Isolation and in vitro Characterization of Anti-Salmonella Enteritidis Probiotic Potential of Indigenous Lactobacilli from Poultry

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INTRODUCTION

Poultry is an important sector of Pakistan agricultural economy with 5.76, 10.4 and 1.3% contributions in agriculture, livestock and total GDP, respectively. In Pakistan, commercial poultry production was established in 1960s which is now providing a significant protein portion in diet on daily basis and employment to 1.5 million people (Hussain et al., 2015). Problems influencing human health still arise from poultry. One major problem is the contamination of poultry products with enteric pathogens (Kamollerd et al., 2016).

Salmonella Enteritidis is one of the most significant enteric pathogens colonizing poultry gut without clinical symptoms. Prolonged salmonellosis in chickens may lead to bacteremia followed by infection of ovaries, liver and spleen. Due to shedding in feces and vertical transmission, Salmonella disseminate through the entire flock and also cause post slaughter contaminations in poultry products (Deblais et al., 2018). Salmonella result economic loss of billions every year (Wales and Davies, 2011). Thus, Salmonella not only obstacle poultry production but it can also enter into human food chain resulting in food poisoning and gastro-intestinal infections.

Salmonella control from farm to fork is a great challenge due to the emergence of antibiotic resistant strains (Deblais et al., 2018). Traditional poultry production usually supplements feed with sub-therapeutic dose of antibiotics for the prevention and treatment of diseases, and increased growth performance. This may lead to the risk of antibiotic resistance in humans due to the consumption of poultry products, containing antibiotic residues (Kamollerd et al., 2016). Thus, the use of antibiotics and chemicals should be prohibited in Pakistan and novel alternatives should be searched out for controlling infectious diseases (Abbas et al., 2017a, b, c; Abbas et al., 2018; Idris et al., 2017). Probiotics can also serve as an alternative approach to control Salmonella issue in poultry (Amara and Shibal, 2015).

Probiotics, which means “for life” have been described as the living supplement in feed which beneficially affect

ABSTRACT

Aim of the present study was to isolate, identify and characterize new indigenous Lactobacillus strains with probiotic potential against Salmonella Enteritidis. From 84 isolated lactobacilli of indigenous poultry origin, 15 isolates were pre-selected for in vitro characterization on the basis of their activity (6.33±0.57-20.33±1.15 mm) against S. Enteritidis by well diffusion assay. All pre-selected isolates had variable tolerance to acidic pH (2, 3 and 4). All isolates also showed growth in MRS broth supplemented with 0.3, 1 and 1.8% bile salts. Isolates had varying degree of auto-aggregation (27.05±0.72 - 65.87±3.12%) and co-aggregation with S. Enteritidis (6.33±0.11 - 55.70±1.32%) within 2 hours. Safety profile of lactobacilli indicated that IKP 23, IKP 111 and IKP 333 had no acquired antibiotic resistance. IKP 23, IKP 111 and IKP 333 were selected as potential probiotics on the basis of probiotic prerequisites and identified as L. fermentum, L. fermentum and L. salivarius, respectively by sequencing their partial 16S rRNA gene or 16S spacer region. IKP 23, IKP 111 and IKP 333 inhibited Enteritidis (81, 99.3 and 93%, respectively) in co-culture experiments. This study insinuate that IKP 23, IKP 111 and IKP 333 have favourable probiotic potential and may be used for in vivo studies for the development of probiotics against S. Enteritidis.

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the host’s health, when administered in sufficient quantity (Somplang and Piyadeatsoontorn, 2016). Many scientists and researchers suggested Lactic acid bacteria (LAB) as probiotics or bio-therapeutic agents. Probiotics are considered as an alternative to antibiotics especially in poultry industry. Many probiotics have been added in birds’ diet (Popova, 2017). Moreover, probiotics have numerous other benefits in poultry industry including increased nutrient absorbance, better feed conversion ratio (FCR), accelerated production performance, strengthened gut microbiota, improved meat quality, better immune response, increased weight gain and competitive exclusions of pathogens (Gupta and Das, 2013).

