IN VITRO EVALUATION OF SAFETY AND PROBIOTIC ATTRIBUTES OF LACTOBACILLI ISOLATED FROM HUMAN AND GOAT MILK

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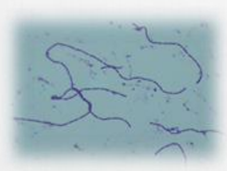
Introduction: Probiotics, globally recognized as "Live microorganisms which when administered in adequate amount confer some health benefit to the host" (FAO/WHO2001), are the beneficial gut microbes that plays a crucial role in maintaining gut and human health (Marchesi et al 2016). Probiotic administration in adequate amount could improve human health through restoration of host normal microflora, re-establishing the intestinal barrier function, immune homeostasis and support of normal digestive functioning by providing several trace elements to the host (Fong et al 2015).The 'probiotic effect' is mainly mediated by suppression of pro-inflammatory cytokines, expression of anti-inflammatory and associated anti-oxidative and anti-microbial activity. The interference of probiotics with adhesion and colonization of pathogens also seems to be involved in probiotic action.

Microbial strains serving as candidate probiotic are mostly isolated from traditional fermented milk products (curd, lassi, cheese(s) etc.) and fermented fruits and vegetables. However, the isolation source varies between studies, regions and do have impact over functionality of isolates (Swain et al 2014). Microbial composition varies between regions, environmental conditions and fermentation type. Although milk, food and vegetables are explored a lot for isolation of probiotic strains, researchers believe that strains with human origin may survive better during human gastric transit compared to those of non-human origin (Ranadheera et al 2014). Keeping this in mind, healthy human milk samples are explored for selection of strains with rich probiotic potential.

Safe transit through stomach and survival and colonization in the intestinal tract are the foremost parameters to qualify as a potential candidate for further screening for probiotic attributes (Kotzamanidis et al 2010). Microbes to be ingested as probiotic strain have to encounter various stress factors during the establishment in the gastro-intestinal tract (GIT). Probiotic bacteria must retain viability during its interaction with stomach acid, bile and high osmolarity in the small intestine (Franz and Holzapfel 2011). Keeping this in view, a bacterial strain to serve as potential probiotic should survive the pH stress of gastric acid. Tolerance towards bile acids is attributed to presence of bile salt hydrolases (BSH), product of *bsh* gene of bacteria (Begley et al 2006). Presence and expression of *bsh* gene is targeted as one of the criteria for probiotic strain selection (Patel et al 2010). After safe transit to the small intestine, probiotic strains need to adhere to the intestinal lining, determined by multiple factors viz. cell surface hydrophobicity (CSH); cell adhesion potential; aggregation. Adhesion to the intestinal epithelial cell lining along with bacterial CSH are among the most important characteristics of lactobacilli for selecting probiotic strains (Ouwehand et al 1999; Younes et al 2012). Higher bacterial hydrophobicity can be directly co-related to their stronger adherence capability (Pan et al 2006). Auto-aggregation potential of bacteria plays an important role in adhesion to intestinal cells (Dunne et al 2001). It tells about the activity of bacterial cells to interact with them in a non-specific way, which is pre-requisite for GIT colonization (del Re et al 2000). Anti-microbial activity is considered to be a significant functional criterion for competitively inhibiting the pathogenic intestinal microflora through production of organic acids, H2O2, bacteriocins etc. LABs possess strong anti-oxidative activity and decrease the risk of reactive oxygen species (ROS) accumulation (Achuthan et al 2012). Oxidative stress is caused by an imbalance between ROS or free radical production and body antioxidant defense, which alters the normal cellular functions, further leading to several clinical situations.


Human and Goat milk

Isolation and Identification


Lactobacillus strains

Probiotic and Safety attributes

Objective: The aim of the present study was to establish the indigenous probiotic strains from human and goat milk along with their safety validation.

