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RESEARCH ARTICLE

Coherence and Colonization Characteristics of Recombinant Lactobacillus under Simulated Gastric Conditions within Chicken GI tract and its impact on Chicken Growth

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ABSTRACT

The gastrointestinal tract (GIT) of chickens is massively occupied by pathogens and lactobacillus where lactobacillus may basically be used as symbiotic member. Particular species of lactobacillus such as *Lactobacillus casei* (Lc393) and *Lactobacillus saerimneri* (M11) regulating the function of immune system, as a probiotics and therapeutic agent produce protein against pathogens. In this study, we constructed a series of recombinant lactobacillus ppG-T7g10-ppT-ompC$_1$-fimA$_1$/Lc393 (POF$_1$/Lc393), ppG-T7g10-ppT-ompC$_1$-fimA$_1$/M11 (POF$_1$/M11), ppG-T7g10-ppT-ompC$_{78}$-fimA$_{78}$/Lc393 (POF$_{78}$/Lc393) and ppG-T7g10-ppT-omp C$_{78}$-fimA$_{78}$/M11 (POF$_{78}$/M11). For Examination, 70 day one old (SPF) chicks were divided into 5 batches (1Lc,78Lc,1M11,78M11 and PBS control) comprising 14 chicks in each batch. At day one, all batches were drenched orally with recombinants of $1.5x10^9$ CFU/ ml, $1.5x10^{10}$ CFU/ ml, $1.5x10^{11}$ CFU/ ml and also drenched control with100ul of PBS respectively. After vaccination, all batches were challenged orally with 0.5ml of *E.coli.* The colonization and adherence ability were then examined in ileum, cecum and colon of chickens at different intervals. Results of recombinant lactobacillus POF$_1$/Lc393 and POF$_{78}$/Lc393 demonstrated that $1.5x10^9$ml, $1.5x10^{10}$ ml and $1.5x10^{11}$ ml concentration of recombinants drenched respectively had no mortality while POF$_1$/M11, POF$_{78}$/M11 showed 20% mortality in all batches. Vaccine treated groups POF$_1$/Lc393 and POF$_{78}$/Lc393 showed significantly higher chicken growth performance ($P<0.05$) as compared with POF$_1$/M11, POF$_{78}$/M11 and control (PBS). The results determined that possibility to construction and colonization of these recombinants to be used as therapeutic agent and growth performance modulator in chickens.

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INTRODUCTION

The livestock industry has become highly global in regard to rapidly increasing demand of meat. In rearing facility of developing countries, economic loss in poultry is due to changes in environmental conditions, high disease rate and hyper circumstances. The segregation and screening of recombinant lactobacillus from GI tract has always been strongly practiced for useful and genetically stable strains for industrial purpose. LAB as probiotics helps stimulating immune response, hindering pathogenic strains of bacteria, treating and preventing from diseases. Probiotics as therapeutic agents, notably proteins to the GIT use for carrier (Bao *et al*., 2013). Probiotics colonization in the intestines of host upon ingestion grants a health benefit and they are safe for human absorption (Vanderhoof, 2000; Kelly and Mulder, 2011) pathogens lack their receptors, potential binding sites in the competition of selected LAB strains in GI tract of chicken. They involve pH reduction of the gut and stimulation in immune response (Bouzaine *et al*., 2005). Colonization of recombinant lactobacillus in the epithelial cells of intestine as one of important factor for probiotic behavior (Yadav *et al*., 2015). Adhesion on epithelial matrix such as collagen,

fibrinogen with corresponds to surface proteins that are present in extracellular compartments (Muñoz-Provencio *et al*., 2009). Further, evaluation is gained by using assessing the strains' capability against acid and bile salt concentration after changing in optical density (Mirlohi *et al*., 2009) also by investigating growth on growing medium and their antibacterial properties. Survival of recombinant lactobacillus is the distinguished factor in order to maintain therapeutic functions. Many factors effect on the stability of probiotic properties of bacteria in the GI tract of broiler chickens such as low pH and high bile salt concentration (Shah, 2000). Recombinant lactobacilli are also orally administered as a delivery vector designed to produce peptides. LAB as live delivery vectors have been practiced in chickens for therapeutic proteins (Wells and Mercenier, 2008; Berlec and Strukelj, 2009). In addition, strain selection of LAB for recombinants has engaged many assays, including acid tolerance, antimicrobial assay, bile salt tolerance, colonization ability on the epithelial cells and cell wall hydrophobicity (Kizerwetter-Swida and Binek, 2005; Taheri *et al*., 2009). Lactic acid bacteria are much more competitor of preventing, treating epidemic disease by yielding bacteriocins and adhesive characteristics to epithelial cells (Xu and Li, 2007). The main objective of this study was to manipulate DNA of *E.coli* serogroups, transfer into desired lactobacillus strain and check colonization of these recombinants in the GIT under severe gastric conditions such as high bile salt concentration, low pH, adhesion capacity to epithelial cells and impact of recombinant LAB colonization on chicken growth. In addition, effect of in vivo fusion expression of inserted genes on growth production was also categorized.

