ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Exploring the microbiota of human milk using the culture-dependent method

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Manuscript Info

Manuscript History:

Received: 12 March 2015
Final Accepted: 22 April 2015
Published Online: May 2015

Key words:

Human milk, Microorganism, Probiotic, Somatic Cell Count, 16S rRNA gene sequencing, Exploring microbiota in the human milk

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Abstract

Breast milk is vital sources of nutrients, also it consist of different antimicrobial compounds, immunoglobulin, immune component cells. Along with, it also contains a diverse microbial population which aid to initiation and development of infant gut microflora hence leads to stimulating growth and development of an infant. These diverse bacterial population also include the probiotic bacteria which stimulating a specific and nonspecific immunity and protect the neonate from intestinal disease, eczema, obesity and other infection due to the colonization of pathogenic bacteria. In the present study 31 milk samples were collected from healthy mother volunteers. Somatic Cell Counts of all samples perform and standardized in order to validate breast health. Based on phenotype and genotype characteristic, 27 bacteria were identified. The probiotic properties of all 27 isolates were checked out of which 12 isolates showed resistant to low pH and 11 isolates illustrated tolerance against 0.3% bile salt. In addition to this indicator organisms like *Escherichia coli*, *Bacillus cereus*, *Bacillus subtilus*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* were found to be most sensitive to 8 different isolates. The information generated from the present study reveals that breast milk is a resource of new life.

INTRODUCTION

Breast milk is the best food for infants as it provides the complete nutritional supplement for their growth. It protects the newborn against intestinal diseases like diarrheal (Salminen, *et al.*, 2004), reduce the risk of eczema in infants (Samuli. *et al.*, 2012), respiratory diseases (Lopez-Alarcon, M. *et al.*, 1997) and reduced enduring risk of obesity (Von, K. R. *et al.*, 1999; Gillman, M.W. *et al.*, 2001). The protective role of human milk seems to be the consequence of a synergistic action of the wide range of health-promoting components such as carbohydrates, nucleotides, fatty acids, immunoglobulins, cytokines, immune cells, lysozyme, lactoferrin, bacteriocine and, other immunomodulatory factors (Boehm, G. e*t al.*, 2008; Penttila, I.A., 2010; Van 't Land, B. *et al.*, 2010; Walker, A., 2010). Breast milk has been described as a source of bacteria influencing the development of the infant gut microbiota. Bacteria that are commonly found in human milk includes *Staphylococci, Streptococci, Lactobacilli, Lactococci*, *Enterococci* and *Bifidobacteria* (Heikkila, M. P. *et al.*, 2003; Martin, R. *et al.*, 2003 and Reviriego, C. *et al.*, 2005); These bacteria may play an important role in reduction of the incidences and severity of infection to the child due to their probiotic properties using specific mechanisms i.e. probiotics are able to secretes antimicrobial substance like bacteriocin which affected as antagonists against pathogenic bacteria and their effectual antagonistic activity alone or synergistically. These antimicrobial compounds can be protein molecules and bioactive peptides. Bacteriocins are significant antimicrobial peptide which has been demonstrated to have efficient therapeutic activity

against intestinal pathogenic infection (Thirabunyanon, M. *et al.*, 2009, Verdenelli M. C. *et al.*, 2009 and Gaudana, S. B. *et al.*, 2010), they also produce metabolites like acetic and lactic acids that decrease the pH in the intestine and making unfavourable environmental for pathogen to survive (Ridwan, B. U. *et al.*, 2008). Probiotics can eradicate pathogens using competitive exclusion and/or blocking the invasion of them at the infection site i.e intestinal epithelium cells thourough competing for the glycoconjugate receptors (Vanderpool, C. *et al.*, 2008). Also Competition for vital nutrient is observed between probiotics and pathogen which depends on the pace of nutrient absorptions, the innate metabolic capacity, the growth rate and the secretion of specific inhibitors (Gram, L. *et al.*, 1999). Although breast milk bacteria may be helpful for the infant's health, but some of the pathogenic bacteria are also present in the milk which may be harmful to the infants or mother.

The human milk microbiome is established by possible mechanisms. Physiological and hormonal changes occur during and after pregnancy leading to increasing gut permeability which help in the migration of gut microflora to the mammary gland. Living bacteria, which cell like Dendritic cells and macrophages also play a role in the transportation of microbes to the mammary gland (Fernandez, L.I. *et al.*, 2013). Beside all above probable mechanisms, the retrograde flux, the mother's skin microbes and infant's oral microbes may contribute to the development of the human milk microbiome (Albesharat, R. *et al.*, 2011; Jimenez, E. *et al.*, 2008c; Makino, H. *et al.*, 2011; Martin, R. *et al.*, 2003; Martín, R. *et al.*, 2006; Matsumiya, Y. *et al.*, 2002).

Somatic cells are mainly milk-secreting epithelial cells that have been shed -from the lining of the gland and white blood cells (leukocytes) that have entered the mammary gland in response to injury or infection (Dairyman's digest, 2009). Milk somatic cells include 75% leucocytes, i.e. neutrophils, macrophages, lymphocytes, erythourocytes, and 25% epithelial cells. Erythourocytes can be found at concentrations ranging from 0 to 1.51×10^6/ml (Paape and Weinland, 1988). Normally, somatic cell count from milk of a healthy mammary gland is lower than 1×10^5 cells/ml, upon bacterial infection can cause it to increase to above 1×106cells/ml (Bytyqi, H. *et al.*, 2010).

In 2003, first description about the bacterial diversity of human milk from healthy women was reported which was based on in vitro culturing methods (Heikella, M. P. *et al.*, 2003). During the last decades, microbiological studies that focused on human milk were restricted to the identification of potential pathogenic bacteria in stored milk or milk retrieved from maternal infected breast milk but microbes present in healthy mother breast milk are unexplored. Also, studies on human milk are carried out in India which was restricted to the isolation of beneficial bacteria from the breast milk or studying oligosaccharides present (Bhatt V. D. *et al.* 2012 and Anandharaj, M. *et al.*, 2013). In the present study, effort are been made to isolates the bacteria present in the healthy breast, characterizing and identified them.

