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**SHORT COMMUNICATION**

# PROBIOTIC CHARACTERIZATION OF THE *LACTOBACILLUS* ISOLATES FROM BUFFALO VAGINA

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## ABSTRACT

This study was conducted to screen and select the potential probiotic bacterial strains from buffalo vagina. Five isolates were identified biochemically and at molecular level. To determine probiotic ability, tolerance to acidic conditions and bile concentration, and antibacterial activity against two indicator pathogens *Salmonella typhii* and *Escherichia coli* were tested. PCR amplification of the isolates using *Lactobacillus* specific primer was carried out followed by sequencing of amplified region. The results indicated that all of the isolates, belonged to different species of *Lactobacillus*, sharing up to 99% homology with sequences of different *Lactobacillus* species in the NCBI database. Three isolates were identified as *Lactobacillus gasseri* and one each as *Lactobacillus bombicola* and *Lactobacillus johnsonii* using specific gene sequence analysis. All of the isolates survived at pH 2 and 0.3% bile concentration and were also found to be active against *S. typhii* and *E. coli* indicating their potential use as probiotic strains.

**Key words:** Acid tolerance, Bile tolerance, Buffalo vagina, Lactic acid bacteria, Lactobacillus, Probiotic.

## INTRODUCTION

The *Lactobacillus* genus has been extensively studied for its probiotic properties and have been isolated from various environments including gastrointestinal tract and vagina of various species, soil, and vegetables etc (Nawaz *et al*., 2011; Meira *et al*., 2012; Asghar *et al*., 2016). Buffaloes (*Bubalus bubalis*) are major milk producers in Pakistan, contributing to 61% of total milk production in the country. However, very few studies describe isolation of Lactobacilli from buffalo vagina (Abd-el-Moez *et al.,* 2008) and no such study has been conducted in Pakistan. This short study was therefore conducted to investigate the probiotic potential of the *Lactobacillus* strains isolated from buffalo vagina.

## MATERIALS AND METHODS

**Sample collection, lactobacilli isolation and identification:** Sterile swabs were used for collecting bacteria from buffalo vagina which were then inoculated on de Man, Rogosa and Sharpe (MRS) agar followed by incubation for 48 hours at 37 °C. The colonies of catalase negative and Gram positive rods were selected for further sub-culturing and preservation in 20% glycerol at -25 °C. DNA was extracted from the isolated *Lactobacillus* strains using Easy DNA Invitrogen Kit (catalog # K1800-01). The *Lactobacillus* specific gene was amplified using following genus specific primer (Amit-Romach *et al*., 2004) as given below:

Forward primer (LAA-F): CATCCAGTGCA AACCTAAGAG

Reverse primer (LAA-R): GATCCGCTTGC CTTCGCA

The final volume of PCR reaction mixture was 30 µL which consisted of 1 µL template DNA, 3 µL of 10X PCR buffer, 1.8 µL each of the four dNTPs and $MgCl_2$, 0.7 µL each of the forward and reverse primer, 0.3 µL of *Taq* DNA polymerase, and 20.7 µL of PCR grade water. The reaction conditions were: initial denaturation at 95 °C for 2 min, denaturation at 95 °C for 1 min, annealing at 54 °C for 1.5 min, extension at 72 °C for 2 min and a final extension at 72 °C for 10 min. Thirty cycles of PCR reaction were performed. The PCR products were visualized on a 1.2% agarose gel along with 1 kb DNA ladder, and later sequenced at Beijing Genomic Institute (BGI). The sequences were analysed for homology using "EzTaxon", "NCBI-BLAST" and "Clustal W".

**Probiotic activity:** To evaluate the survival of the isolated strains at low pH and different bile concentrations, 1 mL from each freshly (active) cultures was used. For pH survival evaluation, MRS broth tubes were prepared and pH was adjusted to 2.0, 3.0 and 4.0 using sterile 5 N HCl. An aliquot of 1 mL from each freshly cultured isolates was transferred to MRS broth and incubated at 40 °C. Viable microorganisms were enumerated after 4 hours using pour plate method. Plates were incubated at 40 °C under aerobic conditions for 48 hours for determination of colony forming units per mL (CFU/mL). For evaluation of tolerance against bile, MRS

broth with 0.2% and 0.3% bile salt concentrations was prepared and inoculated with 1 mL of active cultures of each isolate. After 4 hours, viable colonies were enumerated as described above.

Two pathogens, *Salmonella typhii* and *Escherichia coli*, were used as indicator organisms for agar well diffusion assay. Wells were formed in sterile nutrient agar plates and 1 mL of the broth containing indicator strain was poured and spread to form lawn using sterile swabs. Then 0.5 mL of inoculum from each isolate was transferred to the wells in agar plates (4 strains in each plate) and nutrient agar plates were then incubated at 37 °C under aerobic conditions for 24 hours. At the end of the incubation, the plates were observed for the presence or absence of inhibition zones around the wells.

## RESULTS AND DISCUSSION

The initial screening resulted in selection of five isolates which were subjected to PCR identification and probiotic properties tests. To identify the isolates at genus and species level, PCR amplification of the specific gene for *Lactobacillus* genus was performed. A band of 286 bp was obtained for all the five isolates when the PCR products were run on 1.2% agarose gel. This confirmed that all of the isolates belonged to the genus *Lactobacillus* (Amit-Romach *et al*., 2004). To further identify down to species level, the PCR products were sequenced and then compared using bioinformatics softwares EzTaxon, NCBI-BLAST and Clustal W. The results ( Table 1) indicated that three isolates belonged to the species *Lactobacillus gasseri*, while two others belonged to *L. bombicola* and *L. johnsonii*.