Methods

Isolation and Identification of lactobacilli from Human and Goat Milk

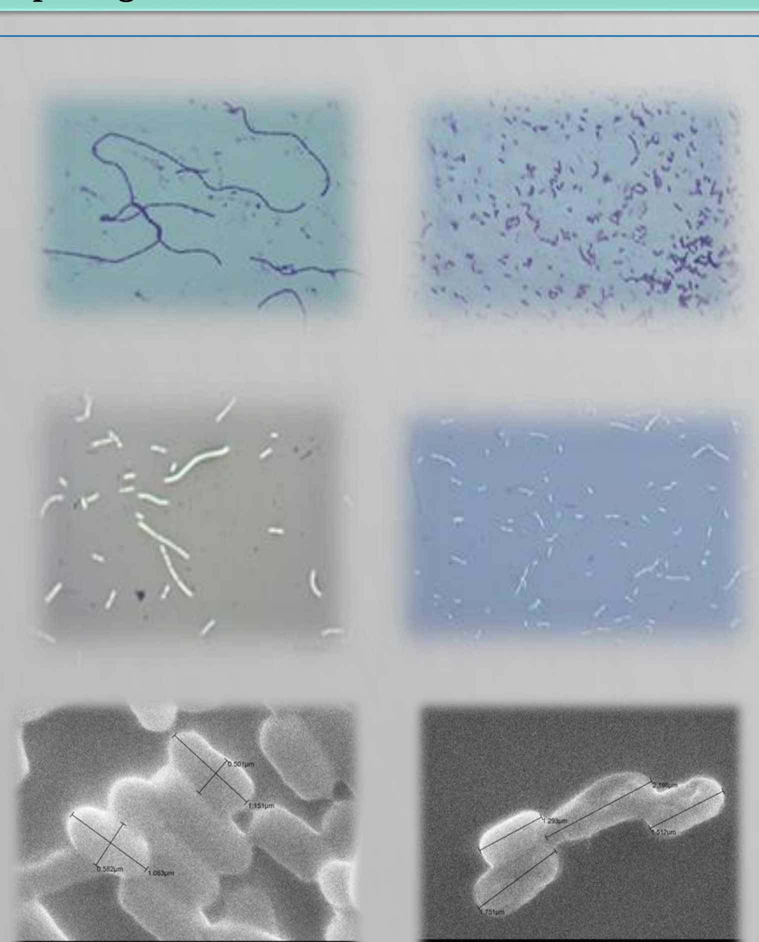
Isolated from milk by standard serial plate standard dilution on MRS Agar

Morphological and Chemical Identification using Catalase test and Gram's staining and scanning electron micrographs

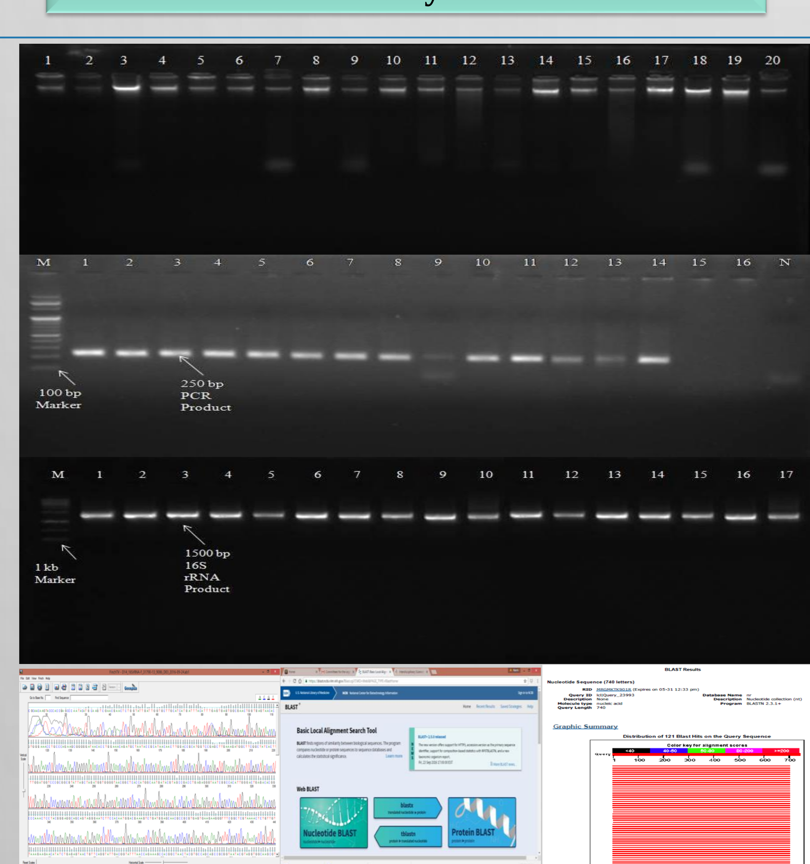
Molecular level identification by polymerase chain reaction (PCR) using *Lactobacillus* genus specific primers and 16S rDNA sequencing and MALDI-TOF

Identified lactobacilli strains were checked for their Probiotic and Safety attributes.