MATERIALS AND METHODS

Bacterial strains and culture conditions: Avian Pathogenic *E.coli* (APEC) O_1 and O_{78} serotypes were purchased from Chinese Veterinary Microbiology Culture Collection Center. These two strains of lactobacillus i.e., *Lactobacillus casei* (Lc393) and *Lactobacillus saerimneri* (M11) were practiced in this study was obtained from Netherlands Institute NIZO and cultured in Man-Rogosa-Sharpe (MRS) medium(Sigma) at 37°C without shaking in anaerobic condition. PMD19-T Simple vector was bought from TAKARA Dalian Company. Recombinant lacto-bacillus ppG-T7g10-ppT-OmpC_1-FimA_1/Lc393 (POF_1/ Lc393), ppG-T7g10-ppT-OmpC_1-FimA_1/M11 (POF_1/M11), ppG-T7g10-ppT-OmpC_{78}-FimA_{78}/Lc393 (POF78/Lc393) and ppG-T7g10-ppT-OmpC_{78}-FimA_{78}/M11 (POF_{78}/M11) were empirically constructed, transformed and used as colonization in the gut of broiler chicken (Li *et al*., 2012).

Primers: According to published genome sequence of lactobacillus into NCBI were designed as 27F and 1452R, by using primer premier 5.0 software and cross checked by Gold Intellectualism Biological Co., Ltd.

DNA manipulation: Genome from O_1 and O_{78} serotypes was extracted by using genome extraction kit and used as template for amplification of FimA and OmpC gene. Primer pairs A1, A2 and C1, C2 (Table 1) were used to amplify these genes according to (Xu and Li, 2007) respectively. The products of PCR amplification were ligated with PMD19-T simple vector and transformed into *E.coli* (TG1) followed by double digestion of Restriction enzymes using Sac1 and Apa1.

Identification and transformation of fusion genes: For the identification of plasmid, isolate PMD19-T-OmpC-FimA from overnight culture, amplified by using C1 and A2 primers with the specific reaction conditions. Recombinant plasmid PMD19-T-OmpC-FimA digested by restriction enzymes and ligated with PPG-T7g10-ppT vector, harvested and digested by double digestion using two restriction enzymes sac1 and Apa1, rescued 4900bp vector. This plasmid transformed into two *Lactobacillus* species *Lactobacillus casei* (Lc393) and *Lactobacillus saerimneri* (M11) respectively.

Experiment design

Animal testing: 1 day old (SPF) chickens were purchased from a veterinary research institute in Harbin Heilongjiang province China for the diet growth correlation and identification and colonization of recombinant *Lactobacillus* performance in GIT.

Isolation of chicken intestinal recombinant lactic acid bacteria: After appropriate intervals, chickens were sacrificed from each group with equity. The intestinal contents were isolated from ileum, cecum and colon of challenged chickens for the case study of colonization of recombinant *Lactobacillus* probiotics properties. These contents were scraping with a clean slide 0.5 g of intestinal mucosal sample into 4.5ml sterile saline tubes serially diluted 10 folds 10:1 to 10:8 and subjected to MRS-Caco3 plates with 10ug/ml cm+.

Recombinant *Lactobacillus* colonization capacity in chicken gut: A total 70 one day old chickens were randomly distributed into 5 groups with 14 birds in each batch. Administered each batch with 100ul of POF_1/ Lc393, POF_{78}/ Lc393, POF_1/ M11 and POF_{78}/M11 containing10^9, 10^{10} 10^{11} CFU/ml also drenched 100ul of phosphate buffer saline (PBS) to control group. In whole trail periods at days 1, 3, 7, 14, 21, 28 two birds from each group select randomly were necropsied for sample collection and examine the ability of colonization in ileum, caecum and colon. Enumeration of recombinants was evaluated by plate count method Herigstad *et al*. (2001).

