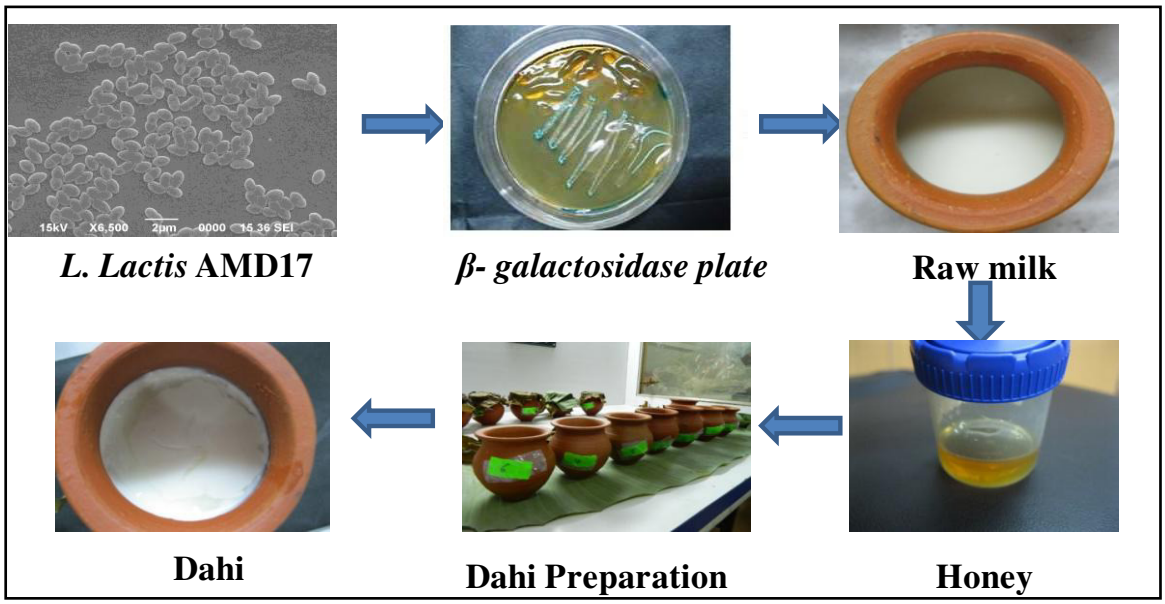
CHAPTER 3

ASSESSMENT OF GOAT MILK-DERIVED POTENTIAL PROBIOTIC *L. LACTIS* AMD17 AND ITS APPLICATION FOR PREPARATION OF DAHI USING HONEY



Graphical Abstract

ASSESSMENT OF GOAT MILK-DERIVED POTENTIAL PROBIOTIC *L. LACTIS* AMD17 AND ITS APPLICATION FOR PREPARATION OF DAHI USING HONEY

3.1. Abstract

Lactococcus lactis AMD17 isolated from free range goat milk was screened for potential probiotic attributes based on functional traits such as resistance to simulated gastric acid and bile salts, antimicrobial activity and inhibition of pathogens adhesion to intestinal epithelium cell line Caco-2. The isolate significantly reduced the adherence of isolated food borne pathogen *Listeria monocytogenes* AMDK2 (47.46 ± 0.17 %) to Caco-2 cells. Honey was used as an adjuvant of *L. lactis* AMD17 for preparation of dahi (curd) from buffalo milk and was found to support its survivability during storage (P <0.05). Sensory evaluation studies revealed dahi prepared with *L. lactis* AMD17 with addition of 3% honey exhibited highest score in taste and color. The texture characteristics were found superior to dahi prepared with only *L. lactis* AMD17. Moreover, the Nisin gene was amplified and showed a similarity of 100% to other NisR-producing *L. lactis* strains. The present study suggests that dahi prepared using honey enriched milk with nisin-producing probiotic strain *L. lactis* AMD17 imparts health benefit and combats food borne pathogens possibly due to the antibacterial features of nisin peptide.

3.2. Introduction

Fermented foods play a significant role in diets since they contain enormous quantities of nutritious constituents with a wide diversity of microbes, aroma, flavor and texture[1]. Dahi is a popular fermented dairy product of South Asia and it has an appearance similar to that of yoghurt and plays an important role in the Indian diet. About 9% of the total milk produced in India is converted into fermented milk products[2]. For dahi production, a small portion (as starter culture) of previously fermented product containing live culture is added to lukewarm milk. However, production of dahi with an individual culture of *Lactococcus lactis*[3] or a combination of cultures containing *lactobacilli* and *lactococci*[4] have been reported. Strains belonging to *Lactococcus* species have been well documented in dairy industry for contributing typical taste and flavor to a variety of fermented dairy products[5]. Unlike

yogurt fermentation, which is carried out by a specific mixed culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, the starter culture of dahi is not well defined due to numerous species and strains of Lactic acid bacteria (LAB) also present in various traditional fermented milk products consumed by different ethnic communities of India.

Nisin, a natural antimicrobial peptide is a broad spectrum bacteriocin. It inhibits Gram-positive bacteria such as *Staphylococcus aureus, Bacillus cereus*, *Listeria monocytogenes*, *Lactobacillus plantarum*, *Micrococcus flavus*, and *Micrococcus luteus*[6]. Nisin was permitted as a safe food additive in over 50 countries around the world by their regulatory agencies as a generally regarded as safe[7]. In this study, dahi was prepared using buffalo milk with nisin-producing potential probiotic *L. lactis* AMD17 strain isolated from goat milk. The development of dairy products containing probiotic bacteria has higher impact in food industries[8]. Buffalo milk has several benefits and advantages in manufacture of milk products since it does not required fortification with milk powder or addition of thickeners or stabilizers[9]. Different dairy products, such as soft and hard cheeses, butter oil (ghee), butter, ice cream and yoghurt has long been prepared in Asian countries such as India and Pakistan (both producing about 80% of the world's production of buffalo milk) using Buffalo milk[10].