Morphological Identification of Isolated Lactobacilli


Gram's staining (First Row), negative staining (Second Row), and scanning electron micrographs (Third Row) for LAB isolates

Minimal inhibitory Concentration



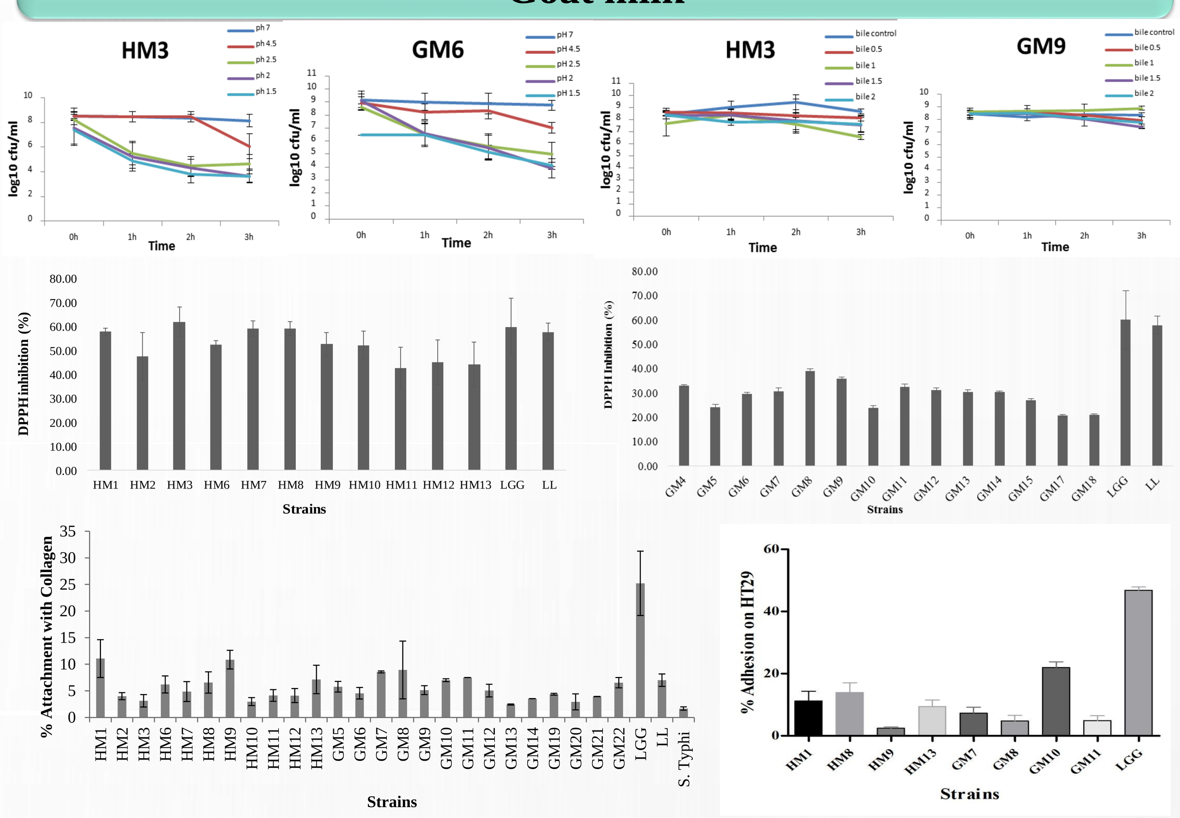
Agarose gel representative for genomic DNA(1st row), Genus specific PCR product for *Lactobacillus* sp.(2nd row), 16S rDNA based PCR product(3rd row); and representative 16S sequence profile, BLAST search tool and BLAST results (last row). Lanes 'M' denotes marker i.e. ladder of 100bp and 1kb, numerical values denotes independent wells and samples

Results

List of confirmed Lactobacillus sp. isolates from both human and goat milk along with their NCBI accession numbers

