., 2012). They inhibit the growth of pathogenic bacteria through different mechanisms such as binding to epithelial cell, modulation of immune system and secretion of antimicrobial compounds (Alonso et al., 2019).

The Limosilactobacillus fermentum has gained significant recognition as one of the most promising probiotics (Rodríguez-Sojo et al., 2021). It is commonly found in gastrointestinal of humans and animals, as well as in plant tissues and dairy products. The beneficial effects of this species are strain specific (Dos Santos et al., 2021). The Limosilactobacillus fermentum strains (PC-10 and PC-76) isolated in our previous study have in-vitro probiotic properties and possess the antimicrobial activity against Salmonella Gallinarum (Mehmood et al., 2023). This study was designed to analyze the effect of these isolates on weight gain, immune response, feed conversion ratio, and gut morphology and inhibition of Salmonella Gallinarum in broiler.

**MATERIALS AND METHODS**

**Experimental design:** Day old chicks (n=90) were purchased from commercial hatchery and housed in experimental shed of the Institute of Microbiology, for a period of 35 days. They were divided in to 6 different groups with 15 birds kept in each group. Group 1 served as negative control and did not receive any bacterial growth. Group 2 was positive control and only challenged with S. Gallinarum (10^5CFU/bird) on day 21. Birds in group 3 and 4 were orally administered Limosilactobacillus fermentum strains PC-10 and PC-76, respectively through drinking water (10^5CFU/bird) from day 1 to day 35 and challenge of S. Gallinarum on day 21. Group 5 received commercial probiotic HAN-LACVET (0.6g/15 birds) from day 1 to day 35 and was challenged with S. Gallinarum on day 21. Group 6 was challenged with S. Gallinarum on day 21, following the infection, started receiving antibiotic florfenicol (30mg/Kg body weight) for five days as given in Table 1. Mortality rate and symptoms of birds in each group were recorded on daily basis.

**Enumeration of Salmonella, coliform and lactobacilli:** On the arrival of chicks and before the S. Gallinarum challenge (day 21) cloacal swabs were collected to ensure the absence of S. Gallinarum. Subsequently, on alternate days following post S. Gallinarum infection, fresh poultry droppings samples were collected (at day 23, 25, 27, 29, 31, 33, 35) from each group to evaluate the effect of different treatments on Salmonella, coliform and Lactobacillus count in broiler. The Lactobacillus count was determined on MRS agar, coliforms were enumerated on MacConkey agar and Salmonella counting was done on SS agar. The bacterial count was calculated as CFU/gram and converted in to log10 values. The mean ± standard deviation of log10 values were calculated for comparison among different groups. The log reduction of coliform was calculated by subtracting the log10 values from negative control group while reduction of Salmonella was calculated by subtracting log10 values from positive control group. The increase in lactobacilli count was calculated by subtracting the log10 values from negative control group (Shi et al., 2022).

**Effect of lactobacilli on weight gain:** Feed conversion ratio and live weight of birds were recorded on different days (1, 7, 14, 21, 28, and 35) with a one-week interval.

**Immune response against ND and IBD vaccine:** The immune modulatory effect of Limosilactobacillus fermentum PC-10 and PC-76 against Newcastle Disease Virus (NDV) and Infectious Bursal Disease (IBD) virus was determined. Chickens in all experimental groups were immunized with NDV "Lasota" (Medion, Bandung Indonesia) via eye drop on day 5 and booster dose was given on day 17 of broiler age. The vaccine for IBD was given to bird on day 11. Blood samples were collected from wing vein of birds (n=5) at different age of birds (14, 21, 28 and 35 days) for determination of antibody titer against ND and IBD (Beard et al., 1975). Serums from blood samples were separated and stored at -20°C till for further analysis. Antibody titer against NDV was determined by Hemagglutination Inhibition (HI) assay and IBD vaccine was determined by commercially purchased ELISA kit (Allan et al., 1978).