MATERIALS AND METHODS

Sample collection & isolation of bacteria from breast milk

Human milk was collected from 31 healthy mother volunteers by concerning them from a different region of Anand, Valsad, and Navsari district of Gujarat, India. Before sample collection nipple and mammary areola were cleaned by swabbing with 70% alcohol. First few drop of milk was discarded then subsequent milk was collected in a sterile tube by manual expression and stored on ice until delivery to the laboratory. The somatic cell count was carried out using an electronic somatic cell counter (Fossmatic minor, Denmark) to differentiate healthy milk sample (Bytyqi, H. *et al.*, 2010). Bacterial species were isolated from the collected milk samples by serial dilution and agar plating method wherein the milk sample was diluted from 10^{-1} to 10^{-5} dilutions, and the diluted milk samples were spread on sterile nutrient agar plates. The inoculated plates were incubated at 37^{o}C for 24 hours. Mixed cultures obtained after incubation were purified by quadrant streaking on sterile NA plates and also colony forming unit (CFU) was calculated. The purity of cultures was cross-checked by gram staining procedure.

Characterization of isolates

Overnight incubated cultures of all the isolates were used to study morphological, physiological and biochemical characteristics of its.

For physiological characteristic, a growth of the isolates was evaluated in nutrient agar at different temperature like 5, 15, 37 and 45 ^{o}C. The growth pattern of all isolates was assessed at different pH i.e. 2.0, 4.0, 7.0, 9.0, 10.0 in nutrient broth by incubating at 37^{o}C. Salt tolerance test was performed by adding 6.0, 10.0, 15.0% (w/v) sodium chloride in nutrient broth. In order to identify the purified cultures tentatively on the basis of Bergey's manual (Aneja, K.R. *et al.*, 2003) various biochemical tests were performed.

Probiotic Properties of Isolates

For the determination of probiotic properties of isolates, the major selection criteria were: Resistance to low pH, tolerance against bile salt and the antimicrobial activity.

Resistance to low pH

Cells were harvested from overnight grown culture by centrifugation at 5000 rpm, at 4 °C for 10 min and pellets were washed with phosphate buffer saline (PBS, pH 7.2). Pellets were resuspended in PBS (pH 3) for 0 to 3 hour. Subsequently to observe the survival rate, every 1 hour interval pellets which suspended in PBS were transferred to the MRS broth and incubated at 37 °C for 24 hour. Optical density (O.D) was measured at 620nm for 0, 1, 2 and 3 hour. (Chou, L.S. *et al.,* 1999 and Çakır, I. *et al.,* 2003).

Tolerance to bile salt

Man Rrogosa Sharpe medium (MRS medium) containing 0.3% bile (Oxoid) was inoculated with active cultures and incubate at 37^{o}C followed by monitoring tolerate at 0, 1, 2, 3, 4 hour by taking the O.D. at 620 nm. (Bhatt, V. D. *et al.,* 2012).

Antimicrobial activity

Antibacterial activity was determined according to Gharieb *et al.* (Gharieb *et al.,* 2005) with slight modification by performing agar diffusion assay. Overnight grown bacterial cultures were centrifuged at 10,000 rpm for 10 min and supernatant was collected to check the antimicrobial activity of the isolates. The pH of the collected supernatant was adjusted to pH=7.0 by 1 M NaOH. A volume of 1 mL of inoculum of each indicator organisms like *Escherichia coli* (MTCC 10312), *Bacillus cereus* (MTCC 9762), *Bacillus subtilus* (MTCC 1789), *Pseudomonas aeruginosa* (MTCC 8076) and *Staphylococcus aureus* (MTCC 9542) were added to slightly warm nutrient agar, mix well then pour into Petri plates separately and allow to solidified. The wells of 7 mm in diameter deep were cut and 300 µl of culture supernatant were poured into each well. The plates were incubated at 37 °C for 24 hour and the clear zones formed around the wells were measured

Antibiotic sensitivity test

Disc diffusion method was used to evaluate the antibiotic sensitivity of the isolates. Nutrient agar was inoculated with 100 µl of overnight grow a bacterial culture and pour in Petri plates as to get lawn growth. Antibiotic disc [Tetracycline (10 µg), Ciprofloxacin (30 µg), Methicillin (10µg), Norfloxacin (10µg), Chloramphenicol (15µg), Erythouromycin(15 µg), Oxacillin (10µg), Cefoxitin (30µg), Meropenem (10µg), Ofloxacin (5µg), Rifamycin (5µg), Trimethoprim (5µg), Vancomycin (30µg), Ampicillin (10µg), Gentamycin (30µg), Kanamycin (1µg), Nalidixic Acid (10 µg), Streptomycin (10 µg), Neomycin (30µg)] (HiMedia Laboratories- Mumbai, India) were placed on the plates with the help of sterile forceps. All the plates were incubated at 37^{o}C for 24 hour. The sensitivity was measured as a diameter of the zone of inhibition surrounding the disc (Bhatt, V. D. *et al.,* 2012).

Molecular characterization

DNA extraction, 16S rRNA gene amplification, and sequencing

Using a proteinase-K-SDS method, genomic DNA isolation was performed according to described by Bhatt *et al.* (Bhatt, V. D. *et al*., 2012). At the end of the procedure, all the samples were checked for integrity using agarose gel electrophoresis and concentration were measured by nanodrop (V 3.6, Thermo Scientific).

Amplification of the 16S rRNA gene was done using the universal set of primer. The primer sequence for the same is forward primer 8F 5'AGAGTTTGATCCTGGCTCAG 3'and reverse primer 926R 5′-CCG TCA ATT CCT TTR AGT TT-3'. PCR reaction mixture was prepared in total of 25 µl which consisting of 1.0 µl of both forward and reverse primers (10 mM), 2.5 µl PCR Taq Buffer with $MgCl_2$, 2.5 µl dNTP mix (25mM), 0.5 µl Taq DNA polymerase (3 U/µl) and 1 µl Template DNA (50ng). Thermo- cycler settings included a 3 min denaturation step at 94 ^{o}C followed by 32 cycles of 94 ^{o}C for 40 sec, 56 ^{o}C for 45 sec, and 72 ^{o}C for 75 sec. A final extension step was done at 72 ^{o}C for 10 min (Bhatt, V. D. *et al*., 2012).

