International Journal of Health Sciences and Research
Vol.12; Issue: 3; March 2022
Website: www.ijhsr.org
Original Research Article *ISSN: 2249-9571*

Isolation, Characterization, and Diversity of Probiotic Microorganisms from Different Postpartum Milk of Various Animals

Suyash Arunrao Kathade[1], Mayur Arjun Aswani[2], Pashmin Kaur Anand[1], Arti Kale[1], Parkhi Shrivastava[1], Siddharth Sharma[1], Ummesaida Badat[1], Jyoti Mohite[1], Jyotish Debbarma[1], Adolia Sangma[1], Bhagyashree Wajravad[1], Suresh Jagtap[2], Niricharan Kunchiraman Bipinraj[1]

[1]Bharati Vidyapeeth (Deemed to be University) Rajiv Gandhi Institute of I.T. and Biotechnology, Pune, Maharashtra.
[2]Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth Deemed to be University, Pune, India.

Corresponding Author: Niricharan Kunchiraman Bipinraj

DOI: https://doi.org/10.52403/ijhsr.20220332

ABSTRACT

Most importantly milk contains many beneficial probiotic microorganisms, hence, it has been sourced for the isolation of many probiotic bacteria. Due to the huge microbial diversity and functionality, there is scope to isolate microorganisms, evaluate them for probiotic potential. It is also important to compare the diversity of probiotic microorganisms in the postpartum periods of milk. In the present study, cultures were isolated from three different postpartum periods milk of Goat, Sheep, Cow, and Buffalo. The 182 cultures were isolated, only 127 were selected by colony characterization and 69 were selected after biochemical analysis such as Gram nature, endospore, catalase, acid-fast, and mannitol fermentation, 54 were non-hemolytic, 47 could tolerate a wide range of pH, bile, temperature, and NaCl. 30 isolates could survive in the presence of a gastric and intestinal environment. These cultures showed inhibitory activity against enteropathogens and did satisfy the need for auto and co-aggregation property. 17 cultures showed BSH and hydrophobicity activity, only 6 cultures showed susceptibility against all types of antibiotics. As per the results from the total potential probiotic cultures from various animal milk samples, 80% from first and second postpartum and 20% cultures from third postpartum milk were isolated, as per the results as milk ages, the probiotic concentration in the milk decreases.

Key Words: Postpartum, Milk, Probiotics, Yeast, *Lactobacillus.*

INTRODUCTION

Milk is the primary source of nutrition for young mammals. It is a rich source of many important biologically active compounds that support the optimal, growth and development of young ones. Owing to its nutritive nature milk from a cow, buffalo, goat, and sheep are consumed worldwide as a major food.

Milk produced immediately after parturition is known as colostrum, which is rich in proteins, fat, growth factors, immunoglobulins oligosaccharides, and another nutritive compound (1,2).

However, as the parturition ages, the concentration of nutrients shows a negative trend (2). Nutritionally, colostrum is rich than the milk produced in later periods of parturition.

Row milk also contains a large number of native microorganisms and many of which have been reported as probiotics.

Probiotics are live microorganisms that confer health benefits to the host when administrated in an adequate amount (3)**.** Probiotics have been known to increase the bioavailability of nutrients and play a key role in the absorption of calcium and magnesium from milk (4). They are also known to reduce intestinal inflammation, antibiotic-induced diarrhoea (5) as well as help to cure and prevent hypercholesterolemia thereby deconjugating bile salts (BSH) to liberate free amino acids such as glycine (6). Ruminant milk is a much-preferred source for isolating probiotics and widely used probiotics such as *Lactobacillus paracasei, Lactobacillus rhamnosus, Lactobacillus acidophilus, Lactobacillus reuteurii, Bifidobacterium bifidum and Saccharomyces boulardii* (7) are isolated from ruminant milk. LAB (Lactic acid bacteria) is a dominant culture present in cow and buffalo milk, which is about 30% and 4.62-5.7x10^4 CFU/ml. Sheep milk contains LAB as a dominant culture, which can be 10^2–10^6 CFU/mL (8). Row buffalo milk contains five dominant *Lactobacillus* cultures of *Lactobacillus acidophilus* (25%), *L. bulgaricus* (21%), *Lactococcus lactis* (40%), and *Streptococcus thermophilus*. Sheep milk constitutes 1.30% of global milk production. Sheep milk contains LAB as a dominant culture, which can be 10^2–10^6 CFU/mL (8).

As the nutritive value of the milk decreases, the microbial concentration shows a negative trend (9). This study tries to understand the change in probiotic concentration along the parturition period, with respect to *Lactobacillus* and probiotic yeasts

MATERIALS AND METHOD

Sample Collection

Colostrum milk of three different postpartum of goat (Osmanadi), sheep (Sangamneri), cow (Khillari), buffalo (Pandharpuri) were collected from Saswad, Pune, Maharashtra, India. in sterile Schott bottles under aseptic condition and transferred to the laboratory. Milk was collected in three different postpartum periods for goat and sheep 1st day, 15th and 30th day and cow and buffalo 1st, 30th and 180th day after birth as the early postpartum period, acute postpartum period, subacute postpartum period, pH and density (g/mL) were calculated for each sample.

Culture Isolation

The milk samples were serially diluted with sterile distilled water and 100µL suspension was spread on MRS (De Man, Rogosa and Sharpe) agar (pH 6.5) and plates, incubated anaerobically for 24h. plated. Serial dilution (10^{-3} - 10^{-6}) at 37°C. Morphological distinct colonies were purified and cultures were inoculated in MRS broth and incubated for 24h., centrifuged and suspended in saline to get 10^7cfu/ml. This suspension (1%) was used as inoculum for all further experiments (10).