Probiotics are commonly used in supplementation of human and animal foods for their health benefits (Shokryazdan et al., 2017). Probiotics can prevent the growth of pathogenic bacteria, such as Salmonella spp. and Escherichia coli, in gastrointestinal tracts (Casey et al., 2004). Mechanism of action of probiotics, not fully elucidated, is dependent upon properties of probiotic strains. Probiotics generally control enteric pathogens by reducing gut pH in microenvironment and secretion of antimicrobial substances (bacteriocins) and strengthening of normal flora (Wang and Gu, 2010). Probiotics also modulate mucosal and systemic immune responses and act as immune boosters (Tsai et al., 2005).

Keeping in mind the importance of S. Enteritidis in food safety and insufficiency of local probiotics, present study was designed as a first step in a multistep project to develop probiotics targeting mitigation of S. Enteritidis from chicken.

**MATERIALS AND METHODS**

**Isolation of lactobacilli:** Samples including caeca (n=50), ileum (n=50) and droppings (n=50) were obtained from indigenous poultry of different areas of the Punjab, Pakistan. Lactobacilli were isolated by plating serially diluted samples (10 fold) on De Man, Rogosa and Sharpe (MRS Agar) supplemented with nystatin (100 µg/100 ml) followed by 48 hours incubation at 37°C. Distinguished colonies were selected, purified and stored in MRS broth supplemented with 15% glycerol.

**Identification of lactobacilli:** Preliminary identification of isolates was achieved by Gram’s staining and catalase test. DNAs of isolates were extracted using GeneAll DNA extraction Kit (GeneAll Biotechnology, South Korea) following the manufacturer’s instructions. Genus specific polymerase chain reaction (PCR) using primers XBS-F (5'-GCCTTGTACACACCGGCGGT-3’) and LbLM1-R (5'-GAAAAACTAAAACAAAGTG-3’) was used for confirmation of lactobacilli. For species identification, 16S rRNA genes of selected isolates were also amplified using universal primers 8FLP-F(5'-GTTTGTGATCCCTGG CTCAG-3’) and XB4-R (5'-GTGTGTAACAGGCCC GG GAAC-3’) as described previously (Nawaz et al., 2011). Amplicons of 16S rRNA amplification (~1400 bp) or 16S-23S intergenic spacer region (~250 bp) were sequenced and submitted to NCBI for obtaining GenBank accession numbers.

**Screening of lactobacilli for anti-Salmonella Enteritidis activity:** Lactobacilli were screened for their activity against Salmonella Enteritidis, using well diffusion assay. Briefly, a lawn of S. Enteritidis (0.5 McFarland) was prepared on Mueller Hinton agar plates, wells were made and sealed with molten agar. Cell free supernatant (80-100µl) of lactobacilli was added. Plates were incubated at 37°C for 24 hours in aerobic condition followed by measuring diameter of zones of inhibition (Bao et al., 2010).

**In vitro characterization of lactobacilli for their probiotic properties**

**Resistance to low pH:** Tolerance of lactobacilli to low pH was determined as described previously (Asghar et al., 2016). Briefly, exponentially growing isolates were re-suspended (1.5x10⁶CFU/ml) in phosphate buffer saline (PBS) at different pH (2, 3, 4, 7) for 90 min. Tolerance to pH was determined by re-culturing 100 µl of pH treated bacterial suspension in 10 ml MRS broth for 24 hours at 37°C, followed by the measurement of optical density (O.D) at 600 nm.

**Resistance to bile salts:** Tolerance of isolates to bile salts was also determined as described previously (Asghar et al., 2016). Briefly, exponentially growing cultures (1%) were inoculated in MRS broth supplemented with different concentrations of bile salts (0.3%, 1.0%, and 1.8%) for 24 hours at 37°C followed by measuring the O.D. at 600 nm.