Sequencing of the amplified products was done by Sanger sequencing at Eurofins, (Bangalore). Further the sequence generated was BLAST against the NCBI nr database and searched for the utmost homology for the sequence identification. The phylogenetic tree was constructed using MEGA 6.0. All the sequences are submitted into Genbank NCBI.

Results and discussion

Isolation of bacteria from human milk

A total of 31 human milk samples were collected from the healthy volunteers' mother. The colony forming unit for all 28 samples was shown below to 1×10^{5} cells/ml which indicates volunteers' mother are healthy. From these, 27 bacteria were isolated based on the difference in the colonies characteristics on nutrient agar plates. All the 27 isolates were further characterized and identified.

Somatic Cell Count

To know that whether the milk samples collected were from healthy or infected breast, Somatic Cell Count was done using an electronic somatic cell counter (Fossmatic minor). It has been reported that infected breast milk gives more than 1000 cells/µl SCC (Arroyo, R. *et al.*, 2010). Out of 31, 29 samples gave SCC in a range 21-221 cells/µl which falls under the normal range whereas 2 samples were showed higher somatic cell count i.e. 2410-3261cells/µl which indicate the infection in the breast. So this 2 sample were not included in further studies. From the result, it can be concluded that 28 volunteer women are healthy.

Result of Gram's staining:

Gram staining is a very important preliminary step in the initial characterization and classification of bacteria. It is also a key procedure in the identification of bacteria based on staining characteristics, enabling the bacteria to be examined using a light microscope. Once stained, the morphology and arrangement of the bacteria may be observed. Gram staining differentiates bacteria by the chemical and physical properties of their cell walls by detecting peptidoglycan, which is present in a thick layer of gram-positive bacteria. Gram-positive bacteria result in a purple-blue colour while gram-negative bacteria in a pink-red colour. In our case out of 27 isolates, 13 gram-positive rods, 7 gram-positive cocci and 7 gram-negative rods occurred.

Physiological & biochemical characterization

Summary of the physiological and biochemical characterization was shown in table 1.

Table 1: physiological and biochemical characterization

Isolate No.	Growth at 10°C , 15°C,60°C	Growth at 37°C, 45°C	Growth at 4 , 6.5, 8 pH	Growt at 9 pH	2%NaCl	4% , 6.5% Nacl	M-R Test	V-P Test	Catalase	Citrate permease	tryptophase	Urease	Casease	amylase	Ammonia Production	Nitrate reductase	Gelatinase	Dehydrogeenaset	desulphurase	Formic hydrolyase	Triple Sugar ion agar test	Phenylalanine deminase	Hemlysins production
DY1	-	+	+	+	-	+	-	-	+		-	-	-	-	-	-	-	-	-	-	LF	-	+
DY2	-	+	+	-	-	+	-	-	+		-	-	+	-	-	-	-	+	-	-	LF	-	-
DY3	-	+	+	-	-	+	-	-	+		-	-	+	-	-	-	-	+	-	-	LF	-	-
DY4	-	+	+	-	-	+	-	-	+		-	-	-	-	+	-	+	-	-	-	LF	-	-
DY5	-	+	+	-	-	+	-	-	+		-	-	-	-	-	-	+	+	-	-	LNF	-	-
DY6	+	+	+	-	-	+	-	-	+		-	-	-	-	-	-	-	+	-	-	LF	-	-
DY7	+	+	+	-	-	+	-	-	+		-	-	+	-	-	-	+	+	-	-	LF	-	-
DY8	-	+	+	-	+	+	-	+	-		-	-	+	+	-	+	+	-	-	-	LF	-	-
DY9	-	+	+	-	-	+	+	-	+		-	+	-	-	+	+	-	+	+	-	LNF	-	-
DY10	-	+	+	+	-	+	-	-	-		-	-	+	-	+	+	-	+	-	-	LF	-	-
DY11	-	+	+	-	-	+	+	-	-		+	-	+	-	+	-	+	+	-	-	LF	-	-
DY12	-	+	+	-	-	+	-	+	+		-	-	+	-	+	+	+	+	-	-	LF	-	+
DY13	-	+	+	-	-	+	-	-	-		-	-	+	-	-	-	-	+	-	-	LF	-	-
DY14	-	+	+	-	-	+	+	-	+		-	-	+	-	+	-	-	+	+	-	LF	-	-
DY15	-	+	+	+	-	+	-	-	-		-	-	-	-	+	-	-	-	-	-	LF	-	+
DY16	-	+	+	-	-	+	+	-	-		-	-	-	-	+	-	+	+	-	-	LF	-	-
DY17	-	+	+	-	-	+	-	-	+		-	-	+	-	+	-	-	+	-	-	LNF	-	+
DY18	+	+	+	-	-	+	-	-	-		-	-	+	-	+	-	-	+	-	-	LNF	-	-
DY19	-	+	+	-	-	+	+	-	-		-	-	+	-	-	+	-	+	+	-	LF	-	-
DY20	-	+	+	-	-	+	+	-	+		-	-	-	-	-	+	+	+	-	-	LF	-	+
DY21	-	+	+	-	-	+	-	+	+		-	-	-	-	-	-	-	+	-	-	LF	-	+
DY22	-	+	+	-	-	+	-	+	+		-	-	+	-	-	+	+	-	-	-	LF	-	-
DY23	-	+	+	-	-	+	-	-	+		-	-	+	-	-	-	+	-	-	-	LF	-	-
DY24	-	+	+	-	-	+	-	+	+		-	-	+	-	-	+	-	-	-	-	LF	-	+
DY25	-	+	+	-	-	+	-	-	+		-	-	+	-	+	-	+	+	-	-	LNF	-	-