Cultural and Colony Characteristics

Cultural and colony characterization of all isolates were performed based on Bergey's manual of systemic microbiology, gram staining was performed as per the method of (Fotou K. at al. 2011) to observe gram character of culture, endospore staining was performed as described by (Reynolds J. at al., 2009) method for observation of spores in cultures, acid-fast staining was performed as per the method of (Reynolds J. at al., 2009) method was used for performing catalase test and to detect the presence or absence of enzyme catalase. For mannitol fermentation bromothymol blue broth base medium containing different carbohydrates (1gm, 1 % w/v) mannitol was used for carbohydrate fermentation assay respectively. After the inoculation, media were incubated at 37°C for 24 h. Anaerobically by keeping it in glass desiccator. The positive reaction was indicated by colour change for carbohydrate fermentation (11).

Toxicity Assay

For toxicity assay isolates was spot inoculated on sheep blood agar plate and

incubated at 37℃ for 24 h. Toxicity was determined by the pattern of haemolysis on a blood agar plate (12).

pH, bile, Temperature Tolerance

For pH tolerance study, media pH was adjusted to 1.5 to 10 using 1N HCl or 1N NaOH. Inoculated media were then incubated at 37℃ for 24 h. and observed for growth. MRS broth containing different concentrations of bile salts 0.3, 0.6, 0.9 and 1.2% were inoculated with culture suspension and growth was compared with control after incubation at 37℃ for 24 h. (13). The culture was incubated in MRS broth at three different temperatures at 28℃, 37℃ and 42℃. Growth was observed after 24 h. of incubation**.**

Auto-Aggregation and Co-Aggregation Assay

The cultures grown in MRS broth for 24h. at 37ºC were collected by centrifugation at 5000 rpm for 5 min. The cell pellets were then suspended in 2 ml of PBS in an Eppendorf tube and 1ml of cell suspension was vortexed with 4ml of sterilized PBS solution in a test tube for 10 seconds. This mixture was then incubated at 37ºC and optical density of the sample were measured at 0, 2, 4 and 24 h. at 600 nm.

For co-aggregation studies, culture was suspended in 0.2 ml of PBS and mixed with an equal amount of pathogen (0.5 OD) and the final volume was adjusted to 5ml. It was then incubated for 24 h. at 37ºC. The OD (600 nm) of the suspension at 2, 4 and 24 h. were then measured and compared with pathogen suspension PBS (14). After 24 h. of incubation, yeast and bacterial cultures were taken and stained by methylene blue and observed under the microscope (15).

Simulated Gastric and Intestinal Juice Tolerance Assay

To determine in-vitro survival of the isolate, culture suspension in PBS (0.2 ml) was added in a mixture of 1 ml gastric juice pepsin (pH 2) or pancreatin (pH 8) and 0.3 ml of sodium chloride (0.5% w/v) (15). Viable count of the culture was checked at 1, 90 and 180 min for gastric and 1, and 240 min for intestinal juice and by spreading it on MRS agar plates. The gastric and intestinal transit tolerance was evaluated by determining the viable count of cells after the incubation period (16,17).

Antimicrobial Assay

Antimicrobial assays were carried against enteropathogens such as; *Escherichia coli* NCIM 3099, *Staphylococcus aureus* NCIM 2408, *Enterococcus faecalis* NCIM 3040 and *Candida albicans* NCIM 3557. The pathogens were spread plated on Muller Hinton agar and plates were incubated at 37ºC for 10 min. Wells were punctured in agar using a punch borer. The isolate was grown in YPD broth at 37ºC for 24 h. The supernatant was collected after centrifugation at 10000 rpm for 20 min at room temperature and added into the agar well. Inhibition zones were measured after 24 h. of incubation at 37ºC (15).

Hydrophobicity Assay

Hydrophobicity assay indicates the ability of probiotics to adhere to human epithelial cells. For hydrophobicity, the cultures (1 OD at A600) were suspended in phosphate buffer (pH 6.5) and treated with xylene in a 5:1 ratio. The suspension was vortexed for 2 min and incubated at 37ºC for phase separation. The decrease in absorbances of the aqueous phase was measured as per cent hydrophobicity (H %) and calculated as H %=[(A0-A)/A0]*100, where A0 and A are the absorbances of the culture in the aqueous phase before and after extraction respectively (18).

Bile Salt Hydrolase (BSH) Assay

BSH assay was performed as per the method described by (Zheng Y. et al., 2013). Isolates were spot inoculated on MRS agar plates supplemented with 0.5% (w/v) sodium salt of taurodeoxycholic acid (Himedia, India) and $CaCl_2$ 0.37 % (w/v).

Plates were incubated anaerobically at 37ºC for 72 h. and BSH activity was determined by the presence of precipitation around colonies (19).

NaCl Tolerance

Tolerance to NaCl was determined by growing the cultures in MRS medium containing different concertation of NaCl, 1-10% after incubation, spectroscopic reading was taken and percentage survival was calculated (20).

Antibiotic Susceptibility Test

Antibiotic susceptibility test was performed according to the Kirby-Bauer antibiotic testing method as described by (Bauer AW., 1959). Accordingly, cultures were plated on MRS agar plates and exposed to antibiotic discs (Antibiotic disc, Hexa G-6, Himedia) containing ampicillin (10 mcg), chloramphenicol (25 mcg), penicillin-G (1 unit), streptomycin (10 mcg), sulphatried (300 mcg) and tetracycline (25 mcg). The plates were incubated at 37ºC for 24 h. before measuring the zone of inhibition around each antibiotic.

RESULT

Sample collection

Milk samples were collected from Saswad, Pune, Maharashtra, India - 18°18'18.3"N 74°00'40.4"E. Average pH and density of milk sample at 1st, 15th, 30th day for goat and sheep at 1st, 30th and 180th day for cow and buffalo were recorded, pH as (goat-6.48, sheep- 6.41, cow-6.71, buffalo- 6.82) and density (g/mL) (goat-1.158, sheep- 1.126, cow-1.026, buffalo-1.182) respectively.