The major problem associated with dahi is rapid deterioration of its quality, lesser stability at low temperature and reduction of its shelf life due to frequent microbial contamination. The incorporation of honey adds nutritional value and enhances stability of dahi. Moreover, the high carbohydrate content of honey is considered as an outstanding energy source and imparts some functional effects on the survivability of probiotic strains.

The present study focused to investigate the potential probiotic features of *L. lactis* AMD17 and to evaluate the stability and consumer acceptability of dahi produced using *L. lactis* AMD17 and fortified with 1.0% to 5.0% (w/v) honey.

3.3. Materials and methods

3.3.1. Isolation of *Lactococcus lactis* AMD17 from goat milk

Potential probiotic strains were screened out from goat milk sample collected from Tezpur, Assam (India). Samples were collected in sterile containers and were appropriately diluted using Ringer solution up to 10^{-8} dilutions. Aliquots of 0.1 ml of each dilution were plated on de Man, Rogosa and Sharpe (MRS) agar (Himedia Labs, Mumbai) as medium for LAB isolation. Plates were incubated at 37 °C for 48 h followed by counting of colonies. Out of 114 LAB strains (Appendix-14) isolated from goat milk, *L. lactis* AMD17 was selected for further studies based on its *in vitro* potential probiotic properties such as resistance to gastrointestinal stress, antimicrobial activity and inhibition of pathogens adhesion to intestinal epithelium cell line.

3.3.2. Strain identification and characterization of bacteriocin gene

Selected isolates were identified by 16S rRNA gene sequence analysis followed by phylogenetic studies. Universal primers 27F (5´-AGAGTTTGATCCTGGCTCAG-3´) and 1492R (5´-GGTTACCTTGTTACGACTT-3´) were used for the amplification of 16S rRNA gene sequence (Guoet al.,2010). The PCR amplification was performed in an Eppendorf thermocycler (Eppendorf, Nexux Gradient, 6331CN705326) in a total volume of 25 µl reaction mixture containing 5 µl of 10X Taq Buffer containing $MgCl_2$, 14.8 µl nuclease free water, 2 µl dNTPs, 1 µl of each primers, 0.2 µl of Taq DNA polymerase, and 1 µl of DNA template for the isolate. Amplification parameters consisted of an initial denaturation step at 95 °C for 5 min, followed by 30 cycles of primer annealing for 30 sec at 53 °C, elongation for 1 min at 72 °C, and final 10 min extension at 72 °C. PCR product was purified after electrophoresis in 1 % (w/v) agarose gel and used for the automated DNA sequencing using 3130 Genetic Analyzer (Applied Biosystem, Rotkreuz, Switzerland). The sequence obtained was subjected to NCBI BLAST search tool in order to retrieve the homologous sequences in Genbank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Nisin gene was amplified using previously reported specific primers[11] (nisRF 5'-CTATGAAGTTGCGACGCATCA- 3' and nisRR 5'-CATGCCACTGATACCCAAGT- 3. Briefly, amplification parameters consisted of

an initial denaturation step at 95 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, primer annealing for 40 sec at 58 °C, elongation for 1 min at 72 °C, and final 10 min extension at 72 °C. PCR product was separated by electrophoresis in 1% (w/v) agarose gel. The PCR products were purified and used for the automated DNA sequencing and the sequences obtained were submitted to NCBI (National Centre of Biotechnology information) Genbank. The deduced amino acid sequence obtained from nucleotide sequence was subjected to homology searching in NCBI database using the BLASTp programme (http://www.ncbi.nlm.nih.gov). The phylogenetic tree was generated by neighbor joining (NJ) method using MEGA 5.05 software[12, 13, 14]. The multiple amino acid sequence alignment was accomplished using ClustalW program of Mega 5.0 software and conserved residues were determined. Secondary structure was predicted using I-TASSER Suite[15]. Domain structure was prepared using DOG 1.0[16].

3.3.3. Screening of probiotic properties

3.3.3.1. In vitro gastrointestinal stress tolerance test

The resistance of isolates to low pH conditions which mimic the human gastrointestinal (GI) tract environment was tested according to the method described by Maragkoudakis et al.[17] with some modifications. Briefly, bacterial cells from an 18 h culture were harvested by centrifugation at 6000 × g for 5 min at 4 °C, washed once with Phosphate buffered saline (PBS) (pH 7.4) before being re-suspended (10^8 CFU ml^{-1}) in different PBS solutions with various pH. In order to test simulated gastrointestinal transit tolerance, bacterial cells were re-suspended in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). SGF was prepared by supplementing sterilized PBS pH 2, 3 and 4 (adjusted with 1N HCl) with pepsin to a final concentration of 3 g l^{-1}. SIF was prepared by supplementing sterilized PBS, pH 6.8 and 8 (adjusted with 1N NaOH/1N HCl) with pancreatin (Sigma Aldrich, USA) to a final concentration of 1 g l^{-1}. The resistance of *L. lactis* AMD17 in every condition was assessed in terms of viable colony count on MRS agar after 0, 1, 2, 3 h for SGF and 0, 1, 2, 3, 4 h for SIF treatment respectively.

3.3.3.2 Bile Salt tolerance

The resistance to bile salts tested according to the slightly modified method described by Kaewnopparat et al.[18] The bacterial cells were re-suspended in PBS, pH 7.4 supplemented with 0.2, 0.3, 0.4 and 0.5% (w/v) oxgall and incubated for 0, 1, 2, and 3 h at 37 ºC. The resistance of *L. lactis* AMD17 in every condition assessed in terms of viable colony count on MRS agar plates after the treatment.