DY26	-	+	+	-	-	+	-	-	+		-	-	-	-	+	-	-	+	-	-	LNF	-	-
DY27	+	+	+	-	-	+	+	+	+		-	-	+	-	-	-	-	-	-	-	LF	-	-

Table 2: Antimicrobial activity of isolates

Isolate no.	Indicator microorganisms				
	Diameter of inhibition zone (mm)				
	E. coli	*B. cereus*	*B. subtilus*	*P. aeruginosa,*	*S. aureus.*
DY1	5	——	——	3	——
DY2	9	——	——	——	——
DY3	4	2	——	——	2
DY4	10	12	6	10	11
DY6	12	15	9	11	17
DY7	6	12	10	8	10
DY14	4	11	12	7	4
DY18	2	9	10	14	10
DY22	5	6	9	4	8
DY25	10	7	13	5	7
DY27	8	12	5	10	3

Table 3: Antibiotic sensitivity test

Isolate ID	Zone of inhibition (mm)																		
	Tetracycline (10 µg)	**Ciprofloxacin (30 µg)**	**Methicillin (10 µg)**	**Norfloxin (10µg)**	**Chloramphenicol (15µg)**	**Erythouromycin (15 µg)**	**Oxacillin (10 µg)**	**Cefoxintin (30 µg)**	**Meropenem (10 µg)**	**Ofloxacin (5µg)**	**Rifamipien (5µg)**	**Trimethoprim (5µg)**	**Vancomycin (30µg)**	**Ampicillin (10 µg)**	**Gentamycin (30 µg)**	**Kanamycin (01 µg)**	**Nalidixic Acid (10 µg)**	**Streptomycin (10 µg)**	**Neomycin (30µg)**
DY1	12	8	4	16	11	12	5	14	11	11	12	7	5	11	7	11	6	6	3
DY2	10	20	2	14	14	11	3	12	1	13	11	8	5	12	8	13	12	16	8
DY3	9	12	5	20	13	12	4	11	2	14	5	5	1	6	5	15	11	13	5
DY4	13	18	1	9	8	1	5	13	4	7	7	11	3	8	11	8	5	12	9
DY5	18	19	3	8	9	3	4	14	9	7	9	14	4	9	14	4	7	18	12
DY6	13	20	4	12	12	5	3	1	13	8	4	6	2	13	6	11	9	19	15
DY7	19	9	0	14	4	6	0	5	13	14	7	8	3	15	8	6	4	22	15
DY8	13	11	3	9	11	4	5	2	12	15	11	11	4	17	11	7	7	26	11
DY9	12	11	0	8	12	11	5	5	15	14	12	12	3	12	12	4	11	21	1
DY10	18	14	3	14	11	13	1	8	7	11	15	15	4	14	15	7	12	18	5
DY11	9	18	4	16	12	11	3	9	5	1	3	3	3	18	3	8	15	6	6
DY12	13	8	0	4	14	12	4	5	9	2	8	8	4	8	8	11	3	8	8
DY13	17	6	4	2	8	14	2	15	11	9	5	5	5	6	5	6	8	12	4
DY14	12	16	5	14	6	15	3	11	16	5	9	9	3	16	9	13	5	17	6
DY15	13	18	3	16	7	8	0	12	14	12	12	12	2	18	12	13	9	20	15
DY16	14	21	2	20	12	9	3	14	13	14	15	15	2	20	15	12	12	3	11
DY17	8	9	2	16	11	3	4	15	6	11	15	15	1	9	15	18	15	6	14
DY18	9	14	0	18	14	4	3	6	8	12	11	11	2	14	11	20	15	16	16
DY19	13	16	2	9	15	11	4	8	4	13	1	1	3	16	1	22	11	20	5
DY20	12	20	3	8	9	12	5	9	11	11	5	5	4	20	5	26	1	18	7

DY21	11	28	4	18	6	14	0	14	14	13	6	6	3	8	6	21	5	16	12
DY22	20	6	3	20	11	15	2	13	11	6	8	8	4	6	8	18	6	20	11
DY23	18	9	4	4	12	6	2	16	14	8	4	4	3	9	4	6	8	6	20
DY24	13	19	3	14	2	7	1	13	16	9	6	6	2	19	4	8	4	3	18
DY25	14	18	2	12	5	9	2	13	13	4	15	15	2	18	6	12	6	20	13
DY26	9	12	2	6	6	4	3	15	14	11	11	11	3	12	15	16	15	14	14
DY27	8	11	0	4	7	15	4	16	7	1	14	14	4	11	11	20	11	6	9