Culture isolation

Total 182 cultures were isolated from goat (62), sheep (40), cow (38) and buffalo milk (51) of three different postpartum periods.

All the isolated cultures were screened for colony characterization, similar to *Lactobacillus* and yeast cultures and out of 127 except 14 yeasts, all 113 cultures similar to Lactobacillus were taken forward for biochemical characterization.

Cultural and colony characterization

Among the 113 cultures only 79 were found to be Gram positive out of which 49 cultures were endospore negative and 30 were positive; 54 cultures were acid-fast negative and 25 were acid-fast positive; 45 cultures were found to be catalase-negative and 34 cultures were catalase-positive; and all screened cultures were mannitol negative. Hence total45 cultures of different postpartum period that showed Gram-positive, Endospore, Acid-fast, Catalase and Mannitol negative were selected for selected for further studies along with 14 yeast cultures as given in (table 1).

Table1: Number of isolates per sample

Sample Milk	Early postpartum (1st)	Acute postpartum (2nd)	Subacute Postpartum (3rd)	Total
Goat	11	7	7	25
Sheep	5	6	4	15
Cow	4	4	6	14
Buffalo	5	5	5	15
Total	25	22	22	69

All 69 isolates designated as from goat 11 (G-a, G-a*, G-b, G-c, G-d, G-e, G-f, G-g, G-h, G-i and G-j), 7 (G-2a, G-2b, G-2c, G-2d, Wa, Wc and Wd), 7 (G-3, G-3a, G-3b, G-3c, G-3d, G-3e and G-3f), sheep 5 (Sm-A, Sm-B, Sm-C, Sm-D and Sm-E), 6 (Sm-1, Sm-3, Sm-6, 7, *1-1 and *2), 4 (Sm-Z, Dg-R, Dg-B and Dg-Z), cow 4 (Ai, Bii, E4 and E-ext), 4 (Cm-a, Cm-h, Cmh-12 and Cm-g), 6 (Cm-1, Cm-2, Cm-3, Cm-4, Cm-5 and Cm-6) and from buffalo 5 (Bm-p, Bm-q, Bm-m, Bmm-3 and Bm-n), 5 (Bm-2a, Bm-2b, Bm-2c, Bm-2d and Bm-2e), 5 (Bm-2, Bm2A-1, Bm-5, Bm-5-1 and Bm-6) from 1st, 2nd and 3rd postpartum milk.

4.2 Toxicity (Hemolytic) Assay

Total of 69 cultures screened, 54 cultures were found to be non-haemolytic as from goat milk 19, sheep 12, cow 9 and buffalo 14 cultures tested showed γ lysis on

sheep blood agar plate and hence eligible for further probiotic characterization (12).

4.2.1 Tolerance to pH and bile salt

Isolated and all screened cultures could tolerate a wide range of pH, range of 1.5 to 10, with maximum growth at pH 7 and 8. From the total of 54 cultures, 47 could survive in a wide range of pH and bile acid except for G-i, Bii, E-ext, Bm-2e, Bm-5, Bm-5-1 and *2.

4.2.2 Effect of Temperature

It is important to find out the optimum temperature required for the growth of probiotic strain were as in present study 47 cultures were able to grow at all three tested temperatures, 28°C, 37°C and 42°C, 6 cultures from first post-partum period milk of goat as (G-a, G-a*, G-e, G-f, G-h, and G-j), 5 from second post-partum (G-2a, G-2b, G-2c, Wa and Wc) and 7 from third post-partum milk as (G-3, G-3a, G-3b, G-3c, G-3d, G-3e and G-3f), 5 cultures from first post-partum milk of sheep (Sm-A, Sm-B, Sm-C, Sm-D and Sm-E), 5 from second post-partum (Sm-1, Sm-3, Sm-6, 7 and *1-1) and only 1 from third post-partum period milk as (Dg-Z), 2 cultures from first postpartum milk of Cow as (Ai and E4), 3 from second (Cm-a, Cm-h and Cmh-12) and 2 from third postpartum milk of cow as (Cm-2, Cm-5) and from buffalo first postpartum 5 cultures as (Bm-p, Bm-q, Bm-m, Bmm-3 and Bm-n), 4 cultures from second (Bm-2a, Bm-2b, Bm-2c and Bm-2d) and 2 from third postpartum milk of buffalo as (Bm2A-1,and Bm-6) Showed good growth in wide range of temperature from 28-42ºC.

4.2.4 Auto-aggregation and Co-aggregation

In the present study 4 cultures from the first post-partum period milk of goat as (G-a, G-f, G-h, and G-j), 5 from second post-partum (G-2a, G-2b, G-2c, Wa and Wc) and 5 from third post-partum milk as (G-3, G-3a, G-3d, G-3e and G-3f), 4 cultures from first post-partum milk of sheep (Sm-A, Sm-C, Sm-D and Sm-E), 5 from second post-partum (Sm-1, Sm-3, Sm-6, 7 and *1-1) and only 1 from third post-partum period milk as (Dg-Z), 2 cultures from first postpartum milk of Cow as (Ai and E4), 1 from second (Cmh-12) and 1 from third postpartum milk of cow as (Cm-5) and from buffalo first postpartum 3 cultures as (Bm-p, Bm-q and Bmm-3), 2 cultures from second (Bm-2a and Bm-2c) and 1 from third postpartum milk of buffalo as (Bm2A-1) cultures have shown good auto-aggregation property.