3.3.3.3. Antibacterial activity

The antimicrobial effects of the bacterial isolates were examined by agar well diffusion method. Briefly, 100 µl of a log-phase culture of the indicator cultures (10^7–10^8 cell as per McFarland standard) were seeded on the surface of Mueller Hinton Agar (for *Bacillus cereus* MTCC 430, *Staphylococcus aureus* MTCC 3160, *Pseudomonas aeruginosa* MTCC 7815, and *Salmonella enterica typhimurium* MTCC 1252) or Trypticase Soy Agar (for *Listeria monocytogenes* KF894986, laboratory isolate). All the indicators strains used in this study were procured from Microbial Type Culture Collection, Chandigarh (INDIA). Using a sterile borer, 8 mm-diameter wells were punched into the surface and 100 µl cell free supernatant was loaded in agar plates. The plates were incubated at 37 °C for 24 h and the zones of inhibition were recorded. The cell free culture supernatant was further neutralized with 0.5 M NaOH / 1 N HCL and treated with proteinase K, pepsin and trypsin respectively at a final concentration of 1.5 mg/ml to check the nature of antimicrobial substances[19].

3.3.3.4. Cell Aggregation

The aggregation assay was measured according to Del Re et al.[20] with slight modifications. 2.0 ml cell suspension was vortexed for 10 s, and incubated at 37 °C. An aliquot of 0.1 ml collected from the upper surface at regular time interval was mixed with 0.9 ml PBS and its absorbance at 600 nm was measured. Autoaggregation percentage was expressed as:

$$\text{Autoaggregation}(\%) = 1 - {}^{A_t}/_{A_0} \times 100$$

where A_t= absorbance at different time interval 2 h, 4 h, 24 h and A_0= absorbance at 0 h.

3.3.3.5. Coaggregation Assay

Equal volume of cell suspensions (1 ml=10^9 CFU/ml) of *L. lactis* AMD17 and pathogen strains were mixed, and incubated at 37 °C. The control contained 2 ml of pure bacterial cell suspension. A_{600} of these suspensions was measured at predetermined time intervals as described above. The co-aggregation (%) was calculated using the equation[21], $100\times[(A_{pat}+A_{Lacto})/2)-(A_{mix})]/[(A_{pat}+A_{Lacto})/2]$, where A_{pat} and A_{Lacto} represent A_{600} of control tubes and A_{mix} represents the A_{600} of the mixture of *Lactococcus lactis* AMD17 and pathogen strains at predetermined time intervals.

3.3.3.6. Cell culture

The human colorectal adenocarcinoma Caco-2 cell line obtained from NCCS, India (National Centre for Cell Science, Pune) was used to study the adhesion capability and the inhibition of food borne pathogenic bacterial adhesion. The cell lines were routinely grown and maintained by following the standard procedures[22]. Briefly, cells were grown in a 25 cm^2 flask using Minimum Essential Medium (MEM) supplemented with 20% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin and cells were incubated at 37 °C in a humidified atmosphere containing 5% CO2. The cell culture medium was changed after every two days and Caco-2 monolayers at late post-confluence, i.e. after 14 d (80% confluence), was used for the study. Media and reagents were purchased from Sigma Aldrich (USA) and Gibco® (Life Technologies).

3.3.3.7. Adhesion and Inhibition of pathogen adhesion to Caco-2 cells by *L. lactis* AMD17

The adhesion study was performed with slight modification by using the procedure described by García-Cayuela et al.[23],Caco-2 cells were seeded (1×10^4 cells/ml) in 24-well tissue culture plates (NEST Biotechnology) and grown for 14 days (post-confluence, 1×10^7 cells/ml) at 37 °C in a humidified atmosphere containing 5% CO2. Overnight cultures of *L. lactis* AMD17 were harvested by

centrifugation (5000 rpm for 5 min), washed twice in PBS 1X and resuspended in MEM medium without antibiotics at a concentration of about 10^8 CFU/ml. *L. rhamnosus* MTCC 1408 was used as a reference strain[24, 25] to study adhesion and inhibition of pathogens to Caco-2 cells. The plates containing cell line monolayers were also washed twice in PBS solution to remove antibiotics before adding the bacterial suspension. For adhesion assay Caco-2 cell monolayers were inoculated with isolated strain (10^8 cfu/ml) and for the inhibition of pathogen adhesion, mixed inoculum of each of the isolate with each of the pathogen indicator strain in MEM (ratio 5:1) and incubated for 1 h and 2 h respectively at 37 °C, 5% CO2. All bacterial strains were previously grown under standard conditions. Wells containing only the food spoilage bacteria were used as controls. After the incubation period, supernatants were discarded and wells were gently washed three times with PBS buffer to remove non-adhered bacteria. Finally, Caco-2 monolayers were trypsinized with 0.25% trypsin-EDTA solution (Sigma) and the number of adherent bacteria was determined by serial dilution plating on MRS agar (for *L. lactis* AMD17). Similarly inhibition of pathogens adhesion to Caco-2 cells monolayer was determined by serial dilution plating on Oxford-Listeria Selective agar (for *L. monocytogenes*) and Bismuth Sulphite Agar (for *S. enterica typhimurium*). Adhesion data were expressed as the percentage of bacteria adhered compared to the total inoculum added (CFU bacteria adhered/CFU bacteria added) and the ability of the isolate *L. lactis* AMD17 for inhibiting the adhesion of the pathogen was calculated as the percentage of pathogen bacteria that adhere in the presence of the isolate compared with the number of pathogen that adhere in the absence of the isolate. All the experiments for each of the strains were performed independently in triplicate. For visualization of adhesion, Caco-2 cell monolayers were washed three times with PBS, dried in air and adherent bacterial isolate were observed under bright field microscopy (100 x) after fixing with 3 % paraformaldehyde and gram stained.

3.3.4. Determination of the antioxidant activities and the phenolic and flavonoid contents in honey

Three different honey samples were collected from traditional wild honey collectors of Tezpur, Assam, India and pasteurized at 70 °C for 15 min, and cooled to

room temperature. The antioxidant activities of the honey samples were measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay[26]. The anti-oxidant activity of each sample was calculated as the percentage of RSA (radical scavenging activity) using the formula: RSA = $[(A_B-A_A)/A_B] \times 100$, where A_B is the absorbance of the DPPH solution and $A_A$is the absorbance of the honey sample solution.