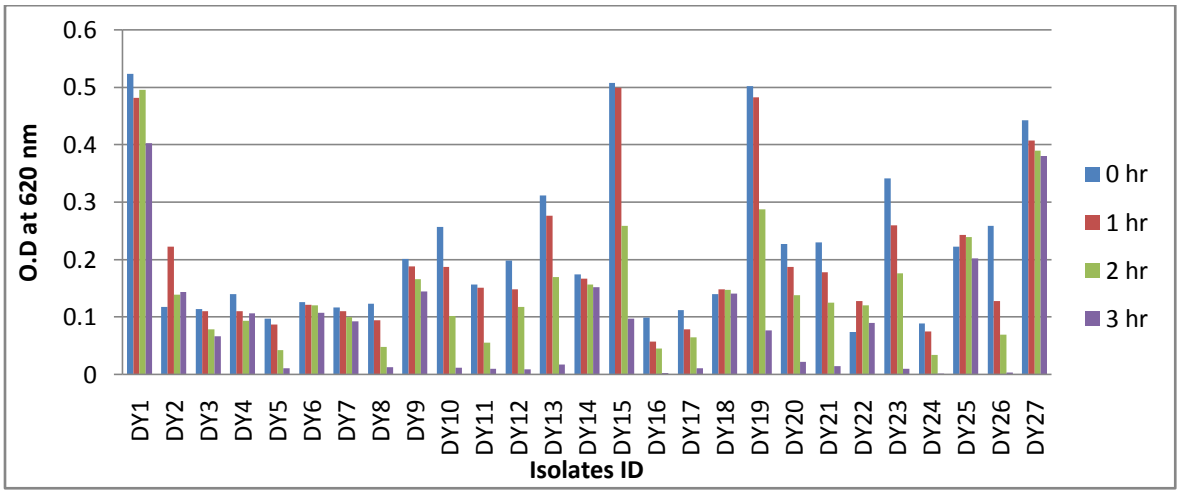

Fig 1: Resistance against the low pH

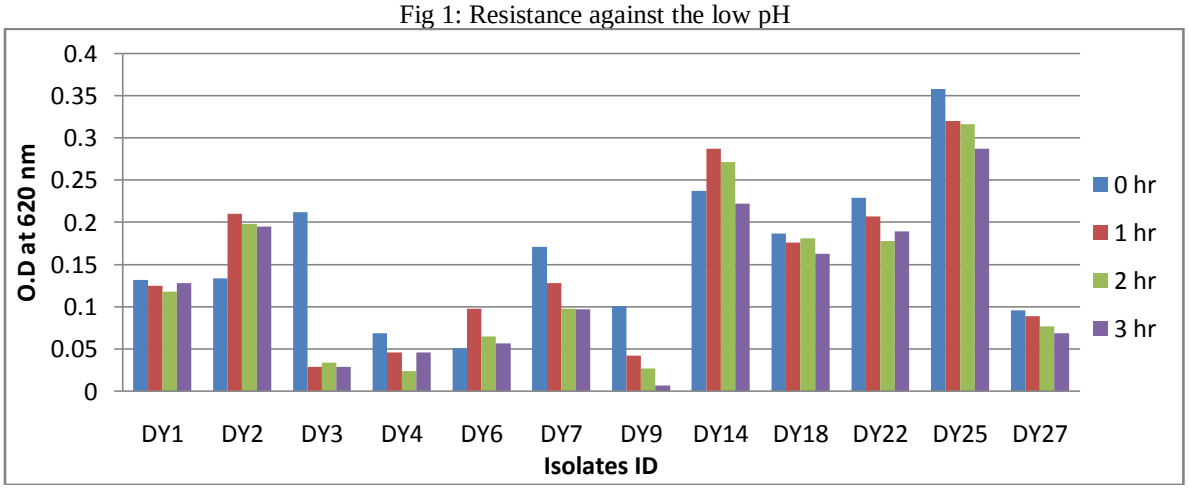

Fig 2: Tolerance against 0.3% bile salts

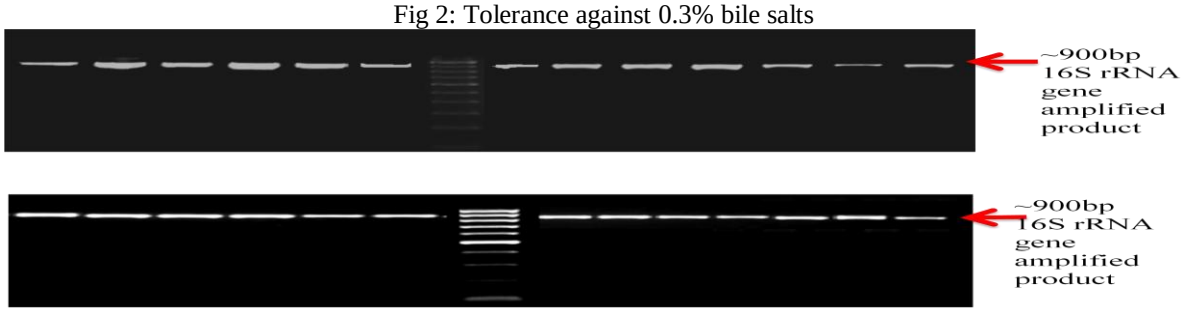

Fig 3: PCR amplified product in 1 % agarose gel

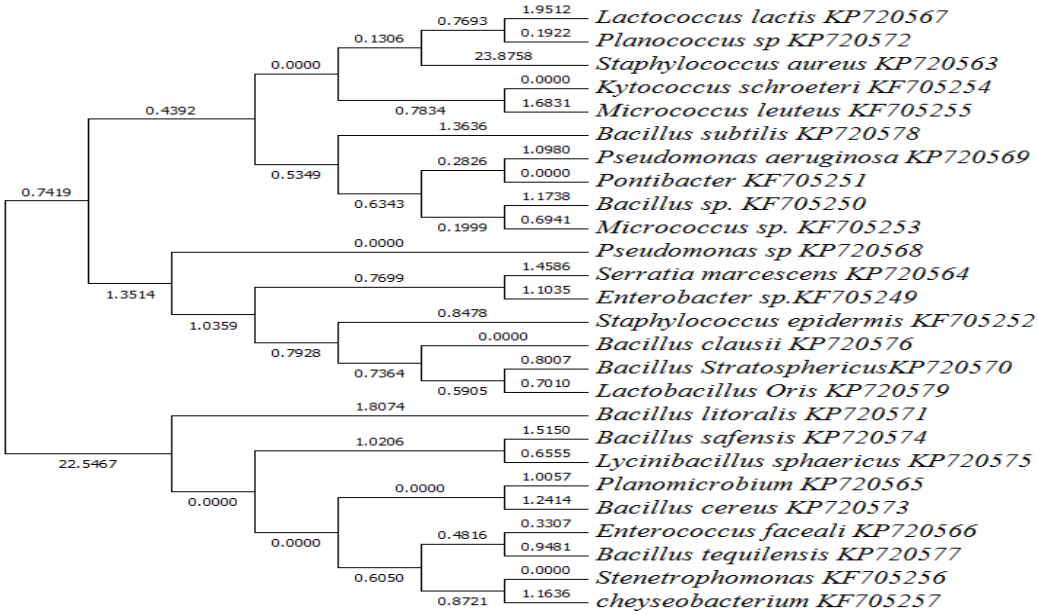

Fig 4: Phylogenetic tree of isolates prepared using mega 6 software and neighbor-joining algorithm

Probiotic property

Resistance to low PH

Resistant to low pH is one of the major selection criteria for probiotic strains (Ouwehand, A. C. *et al.*, 1999, Çakır, I. 2003). Out of 27 isolates, 12 isolates were resistant to low pH (Fig1). As to reach the small intestine bacteria have to pass thourough the stressful conditions of stomach where the pH is ≥ 3 (Chou, L. S. *et al.*, 1999 and Çakır, I., 2003). It is expected that as time taken for digestion as 3 hour in stomach, incubating the culture in PBS for 3 hour will give us the idea whether they can survive at low pH or not (Prasad, J. *et al.*, 1998). In 1999, Jacobsen and co-workers get similar type of results where 6 of the isolates which were probiotics in nature shows resistance to low pH (Jacobsen, C. N. *et al.*, 1999).