All cultures could show considerable co aggregation property against *E.coli* with the best activity from goat milk (G-h, G-j, G-2c, Wc, G-3, G-3a and G-3b), sheep (Sm-A, Sm-C, Sm-D, Sm-E, Sm-1, Sm-6, 7, *1-1, Dg-Z), Cow milk (Ai, Cm-a, Cm-h and Cmh-12) and from buffalo milk (Bm-p, Bmm-3, Bm-n, Bm-2d, Bm2A-1 and Bm-6) Co-aggregation against *E. faecalis*, cultures from goat milk (G-h, G-j, G-2c, Wc, G-3, G-3a, G-3b, G-3c, G-3d, G-3e and G-3f), sheep (Sm-A, Sm-C, Sm-D, Sm-E, Sm-1, Sm-6, 7, *1-1, Dg-Z), cow milk (Ai, Cm-a and Cmh-12) and from buffalo milk (Bm-m, Bmm-3, Bm-2d and Bm2A-1 showed good co aggregation and with *S.aureus* with the best activity from goat milk (G-a*, G-f, G-h, G-j, G-2c, Wc, G-3, G-3e and G-3f), sheep (Sm-A, Sm-C, Sm-1, Sm-6, 7, *1-1, Dg-Z), cow milk (Ai, Cm-a, Cm-h and Cmh-12) and from buffalo milk (Bm-m, Bmm-3, Bm-n, Bm-2d, Bm2A-1 and Bm-6) showed good co-aggregation activity against *S.aureus.*

4.2.3 Gastric and intestinal tolerance

In the present study cultures isolated from three different postpartum milk of goat, out of 18 cultures only 8 could tolerate to the gastric and intestinal environment as (G-f, G-2a, G-2b, G-2c, Wc, G-3, G-3d and G-3e) with the gastric environment for 90 and 180 min (77.5 and 60.9), (72.8 and 53.1), (63.18 and 29.13), (49 and 25.3), (82.3 and 68.5), (71 and 56.2), (78.2 and 44.3), (50.1 and 27.2). and in intestinal environment for 240 min (67, 61, 69, 59, 71, 81, 82, 72) percent tolerance, Out of 11 only

9 cultures from sheep milk could tolerate to GI environment (Sm-A, Sm-C, Sm-D, Sm-E, Sm-1, Sm-6, 7, *1-1 and Dg-Z) with gastric environment for 90 and 180 min (80 and 73), (80 and 62.9), (86 and 77), (75 and 50), (63.3 and 52.1), (64.8 and 47.2), (52 and 41), (68 and 46), (81.6 and 80.1) and in intestinal environment for 240 min (67, 73, 68, 71, 61, 52, 76, 72, 56) percentage, From 7 cow milk cultures 4 could tolerate to GI environment (Ai, E4, Cm-h and Cmh-12) tolerance to gastric environment for 90 and 180 min as (86.6 and 77.7), (85.7 and 57.1), (90 and 48.1), (72.3 and 52) and intestinal environment for 240 min (76, 65, 79, 74) percentage of tolerance were as from buffalo milk from total 11 screened cultures 9 could able to survive in GI environment (Bm-p, Bm-q, Bm-m, Bmm-3, Bm-n, Bm-2a, Bm-2c, Bm-2d and Bm2A-1) with gastric environment for 90 and 180 min (67.9 and 46.5), (94.6 and 77.45), (76.7 and 65.2), (64 and 41), (75.4 and 72.3), (87.3 and 77.1), (96.4 and 69.9), (92.02 and 46.1), (71 and 58.1) and in intestinal environment for 240 min (70, 82, 68, 76, 87, 55, 75, 69, 81) percentage of tolerance in gastric and intestinal condition.

4.2.5 Antimicrobial activity

In the present study bioactive compounds present in supernatant were tested against both gram-positive as well as gram-negative bacteria. The results showed that only Wc, Dg-z, Ai, Cm-h and Bm2A-1 could inhibit *Escherichia coli* NCIM 3099 with 0.6, 0.75, 0.55, 0.8 and 0.7 cm, Culture G-3, G-3d, G3-e and Ai could inhibit *Staphylococcus aureus* NCIM 2408 with 1.1, 0.9, 0.65 and 0.8 cm, G-f, G-3, G-3d, G3-e, *1-1, Cmh-12 and Bm2-A1 could inhibit *Enterococcus faecalis* NCIM 3040 with 0.45, 1.2, 0.95, 0.75, 0.8, 0.65 and 1.0 cm were as G-3, G-3d, G3-e effective against *Candida albicans* NCIM 3557 with 0.5, 0.65 and 0.55 cm zone of inhibition were observed.

4.2.6 NaCl Tolerance

High NaCl acts as an inhibitory compound that may inhibit the growth of micro-organisms (22). This present study showed All 30 cultures could tolerate salt concentration in the range of 2-8% with up to 88.3%, 69.8% survival rate at 6% and 8% NaCl concertation

4.2.7 Hydrophobicity test

The present study showed All cultures Showed good hydrophobicity activity, cultures from goat milk such as G-f, G-2a, G-2b, G-2c, Wc, G3, G-3d and G-3e showed 69.4, 80.1, 80.4, 57.7, 86.7, 78, 85.8, 83.3% hydrophobicity, cultures from sheep milk such as Sm-A, Sm-C, Sm-D, Sm-E, Sm-1, Sm-6, 7, *1-1 and Dg-Z showed 48.3, 39.5, 47.2, 51, 47.5, 20.45, 61, 38.26, 34%, from cow milk Ai, E4, Cm-h and Cmh-12 showed 88.06, 95.79, 96.61, 85.1% were as cultures from buffalo milk as Bm-P, Bm-q, Bm-m, Bmm-3, Bm-n, Bm-2a, Bm-2c, Bm-2d, Bm2a-1 showed as 84.39, 39.1, 93.36, 88, 96.28, 56.93, 65.95, 70.27 and 79.1% of hydrophobicity hence Sm-C, Sm-6, *1-1, Dg-Z and Bm-q were eliminated.

4.2.8 Bile salt Hydrolysis

After 72h of incubation precipitation was observed around 14 cultures (G-f, G-2a, G-2b, Wc, G-3, Ai, Cm-h, Cmh-12, Bm-p, Bm-m, Bmm-3, Bm-2c, Bm-2d and Bm2A-1) indicating bile salt hydrolase activity of the culture (23).