The Folin–Ciocalteu method was used to determine total phenolic content[27]. Briefly, 40 µl honey samples (1g dissolved in methanol) were mixed with 2.4 ml water and 0.2 ml non-diluted Folin-Ciocalteu reagent and 0.6 ml sodium carbonate. After incubation at room temperature for 2 h, the absorbance of reaction mixture was measured at 765 nm against a methanol blank. The total phenolic content was determined using a standard curve with gallic acid (0–1 mg/ml) as the standard. Results were calculated as mg gallic acid equivalents (GAE)/100 g of honey.

The total flavonoid content was measured using the Dowd method, as adapted by Arvouet et al.[28]. Briefly, honey samples was mixed with $AlCl_3$ (0.5 ml) and distilled water. The solution was kept in the dark for 30 min and the absorbance at 425 nm was measured[29]. Quercetin was used as reference for the calibration curve. Finally, the results were expressed as mg quercetin per 100 g honey (mg QE/100 g).

3.3.5. Preparation of dahi with probiotic *L. lactis* AMD17 and their survivability at low temperature

For the preparation of dahi, 1 L of buffalo milk was collected from nearby area of Napaam, Tezpur and heated for 15 min at 90 °C2 with intermittent stirring[30] and fortified with honey at levels of 1.0, 2.0, 3.0, 4.0 and 5.0% (w/v). The *L. lactis* AMD17 starter culture was prepared in autoclaved skimmed milk by sub-culturing once for maintaining its potential activity. It was inoculated with at 7.4 log CFU/ml and incubated at 37 °C until dahi formation. By the time, the milk curdled and became semi-solid and the preparation was considered as dahi. It was firm and of uniform consistency with a smooth and glossy surface. The set dahi samples were stored aseptically in sterile earthen pot at 4 °C until use. Parts of manufactured products

were checked after 1, 7, 14, 21 and 28 days for survivability of *L. lactis* AMD17 under low temperature.

3.3.6. Sensory evaluation

Sensory evaluations were privately conducted after 1 day while participants were seated in a quite area behind a privacy divider in the milk processing lab (Department of Food Engineering and Technology, Tezpur University). A nine-point hedonic scale in which 9 = "liked extremely", 5 = "neither liked nor disliked" and 1 = "disliked extremely" was used by each participant for sample evaluation. A control sample of plain probiotic yogurt was offered and then the remaining five samples were served in a random order.

3.3.7. Texture Profile of probiotic dahi during one month storage

Texture evaluation of the extrudates was performed with texture analyzer (TA-HD-plus, Stable Micro Systems,UK). The pre- and post-test speed of the probe was 2 mm/s, the test speed was 0.2 mm/s during measurements. The distance covered in the sample was 30 mm, using a cylindrical probe of 20 mm diameter. The results were presented as the average of three measurements. Texture properties such as Hardness (N), Springiness (dimensionless), Cohesiveness (dimensionless), and Gumminess (N) were considered.

3.3.8. Statistical analysis

The results were subjected to ANOVA using GraphPad Prism software (version 5.0; GraphPad Software, Inc. CA, USA). Significance differences among the values of survivability (log CFU/ml) or total phenol, flavonoid and antioxidant content are determined by Tukey's Multiple comparison test at $p<0.05$.

3.4. Results and discussion

3.4.1. Strain identification and characterization of bacteriocin gene

Based on potential probiotic characteristics, the isolate was subjected to 16S rRNA gene sequence analysis. 16S rRNA gene sequences were submitted to NCBI GenBank as *Lactococcus lactis* strain AMD17 (KF113841). The Nisin gene amplified was of 610 bp (Fig. 3.1A). The deduced amino acids of Nisin gene showed (Fig.

3.1B) strong similarities (100% identity) to Nisin biosynthesis regulatory protein NisR (Q07597.1) followed by Nisin biosynthesis two-component system, response regulator NisR [*Lactococcus lactis* subsp. *lactis* KF147, 99%], NisR-*Lactococcus lactis* (ACD45086.1, 99%), response regulator [*Lactococcus lactis* (BAG71485.1), 99%]. The conserved amino acid residues in all members of the family and, by sequence identity, in Nisin AMD17 are found to be Phenylalanine, Glycine, Aspartate, Leucine, Isoleucine, Methionine, Cysteine, Arginine, Proline, Valine, Serine, Alanine, Lysine, Glutamine and Glutamic acid (Fig. 3.1C).

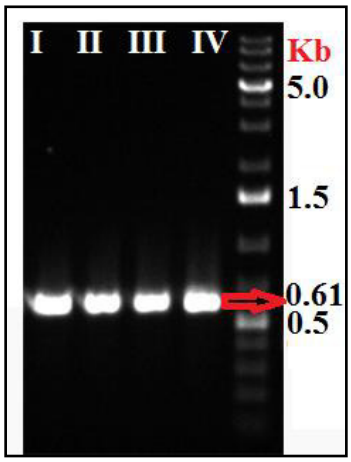


Fig. 3.1 (A) Agarose gel electrophoresis (1%) of Nisin gene. Lane I-IV showing amplicon PCR products (~6 10 bp)

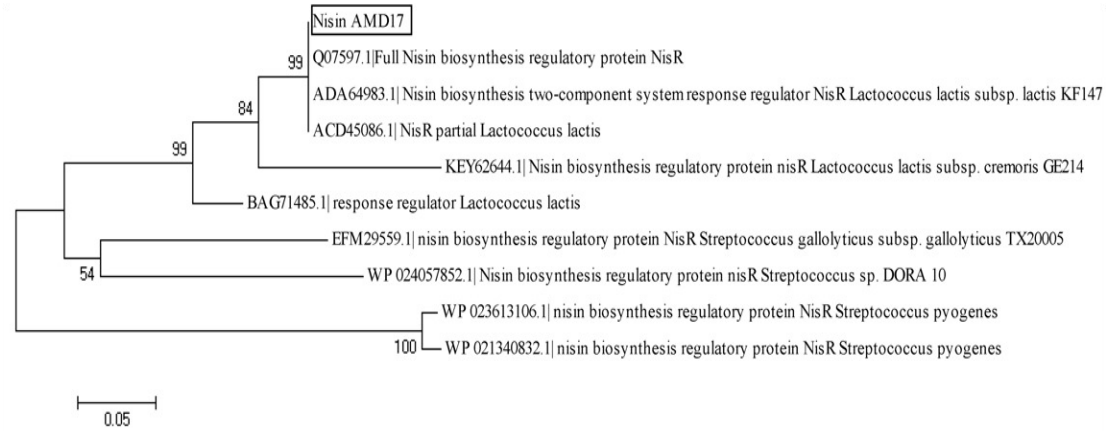


Fig. 3.1 (B) The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Evolutionary analyses were conducted in MEGA 5.0.