**Growth conditions of bacterial strains:** Previously characterized Limosilactobacillus fermentum PC-10 (ON819856) and PC-76 (OP703611) were cultured on De Man Rogosa and Sharpe agar (Mehmood et al., 2023). The test strain, Salmonella Gallinarum (CP116616) isolated from field outbreak was cultured on Salmonella shigella agar.
**RESULTS**

**Lactobacilli effect on Salmonella count in broiler gut:** The probiotic effects of *Limosilactobacillus fermentum* PC-10 and PC-76 on broiler gut were determined by *Salmonella* count, coliform count and total lactobacilli count following the post infection of *S. Gallinarum* in broilers. The results revealed a significantly (P<0.05) higher *Salmonella* count in positive control group (log10 6.88±0.2 CFU/g) at day 35 compared to other experimental groups. However, the groups administered with *Limosilactobacillus fermentum* PC-10 and PC-76 exhibited the significantly less in the growth of *S. Gallinarum* (log10 3.92±0.30 vs log10 3.99±0.22 CFU/g) compared to commercial probiotic (log10 4.90±0.70 CFU/g) and antibiotic treatment group (log10 5.17±0.1 CFU/g) as given in Fig. 1.

On day 35, there was no significant difference in coliform count between groups infected with *S. Gallinarum* (positive control) and negative control (log10 7.35±0.10 vs log10 7.15±0.10) as shown in Table 2. The coliform count was reduced significantly in all probiotic groups while maximum reduction was obtained in the group fed *Limosilactobacillus fermentum* PC-10 (P<0.05) among all other experimental groups.

The groups fed with *Limosilactobacillus fermentum* PC-10 and PC-76 had significantly higher (P<0.05) *Lactobacillus* counts (log10 9.37±0.2 vs log10 9.42±0.44) than negative control group (log10 7.46±0.52) as mentioned in Table 2. The *Lactobacillus* counts decreased progressively in chicken treated with antibiotic and positive control group at the end of experiment on day 35.

**Antibody response to IBD and ND virus in birds challenged with *S. Gallinarum*:** The geometric mean titer of birds in different experimental groups before and after challenged with *S. Gallinarum*.

**Effect of lactobacilli on growth performance of broiler:** The mean weight of birds was calculated in all experimental groups on day 21, 28 and 35 compared with control groups. As indicated in Fig. 2 the higher antibody titer was obtained in group 3 (114.10) and group 4 (110.29) at day 28 as compared to negative control group (90.25). The maximum antibody titer was obtained in group 4 on day 28 against IBD vaccine as given in Fig. 3.

**Fig. 1:** *Salmonella* count in different experimental groups before and after challenged with *S. Gallinarum*

**Fig. 2:** Geometric mean titer of birds in different experimental groups against NDV vaccine at different days

**Fig. 3:** Antibody titer of birds in different experimental groups against IBD vaccine at day 28. A positive result (titer greater than 396) indicates birds are vaccinated with IBD vaccine.
Table 2: Effect of selected lactobacilli on coliform count in broiler gut at day 35

<table>
<thead>
<tr>
<th>Groups</th>
<th>Coliform count Before infection (mean log10CFU/mL)</th>
<th>After infection (mean log10CFU/mL)</th>
<th>Log reduction</th>
<th>Lactobacillus count Before infection (mean log10CFU/mL)</th>
<th>After infection (mean log10CFU/mL)</th>
<th>Increase in Log10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>7.05±0.35a</td>
<td>7.15±0.10d</td>
<td>-</td>
<td>7.20±0.12a</td>
<td>7.46±0.52a</td>
<td>-0.59</td>
</tr>
<tr>
<td>Positive control</td>
<td>7.24±0.19a</td>
<td>7.35±0.10d</td>
<td>-0.2</td>
<td>7.20±0.21a</td>
<td>6.87±0.32a</td>
<td>1.11</td>
</tr>
<tr>
<td>PC-10</td>
<td>7.27±0.46a</td>
<td>5.18±0.40a</td>
<td>1.97</td>
<td>7.18±0.19a</td>
<td>9.37±0.23a</td>
<td>2.19</td>
</tr>
<tr>
<td>PC-76</td>
<td>8.78±0.166</td>
<td>6.01±0.05a</td>
<td>1.14</td>
<td>7.99±0.08a</td>
<td>9.42±0.44a</td>
<td>1.57</td>
</tr>
<tr>
<td>Commercial Probiotic</td>
<td>7.99±0.05b</td>
<td>6.90±0.07b</td>
<td>0.25</td>
<td>7.20±0.17b</td>
<td>8.70±0.16b</td>
<td>1.24</td>
</tr>
<tr>
<td>Antibiotic-Group</td>
<td>7.27±0.19a</td>
<td>5.92±0.50b</td>
<td>1.23</td>
<td>7.18±0.07b</td>
<td>7.39±0.27b</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Table 3: Feed conversion ratio determined on weekly basis in different experimental groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Age of broiler</th>
<th>Day 1</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
<th>Day 35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0.22</td>
<td>0.93</td>
<td>1.10</td>
<td>1.24</td>
<td>1.38</td>
<td>1.36</td>
<td></td>
</tr>
<tr>
<td>Positive control</td>
<td>0.22</td>
<td>0.94</td>
<td>1.09</td>
<td>1.33</td>
<td>1.37</td>
<td>1.67</td>
<td></td>
</tr>
<tr>
<td>PC-10</td>
<td>0.22</td>
<td>0.91</td>
<td>0.94</td>
<td>1.37</td>
<td>1.39</td>
<td>1.70</td>
<td></td>
</tr>
<tr>
<td>PC-76</td>
<td>0.22</td>
<td>0.94</td>
<td>0.98</td>
<td>1.49</td>
<td>1.40</td>
<td>1.74</td>
<td></td>
</tr>
<tr>
<td>Commercial Probiotic</td>
<td>0.22</td>
<td>0.93</td>
<td>1.03</td>
<td>1.37</td>
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<td>1.70</td>
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<td>0.22</td>
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<td>1.74</td>
<td></td>
</tr>
</tbody>
</table>