4.2.9 Antibiotic Susceptibility Test

All cultures when exposed to different antibiotics on MRS agar, showed resistance to ampicillin (10 mcg) while G-3d, G-3e, Ai, Cmh-12, Bmm-3 and Bm2A-1 were susceptible to chloramphenicol (25 mcg), streptomycin (10 mcg), suphatried (300 mcg) tetracycline (25 mcg) and penicillin-G (1 unit) as per the interpretation of zones of inhibition (in mm) for Kirby-Bauer antibiotic susceptibility test as reported in (Reynolds J. at al., 2009). Resistance and susceptibility of strains were considered

according to the breakpoint proposed by the European food safety authority**.**

Table 2: Screening of isolates for probiotic character

Technique	Goat Milk samples			Sheep Milk samples			Cow Milk samples			Buffalo milk samples		
Postpartum	1	2	3	1	2	3	1	2	3	1	2	3
Total Isolates	22	25	15	12	15	13	11	14	13	14	21	16
Colony and Biochemical characterization	11	7	7	5	6	4	4	4	6	5	5	5
Blood haemolysis	7	5	7	5	6	1	4	3	2	5	5	4
pH, Bile Temperature tolerance	6	5	7	5	5	1	2	3	2	5	4	2
Auto and co-aggregation	3	5	5	4	5	1	2	2	1	5	3	1
GI transit	1	4	3	4	4	1	2	1	1	5	3	1
Antimicrobial	1	1	3	0	1	0	2	1	1	0	0	1
NaCl	1	4	3	4	4	1	2	1	1	5	3	1
Hydrophobicity	1	4	3	3	2	0	2	1	1	4	3	1
Bile salt Hydrolases	1	3	1	1	2	0	1	2	0	3	2	1
Antibiotic susceptibility	0	0	2	0	0	0	1	1	0	1	0	1

DISCUSSION

Milk is a rich source of various nutrients and it contains many beneficial microorganisms. It is a well-known fact that the quality and quantity of milk decreases along with parturition period and finally the milk production stops. The present study made an effort to understand the changes in probiotic bacterial count during the parturition.

Total 182 cultures were isolated from various milk samples of different parturition periods and 127 were screened for probiotic potential. Initially the cultures were tested for Gram's nature, endospore, acid-fast, catalase and mannitol fermentation. Toxicity of the cultures was checked by haemolysis on blood agar plate Haemolysis is to determine the toxins produced by bacteria by breaking down or destructing red blood cells. (24). It was followed by pH, salt, temperature and bile tolerance test and bile salt hydrolysis activity. Moreover, auto aggregation and co-aggregation activity were also analysed followed by the antibiotic susceptibility.

In the gastric environment, acidic condition of pH 1.5-2.0 and increased up to 7.8 pH at lower intestine (Çakır, 2003). Probiotics should reach the lower intestinal tract and maintain themselves over there. 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) Prete et al., 2020). Since the human gastrointestinal tract's bile concentration differs, the mean bile concentration of the intestine is assumed to be 0.3 per cent w/v and the duration of stay is suggested to be 4 h (28). The optimum temperature for the growth of most probiotics is between 37°C and 43°C (29,30). Usually, no growth is observed for *Bifidobacteria* at temperatures below 20°C or above 46°C (30). Probiotic *Lactobacilli* can grow well over a similar temperature range though some can grow at up to 44°C and mesophilic temperatures down to 15°C (31).

Auto aggregation is a macroscopic observation of the formation of clusters or microbial clumps and bind to the inorganic or extracellular metrics of the cells this self-binding is termed auto aggregation and is along with surface colonization. It is the first step in the formation of biofilm (32,33). To exert its beneficial effects, colonisation in the intestinal wall is an important desirable feature of probiotic bacteria. Auto aggregation as a function of time which was highest at the 24 h time point auto aggregative percentages between 48% and 69%. And were measured by comparing the initial absorbance at 600 nm. The baseline auto-aggregation percentage for a good probiotic candidate recommended is more than 40%. Co-aggregation is a process where different strains or different species of microorganisms bind together which is used to eliminate pathogenic microorganisms (34–36). Co-aggregation is another important factor for the adhesion of microbial cells to host surfaces. each microbial strain or species has its specific co-aggregation partner. The process of co-aggregation is carried out by stereo-

chemical interactions between specific surface components and interacting microbial cell surfaces, such as lectin-carbohydrate interactions. It has been suggested that the lectin-like components in surface layered proteins (SLP) of *Lactobacillus* play a crucial role in adhesion to the receptors, such as sugar chains of glycoprotein's (37) and on the surfaces of intestinal epithelium cells. Co-aggregation indicates the ability of isolates to bind and inhibit the pathogen. The transit of probiotics through the gastrointestinal tract takes variable times and is submitted to different stressful conditions. The first barrier that bacteria must overcome is the low pH values of the stomach and various proteolytic enzymes such as pepsin with pH values ranging from 1.5 to 2 and mean exposure times of 90 min. Into the duodenum, the pH value rises to 6–6.5, but bile salts are poured from the gallbladder to reach concentrations ranging from 1.5 to 2% during the first hour of digestion and decreasing afterwards to 0.3% w/v or lower (Jernberg et al., 2007). The time of residence in the small intestine up to 50% of the emptying oscillates between 2.5 and 3 hours and the colon transit could take up to 40 h (39,40). In this location pH values are close to neutral (from 5.5 to 7) and the physiological concentration of bile salts is lower. Antimicrobial activity is one of the most critical probiotic selection criteria (41). Probiotic microorganism antimicrobial effects are produced by the processing of certain substances, such as organic acids (lactic, acetic, propionic acids), carbon dioxide, hydrogen peroxide, diacetyl, antimicrobial low molecular weight substances and bacteriocins (25,42).