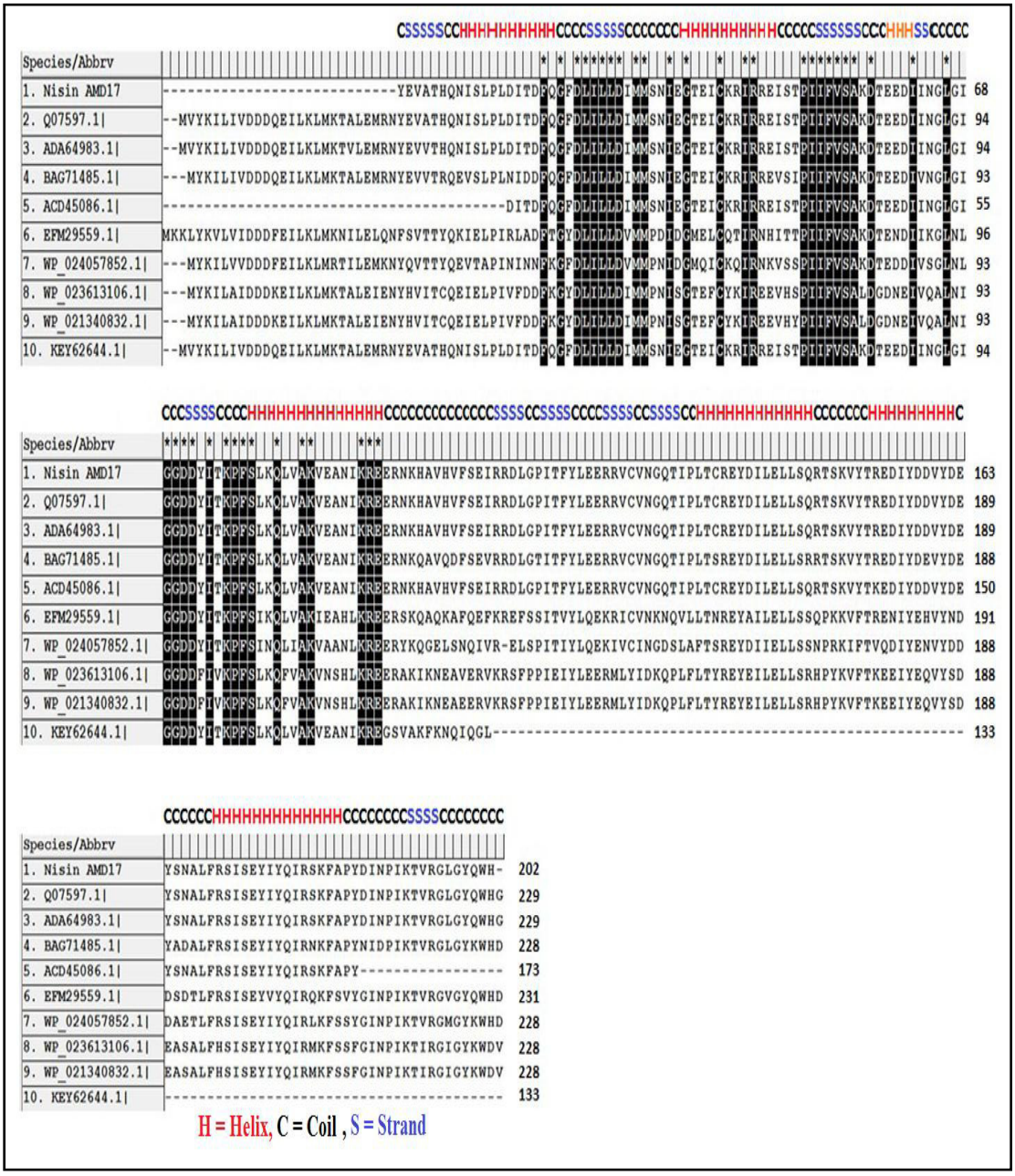


Fig. 3.1 (C) Multiple sequence alignment of *L. lactis* AMD17 Nisin gene with non-redundant protein sequence obtained from databases at the NCBI using the BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Conserved amino acids are indicated in black (100%). Secondary structure were predicted at the top of sequence using I-TASSER Suite

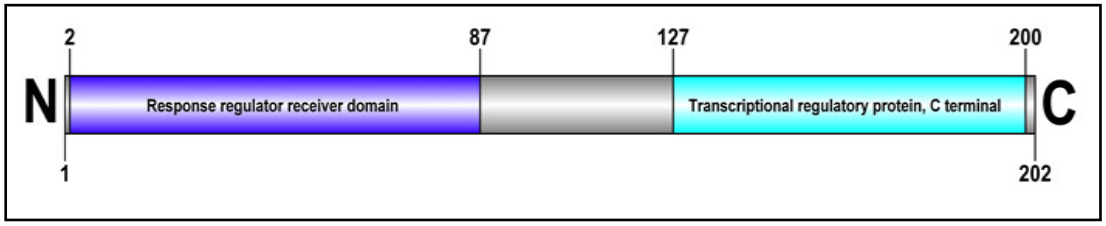


Fig. 3.1 (D) Domain organization of *Lactococcus lactis* AMD17 Nisin gene. Domain structure was prepared using DOG 1.0.