Tolerance against bile salt

Bile acids are synthesized in the liver from cholesterol and sent to the gall –bladder and secreted into the duodenum in the conjugated form (500-700 ml/day). In the large intestine, these acids suffer some chemical modifications (deconjugation, dehydroxylation, dehydrogenation and deglucuronidation) due to the microbial activity. Conjugated and unconjugated bile acids show antimicrobial activity especially on E. coli subspecies, Klebsiella spp., and Enterococcus spp. in vitro. The deconjugated acid forms are more effective on gram-positive bacteria. The mean intestinal bile concentration is supposed to be 0.3% w/v and the staying time is suggested to be 4 hours so probiotic should be tolerate bile salts approx 4 hour (Dunne, *et al.* 1999, Çakır 2003). In our study, Out of 12, 10 isolates were able to tolerate bile salts which shown in Fig 2. In New Zealand's Dairy Research Institute, (NZDRI) was screened 200 strains of Lactobacillus and Bifidobacterium. Out of which they have found thouree isolates from dairy origins and the one was from human origin showed tolerance against bile (Prasad, J. *et al.*, 1998).

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Antimicrobial Activity

The isolates that are able to tolerate low pH and bile salt (DY1, DY2, DY3, DY4, DY6, DY7, DY14, DY18, DY22, DY25, and DY27) were selected for antimicrobial activity. For this purpose, isolates extracellular bacteriocin were tested against the indicator microorganisms *Escherichia coli, Bacillus cereus, Bacillus subtilus, Pseudomonas aeruginosa, and Staphylococcus aureus*. Zone of inhibition obtain is as shown in table 2, except three isolates all other isolates showed antibacterial activity against test organisms. The bacteria present in healthy milk samples are interfered in the growth of many human pathogenic bacteria like *staphylococcus aureus*. (Heikella, M. P. *et al*, 2003). The antimicrobial activities of many (lactic acid bacteria) is mainly due to the production of hydrogen peroxide, which exerts bacteriocidal effects on most pathogens and due to production of organic acids (lactic or acetic acids) (Lindgern, S. E. *et al.*, 1990,). Beside this other antimicrobial components i.e. diacetyl, reuterin, pyroglutamic acid, and especially bacteriocin, are ribosomally synthesized, and extracellularly released proteinaceous antimicrobial compounds which shows antibacterial activities against bacteria (Nes, I. F. *et al.*, 2007

and ouwehand, A. C. *et al.*, 1999). The bacteria present in healthy milk samples may inhibited the growth many pathogenic bacteria like S. *aureus, L.monocytogenes, S. typhimurium, Shigella flexneri, E. coli, Klebsiella pneumoniae, B. cereus, Pseudomonas aeruginosa, and Enterobacter spp*. *enterotoxigenic E. coli, enteropathogenic E. coli, E. faecalis, and Cl. difficile* (Forestier, C. *et al.*, 2001 and Coconnier, M. H. *et al.* 1997).

Antibiotic sensitivity test

A study was carried out to determine the antibacterial susceptibility patterns of isolated bacteria. All isolates were highly sensitive to Ciprofloxacin. Gram negative bacteria are more sensitive to ciprofloxin than the gram positive, it's disrupts the synthesis of peptidoglycan layer of bacterial cell wall (Table 3).

16S rRNA gene sequencing and identification:

After DNA isolation the 16S rRNA gene was amplified by PCR (Fig 3). Afterward, amplified products were sending for sequencing. The sequence obtained was further identified using BLASTn search against the NCBI nr database. Isolates showing the highest homology to the reference sequence and identified. Out of this 27 ,12 isolates were shown probiotic characteristics which shown homology with as *Enterococcus faceali* (DY22), *Bacillus Stratosphericus* (DY25), *Bacillus safensis (DY4), Lactococcus lactis* (DY27), *Lycinibacillus sphaericus* (DY6), *Bacillus clausii* (DY7), *Bacillus tequilensis* (DY14), *Lactobacillus Oris* (DY18), *Bacillus Sp.* (DY23).Phylogenetic tree was prepared using MEGA 6 with Neighbor-joining algorithm. Phylogenetic was tree showing the relationship among obtained sequences.16s rRNA gene shows the relation between all species (Fig 4).

Discussion

The results of recent study afford new scheme for the composition of microbiota in breast milk which are potentially vital for maternal and infant health (Heikella, M. P. *et al*, 2003, Gueimonde, M. *et al.*, 2007, and Perez, P.F. *et al.*, 2006). Our result obtained by culture dependent approach shows much similarity with preceding reported work (martin, R. *et al.*, 2003, Heikella, M. P. *et al*, 2003). The species diversity and the consequence of the common bacterial flora have arriving tiny awareness so far. We explored bacteria diversity in the milk of healthy lactating women. Most of the detected *Staphylococci Lactobacilli, Enteroccoci, Bacilli and Pseudomonas* are known as residents of the human normal bacterial flora, but also some are pathogenic species like *S. aureus* that were also found. In present work *S. aureus*, found in five milk samples, has been reported as a rare contaminant in breast milk of healthy lactating women also in previous studies (Caroll *et al.*, 1979; West *et al.*, 1979; Law *et al.*, 1989; El-Mohandes *et al.*, 1993a). Lactobacilli were the predominant LAB species, but *Lactococcus lactis* was also found. The numerical data was stated that an infant consuming about 800 ml breast milk per day will inserting about $8 \times 10^4 – 8 \times 10^6$ commensal bacteria while suckling. *Staphylococci*, oral *streptococci*, especially S. *epidermidis and S. salivarius*, which were also reported as the predominant bacterial species in breast milk, and have also been identified from stool samples of breast-fed infants. (Millar, M.R. *et al.*, 1996, Kirjavainen, P.V. et *al.*, 2001and Favier, C.F. *et al.*, 2002).