To test for a possible correlation between this physio-chemical property and the ability to adhere to the intestinal mucus, cell-surface hydrophobicity is established. Only bacterial adhesion to n-hexadecane, xylene, and toluene represents hydrophobicity or hydrophilicity of the cell surface (43,44). Determination of hydrophobicity of the cell surface is determined based on the capacity of the microorganisms to partition from phosphate buffer solution into hydrocarbons. Hydrophobic strains were found to be more than 40 per cent hydrophobic (45). Bile is a yellow-green aqueous solution and is synthesized in pericentral hepatocytes of the liver and stored in the gall bladder. The major constituent of bile is bile acids, cholesterol, phospholipids and biliverdin (46). It plays an important role in fat digestion, emulsifying and solubilizing lipids, hence it also acts as a potent anti-microbial compound, as it causes dissolution of the microbial membrane by dissolving the phospholipids, cholesterol, and proteins of cell membranes (6,47). Bile acids, cholic acid and chenodeoxycholic acid are synthesized from cholesterol in the liver and secreted as conjugates of amino acid with amide bond between bile acid carboxyl group and amino group glycine or taurine (6). Normally bile acids are conserved by the process of enterohepatic recirculation, where conjugated and unconjugated bile acids are absorbed by passive diffusion (48), the reabsorbed bile is taken up by the hepatocytes, conjugated and secreted into bile. During this process, a small percentage of bile (5% of the total bile 0.3-0.6g) escapes reabsorption and is exposed to intestinal microbes, where deconjugation occurs, catalysed by bile salt hydrolase enzyme. Bile salt hydrolase, hydrolyse the amide bond liberating glycine or taurine moiety, resulting in deconjugated and unconjugated bile acids (49).

The vast use of antibiotics has been instrumental in spreading/emerging antibiotic-resistant bacteria. Lactic acid bacteria are used as starter cultures for food production and may probably produce antibiotic resistance genes in fermentation technology (50,51). Studies on the selection and dissemination of antibiotic resistance in recent years have focused mainly on clinically relevant bacterial species. More recently, it has been hypothesized that food bacteria can act as reservoirs for genes with

antibiotic resistance (52,53). The European Food Safety Authority (EFSA) has therefore assumed the responsibility of launching the European initiative towards a definition of 'qualified presumption of health' (QPS) which, similar to the GRAS program in the United States, aims to enable strains with an existing background and health status to enter the market without extensive testing requirements (54). The presence of transmissible markers of antibiotic resistance in the evaluation of strains is, therefore, an important safety criterion. According to WHO / FAO, Lactobacilli's antibiotic susceptibility is one of the key safety criteria for potential probiotics because bacteria used as probiotics may serve as a host of antibiotic resistance genes that can be passed to pathogenic bacteria (55,56).

The above study could not clearly show a trend of number of probiotic bacteria along with parturition age, although goat milk showed a clear decrease in the number of probiotic bacteria the trend was reverse in the cow milk, however, the number remain same in buffalo milk.

CONCLUSION

Milk is rich in nutrition along with medicinal properties it contains beneficial probiotic microorganisms. Various studies stated that the milk microbiome benefited humans in various health beneficial activities such as acid-base balance, aid in digestion etc. Due to the huge microbial diversity and functionality, there is scope to isolate microorganisms and evaluate them for probiotic potential and various health benefits. The present study showed isolated culture from three different postpartum period milk of Goat, Sheep, Cow and Buffalo showed that from 182 isolates only 127 were selected by colony characterization and 69 were selected after biochemical analysis such as Gram's nature, Endospore, Catalase, Acid-fast and Mannitol fermentation, among that 54 was non-hemolytic, 47 could tolerate a wide range of pH, bile and temperature, 30 isolates could survive in the presence of a Gastric and intestinal environment. This culture showed inhibitory action against common pathogenic organisms and did satisfy the need for auto-aggregation and co-aggregation properties Also showed NaCl tolerance, 17 cultures showed BSH and hydrophobicity activity, only 6 cultures showed susceptibility against all types of antibiotics.

As per the results from the total potential probiotic cultures from various animal milk samples 40% from first and second postpartum each and 20% cultures from third postpartum milk (table 2), as per the results as milk ages, the probiotic concentration in the milk decreases.

ACKNOWLEDGEMENTS

The authors are indebted to BVDU-Rajiv Gandhi Institute of IT and Biotechnology, Bharati Vidyapeeth Deemed University (BVDU), Pune for allowing them to undertake this work.

Conflict of Interest: None

Source of Funding: None

Ethical Approval: Approved

REFERENCES

1. Hyrslova I. Goat and Bovine Colostrum as a Basis for New Probiotic Functional Foods and Dietary Supplements. J Microb Biochem Technol. 2016 Jan 1;08.
2. Marounek M, Jehlickova K, Kmet V. Metabolism and some characteristics of lactobacilli isolated from the rumen of young calves. J Appl Bacteriol [Internet]. 1988 Jul 1;65(1):43–7.
3. FAO/WHO. Guidelines for the evaluation of probiotics in food. London, Ontario, Food and Agriculture Organization of the United Nations and World Health Organization Working Group Report. 2002.
4. Haghshenas B, Nami Y, Haghshenas M, Abdullah N, Rosli R, Radiah D, et al. Bioactivity characterization of Lactobacillus strains isolated from dairy products. Microbiologyopen [Internet]. 2015;4(5):803–13.