**Antibiotic susceptibility profile:** Antibiotic susceptibility pattern of lactobacilli was determined by disc diffusion method on MRS agar. Lactobacilli (1 McFarland) were swabbed on MRS plates and antibiotic discs were placed. Plates were incubated at 37°C for 24 hours, in anaerobic conditions followed by measuring the diameter (mm) of zones of inhibition. Results were interpreted according to breakpoints adopted from Clinical and Laboratory Standards Institute (CLSI) guidelines or European Food Safety Authority (EFSA, 2012).

**Auto-aggregation and co-aggregation:** Auto-aggregation and co-aggregation of lactobacilli with S. Enteritidis were determined as described previously (Asghar et al., 2016). For auto-aggregation freshly grown Lactobacillus culture was re-suspended in PBS after centrifugation at 6000 rpm, followed by incubation at 37°C. In order to observe co-aggregation, equal volumes of Lactobacillus and S. Enteritidis suspensions in PBS were mixed and incubated at 37°C. OD values were recorded at 600nm after different time intervals (1 hour and 2 hours).

**Inhibition of Salmonella in broth culture:** Selected lactobacilli (IKP23, IKP111 and IKP333) and S. Enteritidis were co-cultured in nutrient broth (10 ml) for 24 hours at 37°C and enumerated at different time intervals (6 hours and 24 hours) on MRS and Salmonella shigella agar, respectively to determine the effect of lactobacilli on Salmonella growth kinetics.

All data were expressed as Mean ± Standard deviation and compared by One way ANOVA followed by Tukey’s multiple comparison test (P≤0.05).
RESULTS

A total of 84 lactobacilli were isolated from indigenous poultry droppings, ileum and caeca and subjected to screening for anti-Salmonella Enteritidis potential. All isolates were Gram positive rods and catalase negative. Genus specific PCR amplification of ~250 bp amplicons confirmed all isolates as lactobacilli. Only 15 isolates, showing anti-microbial activity against S. Enteritidis, were selected for further analysis. As indicated in Table 1, IKP 111, IKP192 and IKP402 showed strongest activity (20.33±0.57 mm) against S. Enteritidis. All fifteen isolates showed varying degree tolerance to pH 4, 3 and 2 as presented in Table 2. IKP07 and IKP76 showed poor growth after exposure to acidity (pH 2, 3 and 4). All isolates were more tolerant to pH 4 as compared to pH 3 and pH 2. Similarly, all isolates were more resistant to 0.3% bile salts as compared to 1% and 1.8% bile salts (Table 3). Antibiotic susceptibility profiles of selected isolates against different antibiotics are presented in Table 4. Isolates containing acquired antibiotic resistance to penicillin (IKP183, IKP229 and IKP271), ampicillin (IKP94 and IKP402), erythromycin (IKP76, IKP94 and IKP402) and tetracycline (IKP138, IKP 271, IKP162 and IKP387) were considered safety risk and excluded from further analysis. IKP23, IKP111 and IKP333 had no acquired antibiotic resistance. Auto-aggregation and co-aggregation of lactobacilli with S. Enteritidis is given in Table 5. Isolates had varying degree of auto-aggregation (27.05±0.72 - 65.87±3.12%) and co-aggregation with Salmonella (6.33±0.11 to 55.70±1.32%) in two hours. IKP138 showed highest auto-aggregation (65.87±3.12%) while IKP111 has highest co-aggregation (55.70±1.32%) against S. Enteritidis. Co-culture experimentation of selected isolates (IKP 23, IKP 111 and IKP 333) with S. Enteritidis revealed highest inhibition (99.3%) by IKP 111, as indicated in Table 6. IKP 23, IKP 111 and IKP 333 were selected as potential probiotics on the basis of all probiotic prerequisites and identified as L. fermentum, L. fermentum and L. salivarius, respectively by sequencing. NCBI GenBank accession numbers of L. fermentum IKP23, L. fermentum IKP111 and L. salivarius IKP333 are MK350329, MK350330 and MK346270, respectively.