The motif analysis using motif search (http://www.genome.jp/tools/motif/) reveals that Nisin gene contains response regulator receiver domain at N terminal and transcriptional regulatory protein at C terminal (Fig. 3.1D). Previous studies reported that NisR can activate the transcription of *nisABTCIP* and *nisFEG* after phosphoryl group is transferred to an aspartate residue on the regulator protein[31].

3.4.2. Screening of probiotics

3.4.2.1. In vitro gastrointestinal stress and bile tolerance test

The gastrointestinal stress tolerance of *L. lactis* AMD17 differs in simulated gastric fluid and simulated intestinal fluid. It should be taken into account that the *in vitro* conditions used in the experiment mimic the physiological condition of human gastrointestinal tract. The tolerance patterns of *L. lactis* AMD17 have been depicted in Fig. 3.2. The viability was decreased to approximately 4 log unit upon exposure to pH 2.0 and pH 3.0 after 3 h. However, isolate showed better survivability at pH 4.0 and the cell populations remained over 6 log CFU/ml after 3 h (Fig. 3.2A). The sudden reduction in the viability at pH 2.0 at 1 h may be due to stress of acidic environment exposed to the cells. In case of simulated intestinal transit tolerance (Fig. 3.2B) at pH 6.8, the isolate showed higher viability (>7 log CFU/ml) after 4 h whereas at pH 8.0, it showed reduction in cell viability upon prolonged exposure.

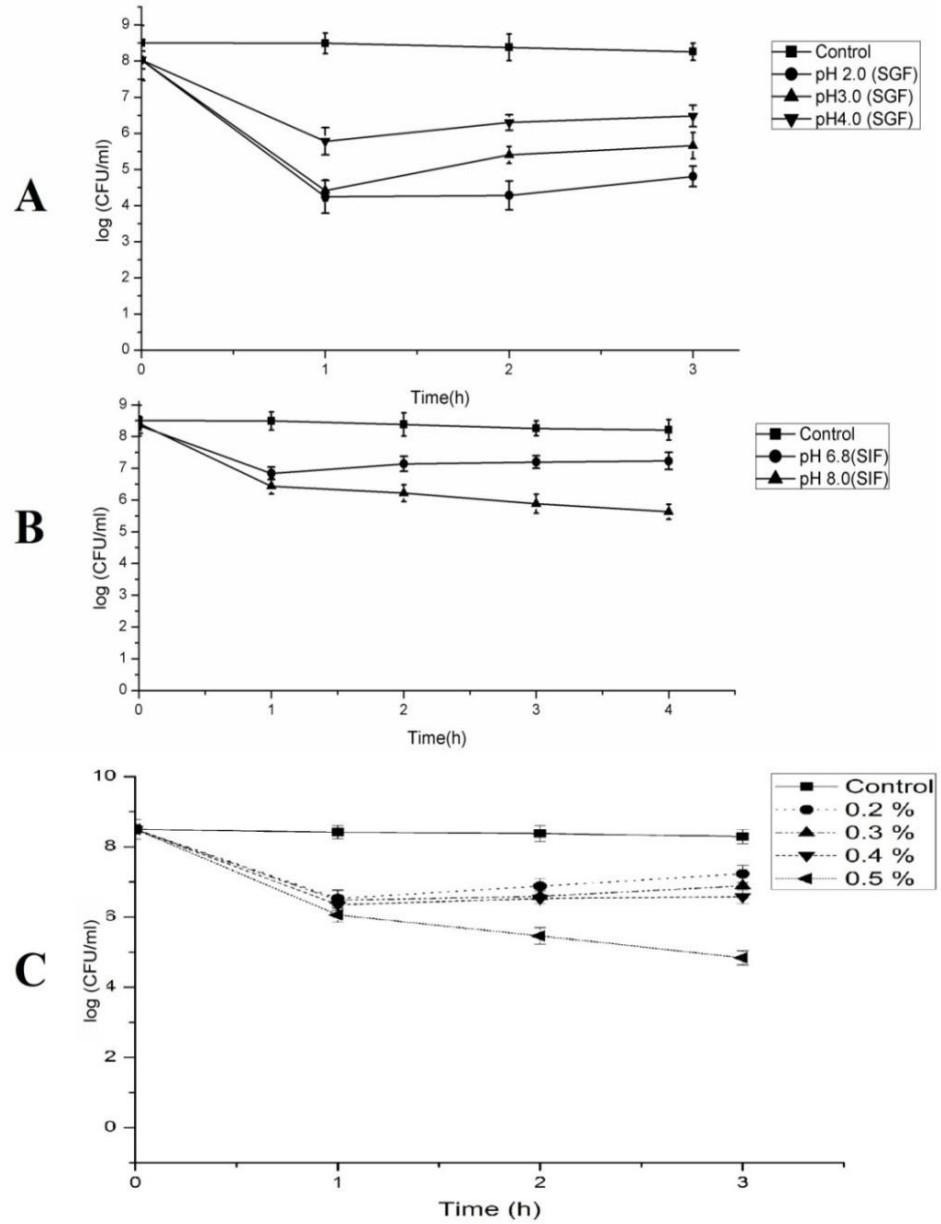


Fig. 3.2 Survival of *Lactococcus lactis* AMD17 in simulated human GI tract conditions: (A) simulated gastric fluid (SGF) pH 2.0, (SGF) pH 3.0 and (SGF) pH 4.0 (B) simulated intestinal fluid (SIF) pH 6.8 and (SIF) pH 8.0; (C) different concentration of bile (Oxgall).

The survivability of *L. lactis* AMD17 were not inhibited to a greater extent and showed considerable resistant to the artificial gastrointestinal stress conditions although their viability decreased from log 8.5 to about log 4.2 CFU/ml. This is in accordance with the findings of Faye et al.[32] who also reported reduction in viability of *L. lactis* at lower pH. *L. lactis* has been widely used in fermented milk products

though it shows relatively lesser viability than *Lactobacillus* spp. to gastrointestinal stress condition[33]. The *L. lactis* AMD17 showed relatively better survivability under different concentration of bile salts treatment as usual bile salt concentration present in human stomach is 0.3% [34].