5. Shokryazdan P, Sieo CC, Kalavathy R, Liang JB, Alitheen NB, Faseleh Jahromi M, et al. Probiotic potential of Lactobacillus strains with antimicrobial activity against some human pathogenic strains. Biomed Res Int. 2014;2014:927268.
6. Prete R, Long SL, Gallardo AL, Gahan CG, Corsetti A, Joyce SA. Beneficial bile acid metabolism from Lactobacillus plantarum of food origin. Sci Rep [Internet]. 2020;10(1):1165.
7. Saikot F, Rahman SM, Forhad M. Probiotic properties analysis of isolated lactic acid bacteria from buffalo milk. Arch Clin Microbiol. 2015 Dec 21;7:5–10.
8. Fotou K, Tzora A, Voidarou C, Alexopoulos A, Plessas S, Avgeris I, et al. Isolation of microbial pathogens of subclinical mastitis from raw sheep's milk of Epirus (Greece) and their role in its hygiene. Anaerobe. 2011 Dec;17(6):315–9.
9. Lima FS, Oikonomou G, Lima SF, Bicalho MLS, Ganda EK, de Oliveira Filho JC, et al. Prepartum and Postpartum Rumen Fluid Microbiomes: Characterization and Correlation with Production Traits in Dairy Cows. Griffiths MW, editor. Appl Environ Microbiol [Internet]. 2015 Feb 15;81(4): 1327 LP – 1337.
10. Kathade SA, Aswani MA, Anand PK, Jagtap S, Bipinraj NK. Isolation of Lactobacillus from donkey dung and its probiotic characterization. Korean J Microbiol. 2020;56(2).
11. Desai A. Strain identification and probiotics properties of Lactobacillus casei. Thesis PhD. 2008;1–228.
12. Pino A, Bartolo E, Caggia C, Cianci A, Randazzo CL. Detection of vaginal lactobacilli as probiotic candidates. Sci Rep [Internet]. 2019;9(1):3355.
13. Chanthala L, Appaiah A. In vitro probiotic characterization of yeasts of food and environmental origin. Int J Probiotics Prebiotics. 2014 Jan 1;9:87–92.
14. Ogunremi OR, Sanni AI, Agrawal R. Probiotic potentials of yeasts isolated from some cereal-based Nigerian traditional fermented food products. J Appl Microbiol [Internet]. 2015 Sep 1;119(3):797–808.
15. Chelliah R, Ramakrishnan SR, Prabhu PR, Antony U. Evaluation of antimicrobial activity and probiotic properties of wild-strain Pichia kudriavzevii isolated from frozen idli batter. Yeast. 2016 Aug;33(8): 385–401.
16. Aditi Sourabh,. In vitro characterization of Saccharomyces cerevisiae HM535662 obtained from an indigenous fermented food "Bhaturu" of Western Himalayas. African J Biotechnol. 2012;11(52):11447–54.
17. Aswani MA, Kathade SA, Anand PK, Kunchiraman BN, Dhumma PR, Jagtap SD. Probiotic Characterization of Cholesterol-Lowering Saccharomyces cerevisiae Isolated from Frass of Pyrrharctia isabella Caterpillars. Appl Food Biotechnol. 2021; 8(3): 189–98.
18. Kathade S, Aswani M, Anand PK, Nirichan B. Probiotic characterization and cholesterol assimilation ability of pichia kudriavzevii isolated from the gut of the edible freshwater snail "pila globosa." Egypt J Aquat Biol Fish. 2020;24(7 Special issue).
19. Toit M Du, Dicks LMT, Holzapfel WH. Identification of heterofermentative lactobacilli isolated from pig faeces by numerical analysis of total soluble cell protein patterns and RAPD-PCR. Lett Appl Microbiol [Internet]. 2003 Jul 1;37(1):12–6.
20. Ragavan ML, Das N. Molecular identification of probiotic yeast strains and their characterization. Asian J Pharm Clin Res. 2017;10(10):339–43.
21. BAUER AW, PERRY DM, KIRBY WM. Single-disk antibiotic-sensitivity testing of staphylococci; an analysis of technique and results. AMA Arch Intern Med [Internet]. 1959 Aug;104(2):208—216.
22. Billah MM, Hoque MZ, F A, Hossain KM, MSM R, Islam KMD. Isolation, Identification and Analysis of Probiotic properties of Lactobacillus Spp. from selective regional yoghurts. World J Dairy Food Sci. 2010 Jan 1;5:39–46.
23. Zheng Y, Lu Y, Wang J, Young L, Pan C, Huang Y. Probiotic Properties of Lactobacillus Strains Isolated from Tibetan Kefir Grains. PLoS One. 2013 Jul 22;8:e69868.
24. Pelczar M. J. Jr., Reid R. D. CECS. Microbiology, 4th Ed. Tata McGraw-Hill Publishing Company Ltd, New Delhi.; 1977.
25. Çakır İ. Determination of some probiotic properties on Lactobacilli and Bifidobacteria. Ankara University; 2003.
26. Sanders ME, Merenstein DJ, Reid G, Gibson GR, Rastall RA, Markowiak P, et al.

A review of probiotic supplementation in healthy adults: helpful or hype? Appl Environ Microbiol [Internet]. 4th ed. 1999 Jan 1;7(1):53–61.