Table 1: Sequence of Primers used in this work

Primers	Sequence（5′–3′）
27 F	5′-AGAGTTTGATCCTGGCTCAG-3′
1495 R	5′-CTACGGCTACCTTGTTACGA-3′
A1	GTGCACGGTGGCGGTGGCTCAGGTGGCGGTGGCTCAGGTGGCGGTGGCTCAACGACTGTAAATGGTGGGACCGT
A2	GGGCCCTTATTATTGATACTGAACCTTGAAGGTCGC
C1	GAGCTCATGGATTACAAGGATGACGACGATAAGGCTGAAGTTTACAACAAAGACGGCA
C2	GTGCACGAACTGATAAACCAGGCCCAGA

Note: The underlined part represents the restriction sites.

Identification of recombinant *Lactobacillus* by PCR magnification: We enumerated recombinant colonies on the plate were among 30-300. After enumeration, 10 colonies were selected from each plate for PCR amplification of recombinants in the GIT. The primer sequence (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse (5'-CTACGGCTACCTTGTTACGA-3').

Ecological performance of recombinant *Lactobacillus* in chicken GIT

Acid hindrance test: Recombinant lactobacillus of POF_1/Lc393, POF_{78}/Lc393, POF_1/M11 and POF_{78}/M11 were harvested in 10% sterile PBS at pH values 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 for 2 hours.

Bile tolerance test: MRS medium was inoculated with relevant mass fraction of bile salt 0.05, 0.1, 0.2, 0.3 and 0.4% (w/v) as described by dunne *et al*. (2001). Recombinant strains of POF_1/Lc393, POF_{78}/Lc393, POF_1/M11 and POF_{78}/M11 were inoculated with 1% (v/v) various concentration bile salt anaerobically at 37°C for 8 hours to examine survival rate of recombinants via plating method.

RESULTS

Development of expression vector: The $OmpC_{1,78}$ genes was successfully ligated with PMD18-T-$FimA_{1,78}$ followed by single and double digestion. Further it was harvested into plasmid of 4315bp (plasmid), 2692bp (cloning vector) and 1623bp (recovered OmpC gene ligated with PMD18-T-FimA) respectively (Fig. 1). These fusion genes were fasten with ppG-T7g10-ppT expression vector with enhancer, electroporated into *L. casei* (Lc393) and *L. saerimneri* (M11).These recombinants screened by PCR and digested by Sac1 and Apa1 restriction enzymes produced two bands on gel of $POF_{1,78}$/Lc393 and $POF_{1,78}$/M11 (Fig. 2). PCR amplification of $POF_{1,78}$/Lc393 and $POF_{1,78}$/M11 by using primers (C1, A2 was listed in Table 1) attained 1623bp target gene bands as shown in (Fig. 3). In the recent study, colonization and dynamic distribution of recombinants were determined by identifying the target genes of recombinant lactobacillus in the different parts of GI tract.

Recombinant *Lactobacillus* colonization ability in the chicken GIT: The colonization of recombinant *Lactobacillus* in GI tract of day 1to 28 chicks was examined in ileum, cecum and colon. After oral administration of a single dose of POF_1/Lc393, POF_{78}/Lc393, POF_1/M11 and POF_{78}/M11 were tested respectively. These recombinants were found into ileum, cecum and colon relatively in large amounts but distribution was not even in GI tract. In fact, huge concentration of recombinants in cecum and colon than in ileum were noted. Although with the effect of metabolism in the GIT showed downwards trend at day 7 to 28 respectively. The abundance order in ileum, cecum and colon largely remains untouched till 72 hours after oral immunization. After 72 hours, only very few recombinants were found in the GI tract. The concentration of POF_1/Lc393 and POF_{78}/Lc393 increased in ileum, cecum and colon than POF_1/M11 and POF_{78}/M11 at day 1 respectively. This distribution pattern of colonization becomes slower from day 7 to onwards. The data was evaluated by using SPSS software.