DISCUSSION

Salmonella is major pathogen of human and poultry. It can transmit horizontally as well as vertically in poultry and eventually to humans (Gole et al., 2014). The prophylactic use of antibiotics to control Salmonella infections in poultry may cause alterations in gastrointestinal flora and promote emergence of antibiotic resistant strains (Saleem et. al., 2018). It is dire need of time to search for alternative approaches like probiotics. As defined by Food and Agriculture organization (FAO), Probiotics are the live microbes which confer health benefits to host when ingested in adequate amounts. Lactic acid bacteria are more appropriate as probiotics because they have GRAS (Generally recognized as Save) status. World Health Organization (WHO) and FAO have already recommended the use of lactic acid bacteria strains as probiotics in animals and humans (Amara and Shihb, 2015).

| Table 1: Antibacterial activity of selected lactobacilli against Salmonella Enteritidis |
|-----------------------------|-----------------------------|-----------------------------|
| Isolate  | Isolate source | Antimicrobial activity (mm) expressed as Mean ± S.D. |
| IKP07  | Poultry dropping | 6.33±0.57^a |
| IKP23  | Poultry dropping | 17.32±0.57^a |
| IKP41  | Poultry dropping | 16.32±1.52^a |
| IKP76  | Poultry dropping | 18±0.1^a |
| IKP94  | Poultry dropping | 11.66±0.57^a |
| IKP11  | Poultry dropping | 20.33±0.57^a |
| IKP138 | Poultry dropping | 14.66±1.15^a |
| IKP162 | Poultry dropping | 9.32±0.57^a |
| IKP183 | Poultry cecum | 13.33±0.57^a |
| IKP192 | Poultry cecum | 20.33±0.57^a |
| IKP229 | Poultry cecum | 8.66±0.57^a |
| IKP271 | Poultry ileum | 12±1.5^a |
| IKP333 | Poultry ileum | 16.96±1.15^a |
| IKP387 | Poultry ileum | 17±1.5^a |
| IKP402 | Poultry ileum | 20.33±1.5^a |

^a^ Different superscripts in different rows of same columns show statistically significant difference at P≤0.05.

<p>| Table 2: pH tolerance in lactobacilli |
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<th>Lactobacilli</th>
<th>pH tolerance in lactobacilli</th>
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<td><strong>pH</strong></td>
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| 7  | 1.8% bile salts (Table 3). Antibiotic susceptibility profiles of selected isolates against different antibiotics are presented in Table 4. Isolates containing acquired antibiotic resistance to penicillin (IKP183, IKP229 and IKP271), ampicillin (IKP94 and IKP402), erythromycin (IKP76, IKP94 and IKP402) and tetracycline (IKP138, IKP 271, IKP162 and IKP387) were considered safety risk and excluded from further analysis. IKP23, IKP111 and IKP333 had no acquired antibiotic resistance. Auto-aggregation and co-aggregation of lactobacilli with S. Enteritidis is given in Table 5. Isolates had varying degree of auto-aggregation (27.05±0.72 - 65.87±3.12%) and co-aggregation with Salmonella (6.33±0.11 to 55.70±1.32%) in two hours. IKP138 showed highest auto-aggregation (65.87±3.12%) while IKP111 has highest co-aggregation (55.70±1.32%) against S. Enteritidis. Co-culture experimentation of selected isolates (IKP 23, IKP 111 and IKP 333) with S. Enteritidis revealed highest inhibition (99.3%) by IKP 111, as indicated in Table 6. IKP 23, IKP 111 and IKP 333 were selected as potential probiotics on the basis of all probiotic prerequisites and identified as L. fermentum, L. fermentum and L. salivarius, respectively by sequencing. NCBI GenBank accession numbers of L. fermentum IKP23, L. fermentum IKP111 and L. salivarius IKP333 are MK350329, MK350330 and MK346270, respectively.