The survival rates of the isolate up to 0.4% bile did not change significantly after exposure to 3 h and the cell populations remained over 6 log CFU/ml (Fig. 3.2C). However, the isolate showed lesser survivability (4.8 log CFU/ml) at 0.5 % bile salt concentration after exposure to 3 h. Our results were in accordance with the earlier report that showed high bile salts tolerance at 0.3% [35]

3.4.2.2 Antibacterial activity

The *L. lactis* AMD17 showed antagonistic effect against indicators strains tested. The isolate showed highest zone of inhibition against *Listeria monocytogenes* AMDK2 (13 ± 1.0 mm) followed by *Bacillus cereus* MTCC 430 (Table 3.1). Antimicrobial substance is a bacteriocin-like peptides produced by our isolate since cell free culture supernatant after treatment with proteinase K, pepsin and trypsin showed no zone of inhibition, which corroborate the findings of Kelly et al.[36]. These observations are evidence that *L. lactis* AMD17 are nisin producing strain. Thus, *L. lactis* AMD17 can reduce the number of undesired microorganisms in fermented dairy product and enhance safety of a food product for human consumption.

Table 3.1 Antimicrobial activity of *L. lactis* AMD 17 cell free culture supernatent against different indicator strains

Microorganisms	**Zones of inhibition (mm)**
Bacillus cereus MTCC 430	12.6 ± 1.1
Staphylococcus aureus MTCC 3160	12.3 ± 1.5
Listeria monocytogenes AMDK2 (KF894986)	13 ± 1.0
Pseudomonas aeruginosa MTCC 7185	–
Salmonella enterica typhimurium MTCC 1252	–

The measurement expressed in mm is the mean of three replicates ± SD;

– indicates no zone of inhibition.

3.4.2.3 Auto-aggregation and Co-aggregation

Auto-aggregation determines the ability of a bacterial strain to interact with itself in a nonspecific way, which is known as prerequisite for colonization and infection of the gastrointestinal tract by pathogens through adhesive ability[20]. Whereas co-aggregation determines the capacity to form biofilms that protect the host by preventing colonization by pathogens. This realization reinforces the importance of therapeutic manipulation of intestinal microbiota[37]. It was found that the auto-aggregation ability increases significantly ($p<0.05$) with increase in incubation time and observed as 75.5± 3.0 % (Table 3.2) after 24 hours. *L. lactis* AMD17 was able to co-aggregate with the tested enteropathogens and the maximum co-aggregation was observed with *S. enterica typhimurium* (55.84 ± 1.2 %) in 24 hours.

Table 3.2 Autoaggregation and Co-aggregation

Time	Autoaggregation	Co-aggregation (%)	
		***Salmonella enterica typhimurium* MTCC 1252**	***Listeria monocytogenes* AMDK2 (KF894986)**
2 h	10.05 ± 0.5	11.87 ± 1.4	12.34 ± 0.7
4 h	25.60 ± 2.5	19.35 ± 0.9	24.61 ± 1.5
24 h	75.5± 3.0	55.84 ± 1.2	43.94 ± 0.6

The measurement expressed in percentage is the mean of three replicates ± SD

3.4.2.4. Adhesion and Inhibition of pathogen adhesion to Caco-2 cells by *L. lactis* AMD17

Bacterial adhesion to epithelial cells such as Caco-2 cell monolayer has been considered as one of the selection criteria for probiotic strains. The *L. lactis* AMD17 strain was examined for its capability to adhere to Caco-2 cells using *L. rhamnosus* MTCC 1408 as a reference strain (Fig. 3.3). A microscopic study clearly shows the adhesion between Caco-2 cell culture and probiotic strain (Fig 3.3A). The results suggested that *L. lactis* AMD17 (14.20 ± 2.74 %) adhered relatively better than *L. rhamnosus* MTCC 1408 (Fig 3.3B). The high adhesion capacity observed in *L. lactis* AMD17 is in agreement with other reports where strains of dairy origin attached to

Caco-2 cell lines with high efficiency[38]. Probiotics are also known to reduce adherence of pathogens to epithelial cell lines[39].