Fig. 2 explains that Chickens were nurtured with recombinants POF_1/Lc393 and POF_{78}/Lc393 colonizing in intestines. Animals were killed on days 1, 3, 7, 14, 21 and 28 after inoculation. The intestinal mucosa was diluted and covered on MRS plate with Cm+. Control group were fed with PBS. Enumerated and compared with control.

Fig. 3 explains Chickens were nurtured with recombinants POF_1/M11 and POF_{78}/M11 colonizing in intestines. Animals were killed on days 1, 3, 7, 14, 21 and 28 after inoculation. The intestinal mucosa was diluted and covered on MRS plate with Cm+. Control group were fed with PBS. Bacteria were examined on plates and fed with recombinants on day 1 in ileum, cecum and colon. Enumerated and compared with control. The statistical significance was calculated by SPSS $P<0.05$.

Resistance of recombinant lactobacillus to acid test: The survival rate of all isolates observed at different pH values for 24 hours at 37°C and 42°C as shown in Fig. 4. The viability of recombinant increase significantly after 1 and 3 hours of pH treatment but below pH=2 numbers of recombinants lower because acidic condition. Both strains were significantly increased while pH=3 but with the increasing pH, two strains grow normally. At Ph≤2.0 none recombinants were observed after very bit intervals; all isolates were executed by critical pH concentration.

Tolerance of recombinant *Lactobacillus* to bile salt concentration: The most substantial strains for bile salt concentration were POF_1/Lc393, POF_{78}/Lc393 and POF_1/M11, POF_{78}/M11 while much more effected by bile salt than effect of $pH<3.0$. It seems that recombinant *Lactobacillus* POF_1/Lc393, POF_{78}/Lc393 were significantly more sound than POF_1/M11 and POF_{78}/M11 to bile salt. POF_1/M11 and POF_{78}/M11 could not exist at 0.3% bile concentration.

Recombinant *Lactobacillus* impact on chicken growth production: The initial weight of experimental birds, weights of birds during feeding was performed by using SPSS statistical software as shown in table 2. There were no differences in initial weights of experimental chicks

Table 2: Determination of increased body weight of chickens after oral administration of POF_1/Lc393, POF_{78}/Lc393, POF_1/M11 and POF_{78}/M11. The statistical significance was calculated by using SPSS statistical software ($P<0.05$)

Treatments	1day	3day	7day	14day	21day	28day	1-21day
$POF7_1$/Lc393	35.24±1.3851	44.75±2.1507	70.59±2.8923	126.52±1.7695	221.89±8.5891	325.00±4.2985	298.72±3.6332
POF_{78}/Lc393	36.34±1.4669	43.25±4.2514	67.31±4.1651	136.61±7.8480	210.69±11.9189	340.87±5.7127	325.11±13.8431
$POF7_1$/M11	36.08±0.8994	40.46±3.5399	66.70±2.2446	127.75±4.5553	193.15±13.9323	347.81±12.9785	278.27±7.2822
$POF7_{78}$/M11	37.05±o.9527	42.23±3.6093	69.01±3.5899	132.92±3.9051	185.18±14.5986	342.78±6.9320	276.00±2.5233
Control	36.56±o.9438	39.66±2.8823	65.91±3.6257	133.09±4.6261	175.98±13.8721	316.80±5.3589	266.52±5.1970
P Value	0.496	0.221	0.224	0.025	0.042	0.000075	0.00012