**Clinical findings and mortality:** Birds in all experimental groups were monitored twice a day for any types of clinical signs and mortality. In control group of broilers not infected with S. Gallinarum, no clinical signs and mortality were recorded throughout the experiment. In positive control and antibiotic group, initial signs of dullness and decreased feed intake were observed on the 3rd day of post infection. Subsequently, on 5th to 6th days after infection, these groups exhibited the additional clinical signs such as droopy wings, ruffled feathers and yellowish green diarrhea. Higher mortality was recorded in positive control group (46.66%) due to S. Gallinarum infection in comparison to all other groups.

**DISCUSSION**

Lactobacilli have been used widely as an alternative to antibiotics to prevent Salmonella infection in chicken (Wang et al., 2023). They are main residents of gastrointestinal tract where they protect the host by competing for nutrients and releasing substances that inhibit the growth of harmful pathogens (Chen et al., 2007). In a previous study, we isolated the Limosilactobacillus fermentum strains (PC-10 and PC-76) from poultry caeca which showed >99% reduction of S. Gallinarum in vitro in co-culture assay. In this study, we investigated the in vivo effect of these strains on reducing the growth of S. Gallinarum in broiler and further analyzed their effect on weight gain, feed
conversion ratio and increase in immune response against ND and IBD vaccine. The efficacy of these strains was compared with commercial probiotic product (HAN LACVET) and antibiotic (florfenicol) routinely used in poultry farms to prevent the Salmonella infections.

The study findings demonstrated the significant decrease (p<0.05) in Salmonella in fecal dropping of broilers that were administered with Limosilactobacillus fermentum PC-10 and PC-76 at day 35. Our results are in line with previous findings, indicating that administration of probiotics via oral route in poultry inhibited the attachment of Salmonella Enteritidis in poultry gut (Shi et al., 2022). This inhibitory effect is likely attributed to production of various metabolites by lactic acid bacteria such as bacteriocins, lactic acid and hydrogen peroxide that inhibit the growth of pathogenic bacteria. Moreover the lactic acid bacteria can competitively exclude the pathogen in gut by reducing the pH in gut and competing them for nutrients and attachment (Kulkarni et al., 2022).

The Lactic Acid Bacteria (LAB) belong to Lactobacillus play a key role in regulating and maintaining the intestinal microbial flora and exert their beneficial effects by inhibiting the adhesion of pathogenic bacteria and facilitating the colonization of beneficial microorganisms (Hai et al., 2021). Probiotic administration on daily basis helped to increase the population of beneficial microbes in GIT (Khan et al., 2019). The current study also reported the higher microbial count of lactobacilli in experimental groups fed with potential lactobacilli on daily basis. A significant increase in lactobacilli (~2log10CFU/mL) was observed in probiotics groups PC-10, PC-76 and commercial probiotic (HAN-LACVET) supplemented group (day 01 to 35). These probiotics groups exhibited a significant difference (P value<0.05) compared to other experimental groups. Challenge control group which was only administrated with S. Gallinarum reported less lactobacilli count (6.87±0.2) at day 35.