Resistance against the low pH and bile are important criteria of selection for probiotic strains. In the current study, two lactobacilli isolates (Figure 1), HVS-2 and HVS-3 most successfully tolerated the low pH showing growth of l.85 log CFU/mL. Though, the bacteria isolated from vagina are not expected to tolerate bile but all the isolates showed good bile tolerance as shown in Figure 2. Similar results regarding acid and bile tolerance of lactobacilli isolates have been reported by previous researchers (Meira *et al.*, 2012). Studies have shown that the members of the genus *Lactobacillus* are intrinsically tolerant to low pH (Tannock, 2004), and their bile tolerance is due to the presence of bile salt hydrolase enzymes that specifically hydrolyze bile salts (McAuliffe *et al*., 2005). Therefore, lactobacilli are considered most suitable for use as probiotics.

In the present study, all the five selected strains from buffalo vagina not only exhibited sufficient acid and bile resistance but also showed inhibitory effects against *S. typhii* and *E. coli* (Table 2). The inhibitory effect was especially prominent against *E. coli* as indicated by larger zones of inhibition. Similar to these results, Abd el Moez *et al*. (2008) noted that 80% of the *Lactobacillus* strains isolated from buffalo vagina showed antimicrobial activity against *E. coli*. Also, the isolates having antimicrobial activity showed inhibition zones ranging from 6 to 16 mm against *E. coli*, *Micrococcus spp*., *Yersinia enterocolitica*, *Enterococcus faecalis*, *S. aureus* and *Bacillus spp*. Overall, the noted inhibition of the indicator pathogens in ours and previous studies could be due to bactericidal or bacteriostatic activities of the bioactive substances produced by the tested strains, such as bacteriocins, organic acids, and low molecular weight peptides etc.

**Table 1. Homology analysis of the Lactobacillus strains from buffalo vagina.**

| Isolates | Closest Match | Similarity % |
|---|---|---|
| HVS-1 | *Lactobacillus bombicola* | 90.0 |
| HVS-2 | *Lactobacillus gasseri* | 96.9 |
| HVS-3 | *Lactobacillus johnsonii* | 97.4 |
| HVS-4 | *Lactobacillus gasseri* | 98.5 |
| HVS-5 | *Lactobacillus gasseri* | 98.1 |

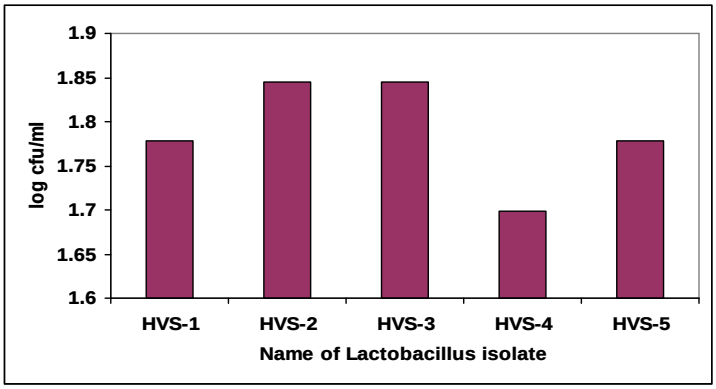


**Figure 1. Viability of selected *Lactobacillus* strains after exposure to pH 2 for four hours.**

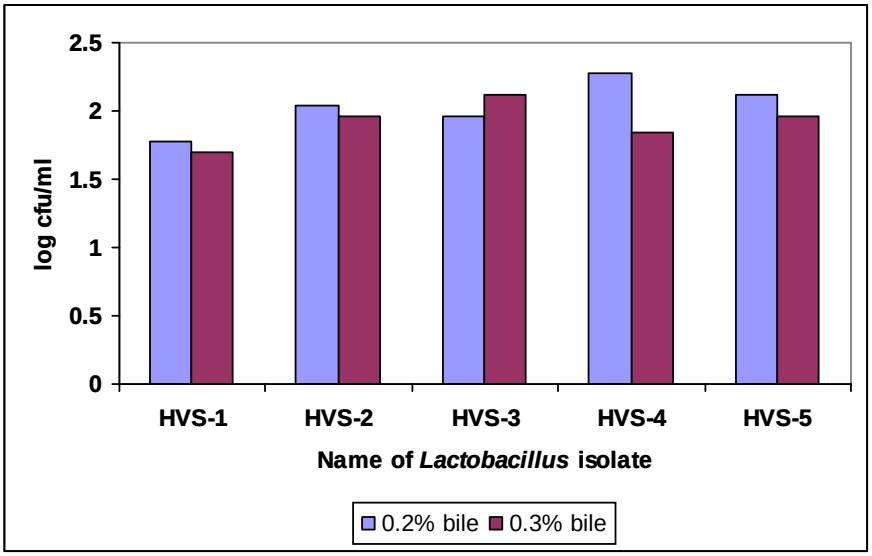


**Figure 2. Viability of selected *Lactobacillus* strains after exposure to different bile concentrations for four hours.**

**Table 2. Antimicrobial activity against selected indicator pathogens in agar well diffusion assay.**

| Name of isolate | *Salmonella typhii* | *Escherichia coli* |
|---|---|---|
| HVS-1 | ++ | +++ |
| HVS-2 | ++ | +++ |
| HVS-3 | +++ | +++ |
| HVS-4 | ++ | +++ |
| HVS-5 | +++ | ++ |

++ means a zone of inhibition ≤ 10 mm; +++ means a zone of inhibition ≥ 10 mm

In conclusion, the present results indicate that buffalo vagina could be a potentially suitable site for searching probiotic strains for use in vagina and in the intestine. Further studies are needed in this direction for development of probiotic products.

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