Acknowledgement

We are greatly thankful to ARIBAS and Charutar vidya mandal (CVM) for providing platform to do Research work. Our team also thankful to Dr. D.S. Nauriyal, Professor and Head, Department of Veterinary Medicine for supporting to used electronic somatic cell counter.

References

1. Albesharat, R., Ehourmann, M.A., Korakli, M., Yazaji, S. and Vogel, R.F., (2011) Phenotypic and genotypic analyses of lactic acid bacteria in local fermented food, breast milk and faeces of mothers and their babies. Syst. Appl. Microbiol; 34, 148-155
2. Anandharaj M and Sivasankari B (2013). Isolation of potential probiotic Lactobacillus strains from human milk. Int. J. Res. Dev. Pharm. L. Sci; 1(1),26-29.
3. Aneja K R, (2003) Experiments in microbiology, plant pathology and biotechnology, New Age International (p). *Ltd*., Fourth edition. ISBN no. 978-81-224-1494-3
4. Arroyo, R., Martín, V., Maldonado, A., Jiménez, E., Fernández, L., & Rodríguez, J. M. (2010). Treatment of infectious mastitis during lactation: antibiotics versus oral administration of Lactobacilli isolated from breast milk. Clin Infect Dis; 50(12), 1551–1558.
5. Bhatt, V. D.,Vaidya, Y. H., Kunjadia, P. D. and Kunjadia, A. P. (2012). Isolation and charecterization of probiotic bacteria from human milk. Int. J. Pharma Sci Health Care, 2(3): 62-70.
6. Boehm, G. and Moro, G., (2008). Structural and functional aspects of prebiotics used in infant nutrition. J. Nutr. 138: 1818S-1828S.
7. Bytyqi H, Zaugg U, Sherifi K, Hamidi A, Gjonbalaj M, Muji S, Mehmeti H. (2010), Influence of management and physiological factors on somatic cell count in raw milk in Kosova. Veterinarski Archiv; 80:173-183.

8. Çakır, İ. (2003). Determination of some probiotic properties on Lactobacilli and Bifidobacteria. Ankara University Thesis *of Ph.D*.
9. Castagliuoluolo, I., Rıegler, M.F., Valenick, L., Lamont, J.T., and Pothoulakis, C. (1999). *Saccharomyces boulardii* protease inhibits the effects of *Clostridium difficile* toxins a and b in human colonic mucosa. American Society for Microbiology 67(1):302-307
10. Chou, L.S and Weimer, B. (1999). Isolation and characterization of acid and bile tolerant isolates from strains of *Lactobacillus acidophilus*. J. Dairy Sci. 82:23- 31.
11. Coconnier, M. H., Liévin, V., Bernet-Camard, M. F., Hudault, S., Servin, A. L., (1997) An human *Lactobacillus acidophilus strain* LB. Antimicrob. Agents Chemother. 41: 1046-1052.
12. Dairyman's Digest (2009). What you should know about somatic cells. Winter issue.
13. Delgado S, Arroyo R, Martin R and Rodriguez JM, (2008). PCR-DGGE assessment of the bacterial diversity of breast milk in women with lactational infectious mastitis. BMC Infect Dis., 8:51.
14. Favier, C.F., Vaughan, E.E., de Vos, W.M. and Akkermans, A.D.L. (2002) Molecular monitoring of succession of bacterial communities in human neonates. App. Env. Micro. 68, 219–226.
15. Fernández L1, Langa S, Martín V, Maldonado A, Jiménez E, Martín R, Rodríguez JM.(2013) The human milk microbiota: origin and potential roles in health and disease. Pharmacol Research 69(1):110.
16. Fooks, L.J., Fuller, R., Gibson, G.R. (1999). Prebiotics, probiotics and human gut microbiology. Int Dairy J 9:53-61.
17. Forestier, C., C. de Champs, C. Vatoux, and B. Joly. (2001). Probiotic activities of Lactobacillus casei rhamnosus: In vitro adherence to intestinal cells and antimicrobial properties. *Research in microbiology*. 152: 167-173.
18. Gaudana, S. B., Dhanani, A. S. and Bagchi, T., (2010); "Probiotic attributes of *Lactobacillus* strains isolated from food and of human origin", Br J Nutr., 103, 1620-1628.
19. Gueimonde M, Laitinen K, Salminen S and Isolauri E, (2007). Breast milk: a source of bifidobacteria for infant gut development and maturation. Neonatology, 92:64–66.
20. Heikella M.P., Saris PEJ, (2003) Inhibition of *Staphylococcus aureus* by the commensal bacteria of human milk. J Appl Microbiol 95: 471–478.
21. Jacobsen, C.N., Nielsen, V.R., Hayford, A.E., Moller, P.L., Michaelsen, K.F., Paerregaard, A., Sandström, B., Tvede, M., and Jacobsen, M. (1999). Screening of probiotic activities of forty seven strains of *Lactobacillus* spp. by in vitro techniques and evaluation of the colonization ability of five selected strains in humans. Appl Environ Microbiol 65(11):4949 4956.
22. Jiménez, E., Fernández, L., Maldonado, A., Martín, R., Olivares, M., Xaus, J. and Rodríguez, J.M., (2008). Oral administration of Lactobacillus strains isolated from breast milk as an alternative for the treatment of infectious mastitis during lactation. Appl Environ Microbiol74: 4650-4655.
23. Kirjavainen, P.V., Apostolou, E., Arvola, T., Salminen, S.J., Gibson, G.R. and Isolauri, E. (2001) Characterizing the composition of intestinal microflora as a prospective treatment target in infant allergic disease. FEMS Immunology and Medical Microbiology 32, 1–7.
24. Li J., Aroutcheva, A. A., Faro, S., and Chikindas, M. L.. (2005). Mode of action of lactocin 160, a bacteriocin from vaginal *Lactobacillus rhamnosus*. Infect Dis Obstet Gynecol. 13: 135-140
25. Lindgren, S. E. and W. J. Dobrogosz. (1990). Antagonistic activities of lactic acid bacteria in food and feed fermentation. *FEMS Microbiol. Review*. 7: 149-163.
26. Mahony, D. E. (1982). A simple device for growing Clostridium perfringens and its application in bacteriocin studies. Can. J. Microbiol. 28: 709-713.
27. Makino, H., Kushiro, A., Ishikawa, E., Muylaert, D., Kubota, H., Sakai, T., Oishi, K., Martín, R., Ben Amor, K., Oozeer, R., Knol, J. and Tanaka, R., (2011). Transmission of intestinal Bifidobacterium longum subsp. longum strains from mother to infant, determined by multilocus sequencing typing and amplified fragment length polymorphism. Appl Environ Microbiol 77: 6788-6793.
28. Martin R, Heilig HGHJ, Zoetendal EG, Jimenez E, Fernandez L, Smidt H and Rodriguez, J.M. (2007a) Cultivation independent assessment of the bacterial diversityof breast milk among healthy women. Res. Microbiol, 158:31–37.
29. Martin R, Langa S, Reviriego C, Jimenez E, Marin ML, Xaus J, Fernandez L and Rodriguez JM, (2003). Human milk is a source of lactic acid bacteria for the infant gut. J. Pediatr., 143:754–758.
30. Martín, R., Jimenez, E., Olivares, M., Marin, M.L., Fernandez, L., Xaus, J. and Rodriguez, J.M., (2006). Lactobacillus salivarius CECT 5713, a potential probiotic strain isolated from infant feces and breast milk of a mother-child pair. Intl J Food Microbiol. 112: 35-43.