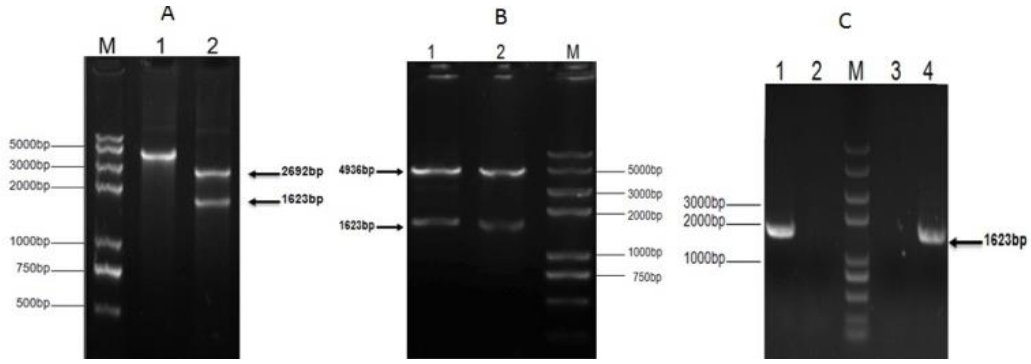

Fig. 1 (A): Digestion of pMD19-T-POF1,78/TG1 Lane 1. Shows Plasmid digested by restriction enzyme Sac, Lane 2. shows Plasmid digested by restriction enzymes SacI/ApaI, Lane M shows DNA marker Trans 2K, **(B)** plasmid digestion of recombinant LAB, Lane 1 shows Plasmid of POF1,78/M11 digested by SacI/ApaI, Lane 2 shows Plasmid of POF1,78/Lc393 digested by Sac I / Apa, Lane M shows DNA marker Trans 2K, **(C)** PCR amplification of POF1,78/Lc393 and POF1,78/M11, Lane 1. PCR result of POF1,78/M11, Lane 2. negative control, M. DNA marker Trans 2K, Lane 3. negative control Lane 4. PCR result of POF1,78/Lc393.

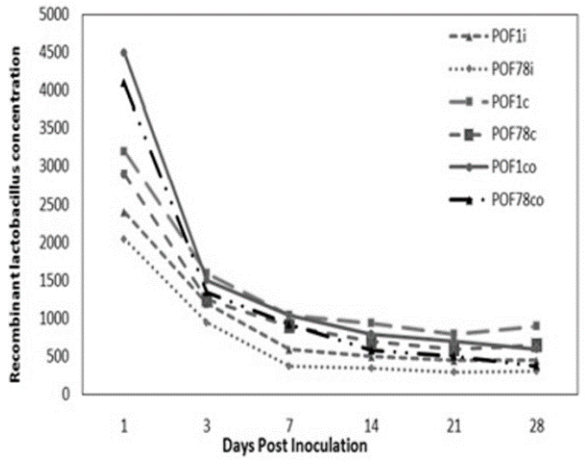

Fig. 2: Colonization of recombinant lactobacillus after oral immunization of POF_1/Lc393, POF_{78}/Lc393 at different compartments in the GI tract of chickens. (*i= ileum, c=cecum, co=colon*).

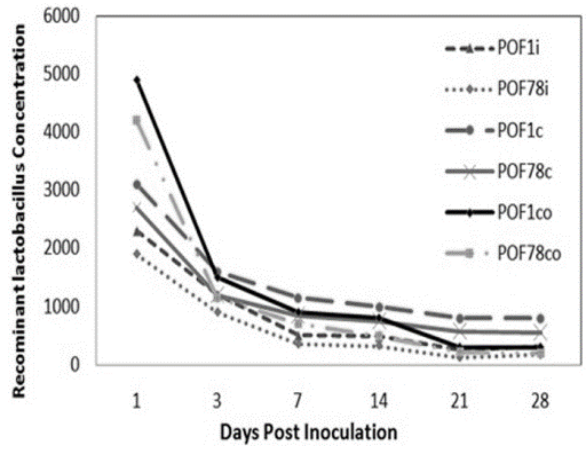

Fig. 3: Colonization of recombinant lactobacillus after oral immunization of POF_1/M11, POF_{78}/M11 at different compartments in the GI tract of chickens.

(P<0.496), non-significant difference in average weight of 7-day old chicks of POF_1/Lc393 and POF_1/M11.There were more significant difference in 28-day old chicks weights (P<0.01) among the experimental groups during feeding of chickens. The average weight of 28-day old birds in the group of POF_1/Lc393, POF_{78}/Lc393 was significantly higher than POF_1/M11 and POF_{78}/M11 and so were those in same groups of 3-day old with non-significant difference. The average value of experimental birds among groups showed significant difference (P<0.01) relatively with difference in average weight of 28-day old birds.

DISCUSSION

Earlier studies regarding, *Lactobacillus* colonization of the chicken gut was mostly involved with mucosal immunity against pathogens (Dalloul *et al*., 2005). In recent study, with colonization of recombinant *Lactobacillus* in the gut of chickens under harsh intestinal conditions and its impact on chicken growth performance as starting points the results were persistent with those of most researches regarding the expression of protein by recombinants. (Nguyen *et al*., 2015). The results determined that recombinants were able of colonization into ileum, cecum and colon with colonization time of at least 28 days.