Code	Identification name	NCBI accession numbers
IPhp-HM1	Lactobacillus casei	KX714820
IPhp-HM2	L. plantarum	KX943021
IPhp-HM3	Lactobacillus sp.	KX301286
IPhp-HM6	L. pentosus	KX301287
IPhp-HM7	L. plantarum	KX301288
IPhp-HM8	L. plantarum	KX301289
IPhp-HM9	L. plantarum	KX714821
IPhp-HM10	L. Plantarum	KX301290
IPhp-HM11	L. Plantarum	KX714822
IPhp-HM12	Lactobacillus sp.	KX301291
IPhp-HM13	L. pentosus	KX301292
IPhp-GM4	L. plantarum	KX943025
IPhp-GM5	L. rhamnosus	KX943026
IPhp-GM6	L. plantarum	KX943027
IPhp-GM7	L. plantarum	KX943028
IPhp-GM8	L. plantarum	KX943029
IPhp-GM9	L. pentosus	KY658473
IPhp-GM10	L. plantarum	KX943030
IPhp-GM11	L. plantarum	KY658474
IPhp-GM12	L. plantarum	KY658475
IPhp-GM13	L. plantarum	KY658476
IPhp-GM14	L. plantarum	KY658477
IPhp-GM15	L. plantarum	KY658478
IPhp-GM17	L. fermentum	KY658479
IPhp-GM18	L. gasseri	KY658480

Probiotic Attributes of Lactobacilli isolated from Human and Goat milk



(1st row left to right)Acid Tolerance profile of human and goat milk, Bile Tolerance of human and goat Milk (2nd row left to right)DPPH radical scavenging activity of human and Goat milk lactobacilli. (3rd row left to right) Percent attachment of test strains to collagen matrix in comparison to *L. rhamnosus* GG. And Percent adhesion test strains to HT-29 cells.

Antibacterial profile of Lactobacilli isolated from human and Goat milk samples

Antibacterial profile of lactobacilli isolated from human milk samples

	S. aureus	B. cereus	S. Mutams	L. monocytogenes	S. enterica Typhi	P. mirabilis	S. flexneri	P. aeruginosa	E. coli	K. pneumonia
HM1	-	++	-	-	+	-	++	++	-	-
HM2	-	+++	-	-	++	-	++	+	-	-
HM3	-	++	-	-	+	-	+	+	-	-
HM6	-	++	-	-	+	-	++	+	-	-
HM7	-	++	-	-	+	-	++	+	-	-
HM8	-	+	-	-	+	-	-	+	-	-
HM9	-	+	-	-	+	-	-	+	-	-
HM10	-	+	-	-	+	-	-	+	-	-
HM11	-	+	-	-	+	-	-	+	-	-
HM12	-	+	-	-	+	-	+	+	-	-
HM13	-	++	-	-	+	-	++	+	-	-
LGG	+	+	-	-	+	-	++	+	-	-
LL	-	+	-	-	-	-	-	-	-	-

>11mm - No activity (-); 12-15mm – Weak inhibition (+); 16-19 mm – Moderate/Average inhibition (++); 20-24 - Strong inhibition (+++); <25mm - Very strong inhibition (++++)

Antibacterial profile of CFSs of lactobacilli isolated from goat milk sample

	S. aureus	B. Cereus	S. mutams	L. monocytogenes	S. enterica Typhi	P. mirabilis	S. flexneri	P. aeruginosa	E. coli	K. pneumoniae
GM4	-	-	-	-	-	-	+	++	-	-
GM 5	-	-	-	-	-	-	++	++	-	-
GM 6	-	-	-	-	-	-	+	++	-	-
GM 7	-	-	-	-	-	+	-	++	-	-
GM 8	-	-	-	-	-	-	+	++	-	-
GM 9	+	-	-	-	-	+	-	++	-	-
GM 10	+	-	-	-	-	+	-	++	-	-
GM 11	+	-	-	-	-	+	-	++	-	-
GM 12	-	-	-	-	-	+	-	++	-	-
GM 13	+	-	-	-	-	-	+	+	-	-
GM 14	+	-	-	-	-	-	+	++	-	-
GM 15	+	-	-	-	-	-	+	++	-	-
GM 17	+	-	-	-	-	-	-	++	-	-
GM 18	++	-	-	-	-	+	+	+++	-	-