31. Martin, V., Maldonado-Barragan, A., Moles, L., Rodriguez-Banos, M., Campo, R.D., Fernandez, L., Rodriguez, J.M. and Jimenez, E., (2012). Sharing of bacterial strains between breast milk and infant feces. J Hum Lact28: 36-44.
32. Matsumiya, Y., Kato, N., Watanabe, K. and Kato, H., (2002). Molecular epidemiological study of vertical transmission of vaginal Lactobacillus species from mothers to newborn infants in Japanese, by arbitrarily primed polymerase chain reaction. J Infect Chemother8: 43-49.
33. Millar, M.R., Linton, C.J., Cade, A., Glancy, D., Hall, M. and Jalal, H. (1996) Application of 16S rRNA gene PCR to study bowel flora of preterm infants with and without necrotizing enterocolitis. J. Clin. Micro. 34, 2506–2510.
34. Montville, T. J. and Y. Chen. (1998). Mechanistic action of pediocin and nisin: Recent progress and unresolved questions. Appl. Microbiol. Biotechnol. 50: 511-519.
35. Nes, I. F., S. S. Yoon, and D. B. Diep. 2007. Ribosomally synthesiszed antimicrobial peptides (bacteriocins) in lactic acid bacteria: A review. *Food Sci Biotechnol*. 16: 675-690.
36. Ouwehand, A. C., P. V. Kirjavainen, C. Shortt, and S. Salminen. (1999). Probiotics: Mechanisms and established effects. Int Dairy J. 6: 43-52
37. Penttila, I.A., 2010. Milk-derived transforming growth factor-β and the infant immune response. J. Pediatr 156: S21-S25.
38. Perez PF, Dore´ J, Leclerc M, Levenez F, Benyacoub J, *et al.,* (2006) Bacterial imprinting of the neanatal immune system: lessons from maternal cells? J. Pediatr 119: e724–e732.
39. Prasad, J., Gill, H., Smart, J., and Gopal, P.K. (1998). Selection and Characterization of *Lactobacillus* and *Bifidobacterium* strains for use as probiotic. Int Dairy J. 8:993-1002.
40. Rescigno M, Urbano M, Valzasina B, Francolín M, Rotta G, Bonasio R,(2001) Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat Immunol. 2:361–7.
41. Reviriego C, Eaton T, Martin R, Jimenez E, Fernandez L, Gasson MJ and Rodriguez JM, (2005). Screening of virulence determinants in *Enterococcus faecium* strains isolated from breast milk. J Hum Lact, 21:131–137.
42. Ridwan,B. U. Koning C. J., Besselink M. G., Timmerman H. M., Brouwer E. C., Verhoef, J., Gooszen, H. G. and Akkermans, L. M., (2008); "Antimicrobial activity of a multispecies probiotic (Ecologic 641) against pathogens isolated from infected pancreatic necrosis", lett. Appl. Microbial., 46, 61-67.
43. Salminen MK, Rautelin H, Tynkkynen S, Poussa T, Saxelin M, Valtonen V, (2004);Lactobacillus bacteremia, clinical significance, and patient outcome, with special focus on probiotic L. rhamnosus GG. Clin. Infect. Dis. 38(1):62–9.
44. Salminen, S. (1999). Probiotics: scientific support for use. Food Technology 53(11):66.
45. Samuli Rautava, Essi Kainonen, Seppo Salminen, Erika Isolauri (2012); Maternal probiotic supplementation during pregnancy and breast-feeding reduces the risk of eczema in the infant. J Allergy Clin Immunol., 130 (6), 1355–1360.
46. Thirabunyanon, M., Boonprasom ,P. and Niamsup, P., (2009) "Probiotic potential of lactic acid bacteria isolated from fermented dairy milks on antiproliferation of colon cancer cells", Biotechnol Lett., , 31, 571-576
47. Van't Land, B., Boehm, G. and Garssen, J., (2010). Breast milk: components with immune modulating potential and their possible role in immune mediated disease resistance. In: Watson, R.R., Zibadi, S. and Preedy, V.R. (eds.) Dietary components and immune function. Springer, Berlin, Germany, pp. 25-41.
48. Verdenelli, M. C., Ghelfi, F., Silvi, S., Orpianesi, C., Cecchini, C. and Cresci ,A, (2009); "Probiotic properties of *Lactobacillus rhamnosus* and *Lactobacillus paracasei* isolated from human faeces", Eur J Nutr., 48, 355-363.
49. Walker, A.,(2010). Breast milk as the gold standard for protective nutrients. J. Pediatr.156: 53-57.