We exhibited the adhesion ability of recombinant *Lactobacillus* to epithelial cells under simulated gastric conditions for at least 28 days. Our research illustrated that colonization of recombinants in GI tract has gained more consideration to be used as a carrier for mucosal immunization against pathogens (Wells and Mercenier, 2008) which is also improved growth performance of chicken. Low levels of *Lactobacillus* colonization in GI tract of birds have enhanced wide range of pathogens exposed to mucosal surface of respiratory and gastrointestinal layers. In previous studies, many efforts have been made to boost colonizing factor and protein level expressed by recombinant *Lactobacillus* in the gastrointestinal tract of chickens. In contrast with previous studies, oral administration of delivery vector significantly increased colonization of POF_1/Lc393, POF_{78}/Lc393 recombinant *Lactobacillus* and growth performance (P<0.05) in severe gastric conditions.

The identification of inserted genes by PCR of isolates from ileum, cecum and colon were strongly colonized in chicken intestines. In earlier studies, *Lactobacillus* colonization in intestines mostly focused on genetics and taxonomy (Tierney *et al*., 2004). Micro-organisms' colonization in the gut of chicken was usually traced by fluorescent labeling (Fortineau *et al*., 2000).

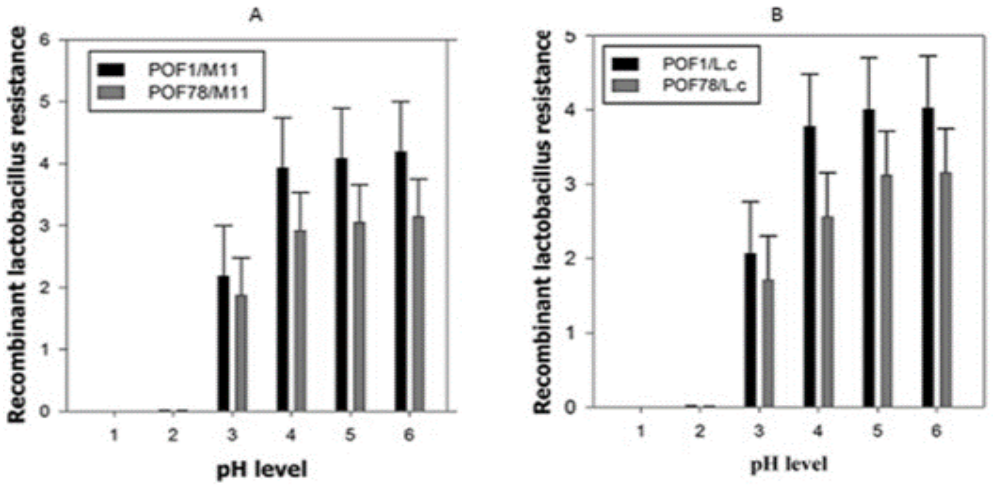

Fig. 4(A): Resistance of recombinants *Lactobacillus* POF_1/M11 and POF_{78}/M11 to pH, **(B)** Resistance of recombinants *Lactobacillus* POF_1/Lc393 and POF_{78}/Lc393 to pH (results are significant at 0.05 level).