Validation of safety parameters of candidate probiotic LAB Isolates

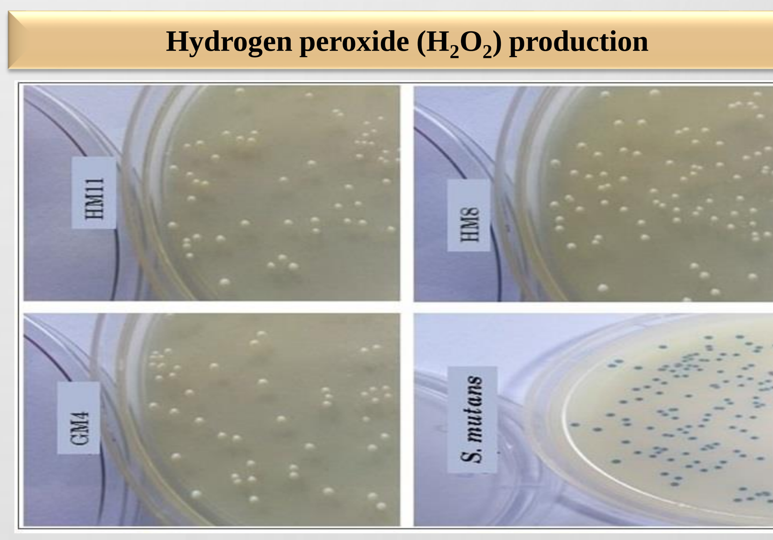
Antibiotic susceptibility assay (Human milk Isolates)

Antibacterial susceptibility profile of human milk lactobacilli to commercial antibiotics

S.No.	Antibiotics	Lactobacillus isolates										
		HM1	HM2	HM3	HM6	HM7	HM8	HM9	HM10	HM11	HM12	HM13
1	Ampicillin	R	S	S	S	S	S	S	S	S	S	S
2	Imipenem	S	S	S	S	S	S	S	S	S	S	S
3	Meropenem	S	S	S	S	S	S	S	S	S	S	S
4	Methicillin	R	R	R	R	R	R	R	R	R	R	R
5	Oxacillin	R	R	R	R	R	R	R	R	R	R	R
6	Penicillin	R	S	15.3 ± 0.57	19 ± 0	S	17.6 ± 0.57	19 ± 1	S	16.3 ± 0.57	16.6 ± 0.57	S
7	Cefuroxime	17.6 ± 0.57	R	R	R	S	16.3 ± 0.57	R	16 ± 0	R	R	R
8	Cefoxitin	R	R	R	R	R	R	R	R	R	R	R
9	Ceftazidime	15.3 ± 0.57	R	R	R	18.3 ± 0.57	17.3 ± 0.57	16.3 ± 0.57	R	16.3 ± 0.57	16 ± 1	R
10	Cefotaxime	15.6 ± 0.57	S	S	S	S	S	S	S	S	S	S
11	Teicoplanin	R	R	R	R	R	R	R	R	R	R	R
12	Vancomycin	R	R	R	R	R	R	R	R	R	R	R
13	Ciprofloxacin	R	R	R	R	R	R	R	R	R	R	R
14	Ofloxacin	R	R	R	R	R	R	R	R	R	R	R
15	Gentamicin	17.3 ± 0.57	16.3 ± 0.57	16 ± 0	15.3 ± 0.57	16.6 ± 0.57	17.3 ± 0.57	17 ± 1	16.3 ± 0.57	15.6 ± 0.57	16.3 ± 0.57	R
16	Streptomycin	R	R	R	R	R	R	R	R	R	R	R
17	Tobramycin	R	R	R	R	R	R	R	R	R	R	R
18	Chloramphenicol	17.3 ± 0.57	S	S	S	S	S	S	S	S	S	S
19	Clindamycin	S	R	R	R	R	R	15.6 ± 0.57	R	R	16.3 ± 0.57	R
20	Erythromycin	17.6 ± 0.57	S	S	S	S	S	S	S	S	S	S
21	Fusidic acid	R	16.3 ± 0.57	17.3 ± 0.57	16.6 ± 0.57	17 ± 1	16.3 ± 0.57	R	17 ± 0	R	17.6 ± 0.57	15.6 ± 0.57
22	Nitrofurantoin	R	S	S	S	S	S	S	S	S	S	S
23	Tetracycline	19.3 ± 0.57	S	17.6 ± 0.57	18.3 ± 0.57	S	19 ± 0	17.3 ± 0.57	18.6 ± 0.57	16.3 ± 0.57	S	19 ± 0
24	Tigecycline	16.6 ± 0.57	S	S	S	S	S	S	S	S	S	S
25	Co-Trimoxazole	R	R	R	R	R	R	R	R	R	R	R
26	Trimethoprim	R	S	R	R	R	R	R	R	R	19 ± 0	18.3 ± 0.57