27. Dunne, C., Murphy, L., Flynn, S., O'Mahony, L., O'Halloran, S., Feeney, M., Morrissey, D., Thornton, G., Fitzgerald, G., Daly, C., Kiely, B., Quigley, E.M.M., O'Sullivan, G.C., Shanahan, F., and Collins JK. Probiotics: from myth to reality. Demonstration of functionality in animal models of disease and in human clinical trials. Antonie Van Leeuwenhoek. 1999; 76:279–92.
28. Jacobsen CN, Rosenfeldt Nielsen V, Hayford AE, Møller PL, Michaelsen KF, Paerregaard A, et al. 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 [Internet]. 1999 Nov;65(11):4949–56.
29. Ahmed M, Prasad J, Gill H, Stevenson L, Gopal P. Impact of consumption of different levels of Bifidobacterium lactis HN019 on the intestinal microflora of elderly human subjects. J Nutr Health Aging. 2007;11(1):26–31.
30. Shu Q, Gill HS. A dietary probiotic (Bifidobacterium lactis HN019) reduces the severity of Escherichia coli O157:H7 infection in mice. Med Microbiol Immunol. 2001 Apr;189(3):147–52.
31. Salminen S, Laine M, von Wright A, Vuopio-Varkila J, Korhonen T and M-ST. Development of selection criteria for probiotic strains to assess their potential in functional foods: a Nordic and European approach. a Nord Eur approach Biosci. 1996;15(2):61-67\.
32. Sorroche F, Spesia M, Zorreguieta A, Giordano W. A Positive Correlation between Bacterial Autoaggregation and Biofilm Formation in Native Sinorhizobium meliloti Isolates from Argentina. Appl Environ Microbiol. 2012 Apr 6;78:4092–101.
33. Kragh KN, Hutchison JB, Melaugh G, Rodesney C, Roberts AEL, Irie Y, et al. Role of Multicellular Aggregates in Biofilm Formation. MBio. 2016 Mar;7(2):e00237.
34. Corno G, Coci M, Giardina M, Plechuk S, Campanile F, Stefani S. Antibiotics promote aggregation within aquatic bacterial communities. Front Microbiol [Internet]. 2014 Jul 1;5:297.
35. Ochiai K, Kurita-Ochiai T KY. Effect of co-aggregation on the pathogenicity of oral bacteria. J Med Microbiol. 1993;39:183–90.
36. Malik A, Sakamoto M, Hanazaki S, Osawa M, Suzuki T, Tochigi M, et al. Coaggregation among nonflocculating bacteria isolated from activated sludge. Appl Environ Microbiol [Internet]. 2003 Oct;69(10):6056–63.
37. Gusils C. Lactobacilli isolated from chicken intestines: potential use as probiotics. J Food Prot. 1999;62(3):252–6.
38. Jernberg C, Löfmark S, Edlund C, Jansson JK. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. ISME J. 2007 May;1(1):56–66.
39. Satokari RM, Vaughan EE, Akkermans AD, Saarela M, de Vos WM. Bifidobacterial diversity in human feces detected by genus-specific PCR and denaturing gradient gel electrophoresis. Appl Environ Microbiol. 2001 Feb;67(2):504–13.
40. Zinniel DK, Lambrecht P, Harris NB, Feng Z, Kuczmarski D, Higley P, et al. Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. Appl Environ Microbiol. 2002 May;68(5):2198–208.
41. Klaenhammer TR, Kullen MJ. Selection and design of probiotics. Int J Food Microbiol. 1999 Sep;50(1–2):45–57.
42. Quwehand, A.C. and Vesterlund S eds. Antimicrobial components from lactic acid bacteria. Lact Acid Bact Microbiol Funct Asp. 2004;
43. Krausova G, Hyrslova I, Hynstova I. In Vitro Evaluation of Adhesion Capacity, Hydrophobicity, and Auto-Aggregation of Newly Isolated Potential Probiotic Strains. Fermentation. 2019 Dec 4;5:100.
44. Bellon-Fontaine M N RJ and van OCJ. Microbial adhesion to solvents: a novel method to determine the electron donor/electron acceptor or lewis acid-base properties of microbial cells. Colloids Surfaces B Biointerfaces. 1996;7:47–53.
45. Boris S, Suárez JE, Vázquez F, Barbés C. Adherence of human vaginal lactobacilli to vaginal epithelial cells and interaction with uropathogens. Infect Immun. 1998 May;66(5):1985–9.

46. De Smet I, Van Hoorde L, Vande Woestyne M, Christiaens H, Verstraete W. Significance of bile salt hydrolytic activities of lactobacilli. J Appl Bacteriol [Internet]. 1995 Sep 1;79(3):292–301.
47. Begley M, Gahan CGM, Hill C. The interaction between bacteria and bile. FEMS Microbiol Rev [Internet]. 2005 Sep 1;29(4):625–51.
48. Boyer JL. Bile Formation and Secretion [Internet]. Comprehensive Physiology. 2013. p. 1035–78. (Major Reference Works).
49. Dawson PA, Karpen SJ. Intestinal transport and metabolism of bile acids. J Lipid Res [Internet]. 2015;56(6):1085–99.
50. Teuber M, Meile L, Schwarz F. Acquired antibiotic resistance in lactic acid bacteria from food. Antonie Van Leeuwenhoek. 1999;76(1–4):115–37.
51. Danielsen M, Wind A. Susceptibility of Lactobacillus spp. to antimicrobial agents. Int J Food Microbiol. 2003 Jan;82(1):1–11.
52. Klein G, Pack A, Reuter G. Antibiotic resistance patterns of enterococci and occurrence of vancomycin-resistant enterococci in raw minced beef and pork in Germany. Appl Environ Microbiol [Internet]. 1998 May;64(5):1825–30.
53. Rowland I, Gibson G, Heinken A, Scott K, Swann J, Thiele I, et al. Gut microbiota functions: metabolism of nutrients and other food components. Eur J Nutr [Internet]. 2018;57(1):1–24.
54. Authority EFS. EFSA Scientific Colloquium Summary Report. QPS: qualified presumption of safety of microorganisms in food and feed. 2004.
55. Chand D, Avinash VS, Yadav Y, Pundle AV, Suresh CG, Ramasamy S. Molecular features of bile salt hydrolases and relevance in human health. Biochim Biophys acta Gen Subj. 2017 Jan;1861(1 Pt A):2981–91.
56. Ashraf R, Shah N. Antibiotic resistance of probiotic organisms and safety of probiotic dairy products. Int Food Res J. 2011 Jan 1;18:837–53.

How to cite this article: Kathade SA, Aswani MA, Pashmin Kaur Anand et.al. Isolation, characterization, and diversity of probiotic microorganisms from different postpartum milk of various animals. *Int J Health Sci Res.* 2022; 12(3): 223-234. DOI: *https://doi.org/10.52403/ijhsr.20220332*

******