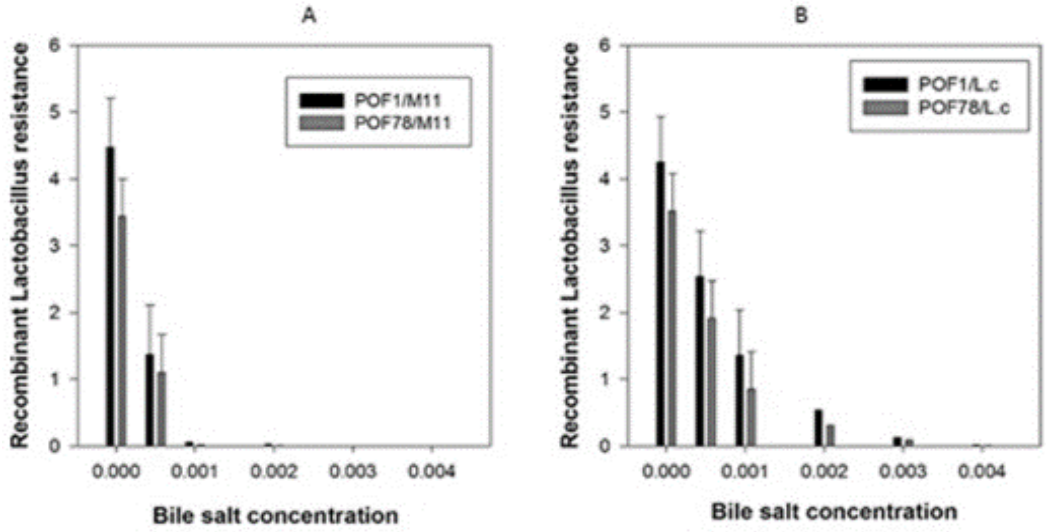

Fig. 5 (A): Resistance of recombinant *Lactobacillus* of POF_1/M11 and POF_{78}/M11 to bile salt **(B)** Resistance of recombinant *Lactobacillus* of POF_1/Lc393 and POF_{78}/Lc393 to bile salt concentration (results are significant at 0.05 level).

In consistent with earlier reports, the dynamic scattering and colonization of recombinants *Lactobacillus* in the ileum, cecum and colon at different intervals were detected by amplification of target genes. Recombinant LAB has been demonstrated to hinder in vitro intestinal pathogenic growth and therapy of broad range intestinal disorder (Rolfe, 2000). Many probiotics have various mechanisms of attachment and colonized on the epithelial cells of stomach (Friedrich, 2013). In this study, we evaluated a significant increase in retention rate of specific *Lactobacillus* strains in GI after gavage. In addition to, birds weight during the whole trail period determined that no antagonistic response because of recombinant *Lactobacillus* colonization. Bile salt in GI is one of the main factors that destroy the structure of cell and colony morphology (Margolles *et al*., 2003; Kurdi *et al*., 2006). Hence, we constructed bile salt resistant recombinant *Lactobacillus* with specific strains; *L. casei* and *L. saerimneri* that can sustain at 3.0% bile concentration. In previous studies, bile salt concentration totally depends upon species, strain of LAB and kind of consumed food (Soccol *et al*., 2010; Fontana *et al*., 2013), about 0.2 to 0.3% bile salt value found in GI of chicken and it may rise up to 2% (w/v) in different individuals. The LAB species used to construct POF_1/Lc393, POF_{78}/Lc393, POF_1/M11 and POF_{78}/M11 recombinants showed more resistance up to 0.3% also was significantly higher than those used in previous research. In addition, probiotics show fluctuating resistance to pH and this unique behavior is species and strain dependent (Dunne *et al*. 2001). The average resistance of selected LAB strains that used in recombinants showed significant difference ($P<0.01$). Both recombinant *Lactobacillus* strains colonize with high survival rates at pH 3.0 and have more adhesion ability on the epithelial cells at critical value. Recombinant *Lactobacillus* must pass through stomach, where average pH from 1.5 to 2.0 and stay alive for long period (Bakari *et al*., 2011). The adhesion properties of recombinants and high hydrophobicity such as a resistance against low pH and high bile salt, capacity to adhere on mucosa of intestine, low chance for pathogens to attack that expressed a hydrophobic property (Ripamonti *et al*., 2011; Pringsulaka *et al*., 2015). In conclusion, our research prefers that construction of

recombinant with selected *Lactobacillus* strains enhances colonization and adherence of recombinant LAB were significantly higher rather than pathogens. In that case, more advantages such as cohering or colonizing approaches could be used to enhance the immunity and growth performance in chickens in future. Further investigations will be required to construction of recombinant from other serogroups of APEC and transformation into more tolerable probiotic lactobacillus strains whether can be provide better colonization in the GI tract of chicken at different stages.

Conclusions: Finally, it can be concluded that the POF_1/Lc393, POF_{78}/Lc393, POF_1/M11 and POF_{78}/M11 recombinants showed more resistance against chicken diseases and impact of these on growth performance has been explored. It suggests that Recombinant DNA vaccine can be used as oral vaccines for the prevention and treatment of colibacillosis. However, further studies are needed to construct recombinants from other serogroups to inhibit various bacterial infections in chickens.

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Authors contribution: SMB has chased the concept, organized a method, analyzed and interpreted the collected data. MI helped in statistical analysis. MS has provided support in experiment handling. HLM provided assistance during the experiment. GA helped in data arrangement and critical revision.TL has provided study design co supervisor. YL has provided study concept as supervisor. IB analyzed the data.

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