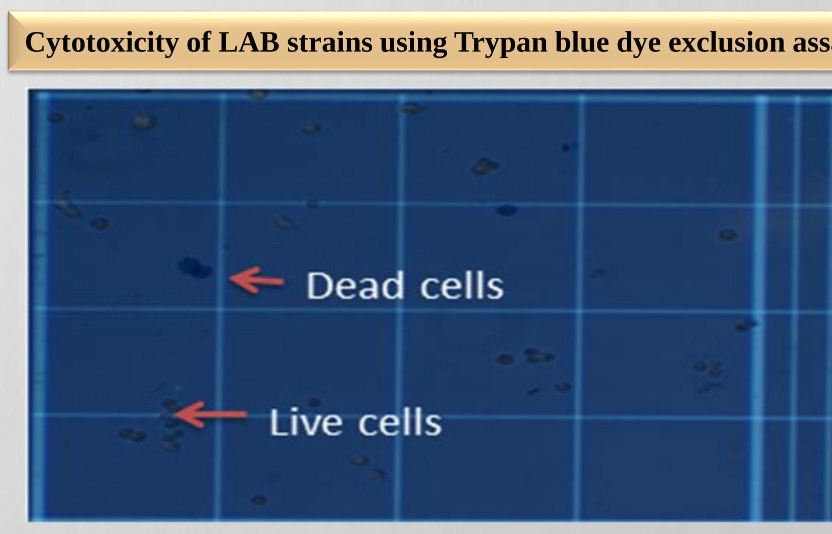
R' signifies resistance; 'S' signifies sensitive; and numerical values ± SD signifies intermediate resistance

Hydrogen peroxide (H_2O_2) production



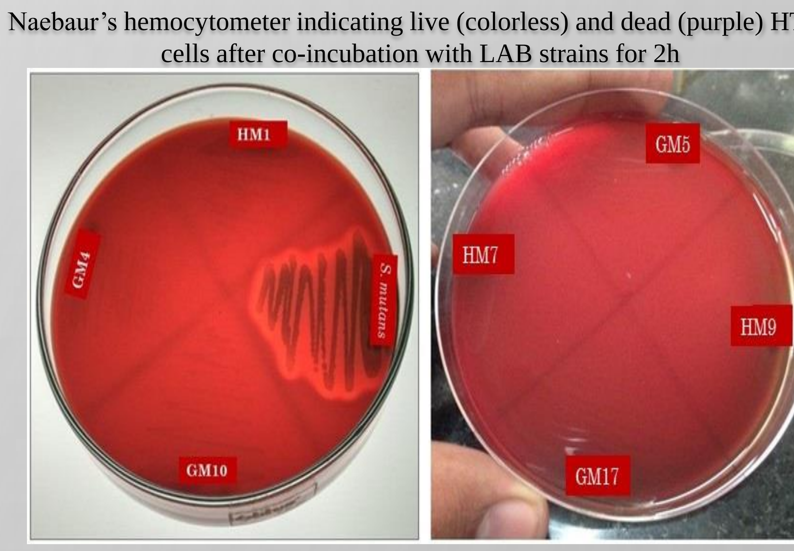
Brucella agar plates showing negative H_2O_2 production by lactobacilli and positive (blue colonies) for *S. mutans*

Cytotoxicity of LAB strains using Trypan blue dye exclusion assay



Naebaur's hemocytometer indicating live (colorless) and dead (purple) HT29 cells after co-incubation with LAB strains for 2h

Hydrogen peroxide (H_2O_2) production



Blood agar plates showing negative hemolysis for lactobacilli and positive for S. mutans

Conclusion

- Both human and goat milk served as a good source of LAB strains falling under different species.
- HM3, HM6, HM8, HM9 and HM10 strains displayed higher acid tolerance for all pH ranges i.e. 7, 4.5, 2.5, 2 and1.5 whereas, among goat milk isolates, GM6 showed highest acid tolerance at lowest studied pH (1.5) after 3h of incubation.
- All human as well goat milk lactobacilli were negative for hemolytic activity and hydrogen peroxide production.
- All the tested human milk lactobacilli displayed sensitivity to β-lactams (imipenem and meropenem), whereas, complete resistance has been observed towards methicillin, oxacillin (β-lactams); teicoplanin, vancomycin (glycopeptides) and ciprofloxacin, ofloxacin (quinolones).
- High percent viability (95% or more) of HT-29 cells was recorded with all the test strains after 2h of incubation, indicating zero toxicity and ensuring safety of LAB strains under in vivo conditions