Binding of probiotics to gut prevent the colonization of pathogenic bacteria and coliform via competitive exclusion process. Newly hatched chicks have incomplete intestinal microbiota. So, early administration of lactobacilli helped to establish the dominant microbiota and prevent the colonization of non-host specific pathogens (Chen et al., 2020). Previous research has also highlighted that using probiotics for prevention rather than treatment can offer better health benefit to host (Chen et al., 2020). The current study findings are similar to previous study, administration of probiotics in new hatched chicks decrease the coliform in broiler gut. In comparison to this, coliform count (7.14±0.36 log10 CFU/g) was high in negative control group and is more than coliform count (5.07±0.15 log10 CFU/g) in PC-10 and other probiotic groups.

Growth performance and weight gain of birds in all experimental groups were monitored on weekly basis. Significant difference in weight was recorded at 3rd week of broiler age and it was observed till the completion of experimental trial. Meanwhile, various previous studies have also reported the similar results (Asghar et al., 2016). In our study the Limosilactobacillus fermentum strain PC-10 and PC-76 significantly improved weight gain of broilers at day 35 groups in comparison to negative control group. Enhancement of weight was also analyzed in probiotic supplemented groups in broilers in comparison with the negative control group (Forte et al., 2018). A related study reported that supplementation of L. fermentum in broiler increased the weight of broiler (Mustafa et al., 2022). Various previous evidences have reported the probiotic effect on weight gain in chicken (Asghar et al., 2016; Chen et al., 2020; Khan et al., 2019).

Probiotics also play an important role in immune modulation in poultry and also enhanced the immune cells to produce natural antibodies (Azad et al., 2018). Probiotics also stimulate the cell mediated and humoral immune response in chicken. L. salivarius is a natural microflora of chicken gut and in mononuclear cell it induced the proliferation of cytokines (Brisbin et al., 2011). Lactobacillus gallinarum PL-53 have improved the immune status of chicken (Khan et al., 2019). In present study immune response of broiler was assessed with elevated antibody titer against NDV vaccine. Probiotics have good influence on immune response in broiler. This study reported that GMT against NDV and IBD was higher in probiotic groups in comparison with those groups which not received probiotics. Hedayati et al. (2022) recorded higher antibody titer against IBD and infectious bronchitis (IB) virus in broiler fed with lactobacilli spores. Immune response of probiotics supplemented groups and control groups against NDV were determined on weekly basis and is mentioned Fig. 2. The highest GMT titer in this study was recorded by Limosilactobacillus fermentum (PC-10) in comparison to all experimental groups at day 35. The results of this experiment are also in agreement with Khan et al. (2019). This study reported that antibody titer was increased at 4th week of broiler age in probiotics groups in comparison with groups challenge control and antibiotic group.

In control group of broilers, which was not infected with S. Gallinarum, no clinical signs and mortality was recorded in throughout the experiment. Birds in positive control and antibiotic group were represented the initial signs of dullness and decrease of feed intake on 3rd day of post infection. Some of the birds died suddenly due to the acute form of illness. Higher mortality was recorded in positive control group (46.66%) after the post infection of S. Gallinarum. Abbas et al. (2021) reported the 48% mortality rate in chicks only challenged with S. Gallinarum and less mortality 24% in birds infected with S. Gallinarum and lactobacilli. Birds in probiotic groups represent the mild sign of dullness after the post challenge of S. Gallinarum and no mortality was recorded in 6 days of post challenge. These results are similar to another study reported, Lactobacillus as probiotic supplementation in poultry reduced the clinical sign against Salmonella infection (Abbas et al., 2021).

**Conclusion:** This study demonstrated that indigenous Limosilactobacillus fermentum strains (PC-10 and PC-76) have potential probiotic properties, regulate intestinal microbiota of poultry, and competitively exclude S. Gallinarum in poultry gut. Moreover, these isolates have significant effect on weight gain and immune response in broiler. The Limosilactobacillus fermentum strains (PC-10 and PC-76) may be used as probiotic to mitigate S. Gallinarum in poultry.
Ethics approval and consent to participate: The study was conducted according to the guidelines and protocols approved by “Ethical Review Committee” of University of Veterinary and Animal Sciences Lahore, Pakistan (vide letter no. DR/1103; Dated: 11 October 2017). Informed consent was obtained from the owners or an authorized agents of poultry farms for the collection of intestinal samples from poultry birds for this study.

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Authors contribution: MN, MR and MHM designed the research project. AM collected samples, performed experiments and MN analyzed the data. AM prepared the manuscript. All authors contributed in manuscript revision and approved final version for submission.

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