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Lactobacilli can kill or reduce pathogen by reduction in gut pH due to lactic acid production, secretion of antimicrobial bacteriocins and H2O2, competitive exclusion of pathogen and strengthening normal flora (Wang and Gu, 2010). Present study searched out 84 indigenous probiotic lactobacilli from poultry birds. Different researches have also reported the isolation of lactobacilli from poultry and fermented food products in Pakistan (Asghar et al., 2016; Arif et al., 2018; Saleem et al., 2018).

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other researchers have declared anti-Salmonella potential of lacticbactocili using different strategies like spot test (Garriga et al., 1998), inhibition of Salmonella invasion using HT29 human intestinal cell line (Casey et al., 2004) and competitive exclusion of Salmonella in gut (La Ragione et al., 2004). Salmonella inhibition capability of isolates is in accordance to the previous studies (Makras et al., 2006). In this way, growth kinetics of S. Enteritidis was determined when co-cultured with lacticbactocili. L. salivarius can produce acetic acid and lactic acid as a result of sugar fermentation in poultry feed, resulting in inhibition of pathogenic bacteria. Another in vitro study also declared probiotic potential of L. salivarius isolated from ducks (Sumarish et al., 2014). In vivo study by Choi et al. (2011), also declared immune enhancing effect of L. salivarius. Complete inhibition of H. pylori by L. salivarius both in mixed cultures and infected gnotobiotic murine model has been also reported previously (Aiba et al., 1998). Similarly, L. fermentum have been also isolated previously from fermented plant material (Morita et al., 2008). Hypocholesterolemic effect of L. fermentum as a probiotic have been also evaluated previously (Pereira et al., 2003).

Acquired antibiotic resistance in lacticbactocili poses a significant threat to public health. Probiotic lacticbactocili should lack resistance against antibiotics so that they may not transfer it to pathogens. Present study revealed resistant lacticbactocili against tetracycline, erythromycin, ampicillin, chloramphenicol, penicillin and vancomycin which were excluded from further analysis. This resistance may be adopted during the course of time by acquiring resistant genes harboring plasmids from other resistant pathogens. Acquired antibiotic resistance have also been previously characterized in lactobacilli (Saleem et al., 2018).

Auto-aggregation and co-aggregation are also important pre-requisites for the selection of probiotics. Auto-aggregation is an indicator of adhesion capacity of lacticbactocili to epithelial cells, resulting in mitigation of pathogen adhesion. Lactobacillus strains showed significant auto-aggregation. Similar results have also been reported previously (Bao et al., 2010; Asghar et al., 2016). Co-aggregation of probiotics indicates their ability to inhibit pathogens. IKP 76 and IKP 387 carried good capability to co-aggregate with S. Enteritidis. Similar co-aggregation pattern of lacticbactocili against Salmonella have been also reported previously (Collado et al., 2007; Asghar et al., 2016). Thus, these isolates can be employed as potential probiotics against S. Enteritidis in poultry after in vivo evaluations. Various studies have determined in vivo probiotic potential of lacticbactocili (Garriga et al., 1998). During in vivo evaluations, effect of lacticbactocili on parameters i.e., body weight, feed conversion ratio, immune response, phosphorus availability and gut morphology may also be examined (Asghar et al., 2016).

**Conclusions:** It is concluded that L. fermentum IKP 23, L. fermentum IKP 111 and L. salivarius IKP 333 may be used as potential probiotics in poultry to control and mitigate Salmonellae as an alternative of antibiotics, after further in vivo evaluations.

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Authors contribution: MN, AAA and MDA designed the project. IK collected samples and executed experiments. MN and IK analyzed data. MN and AAA prepared manuscript. All authors revised the manuscript and approved the final version

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