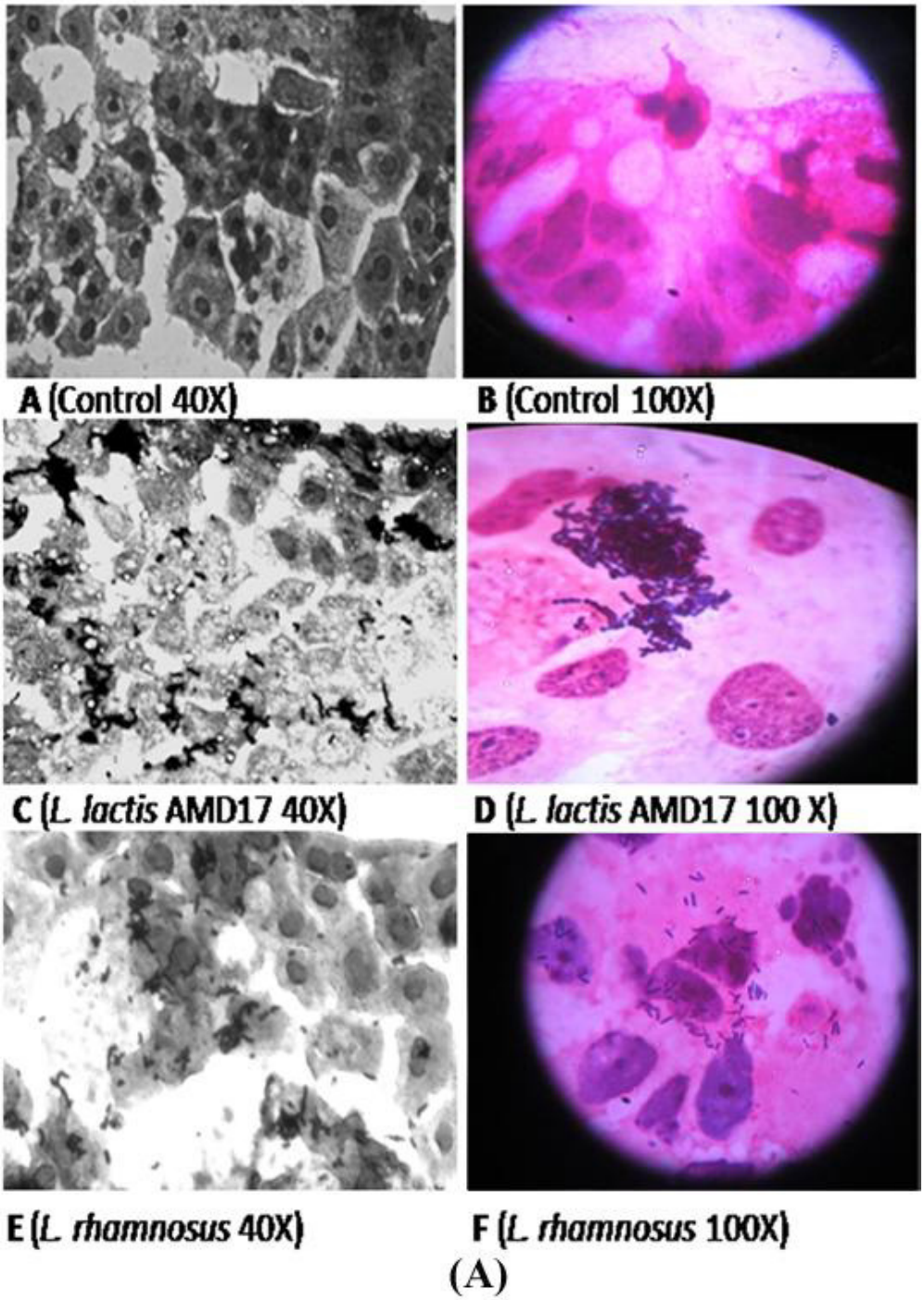


Fig. 3.3 (A) Adhesion to Caco-2 cell line observed under inverted microscope at 40X and by Gram Stain using bright field microscopy at 100x.

Our study suggested that *L. lactis* AMD17 significantly ($P < 0.05$) reduced the adherence of *Listeria monocytogenes* AMDK2 (KF894986, laboratory isolate) and *S. enterica typhimurium* (MTCC 1252) to Caco-2 cells by 47.46 ± 0.17 % and 17.21 ± 1.19 % respectively (Fig.3.3C). *L. rhamnosus* MTCC 1408 also showed inhibition of *Listeria monocytogenes* AMDK2 and *S. enterica typhimurium* (MTCC 1252) adherence by 26.5 ± 0.24 % and 14.25 ± 0.89 % respectively. The presence of *L. lactis* AMD17 may impede the colonization of pathogen to cell surface by competitive exclusion or production of antimicrobial agent[40]. This may explain the reduction of adhesion ability of the pathogens to Caco-2 cells. Thus, Nisin producing *L. lactis* AMD17 could be helpful to reduce colonization of human gut by foodborne pathogens.

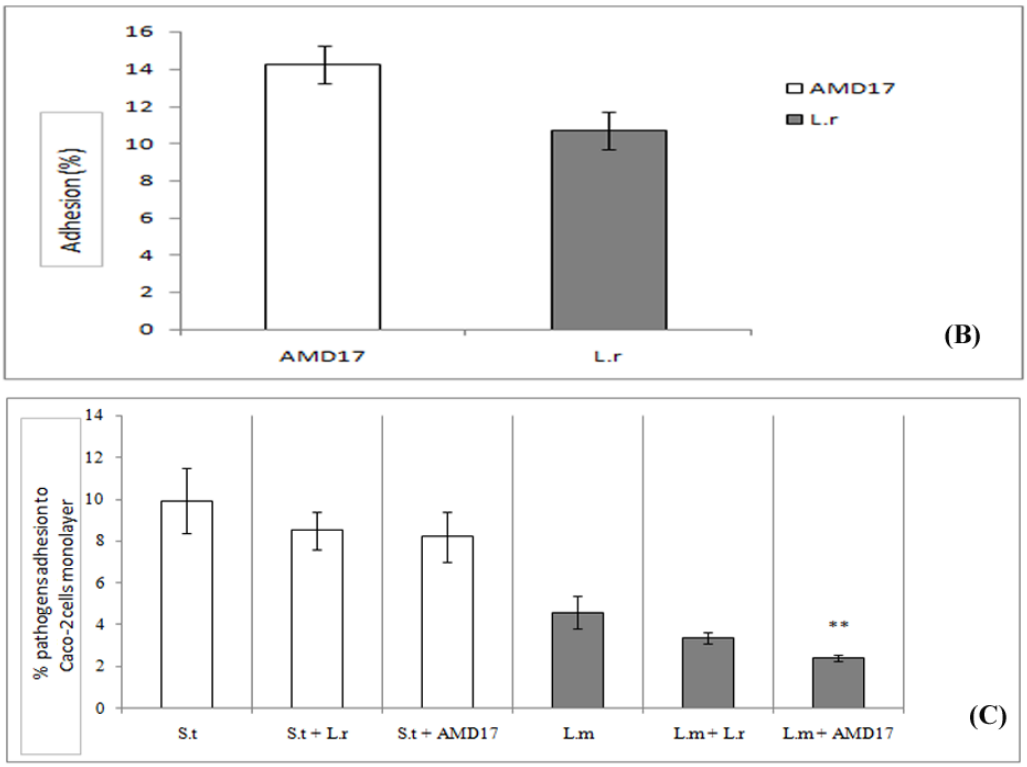


Fig.3.3. **(B)** Adherence (%) of *L. lactis* AMD17 and *L. rhamnosus* MTCC 1408 (L.r) to Caco-2 monolayer. **(C)** Percentage adhesion of *Listeria monocytogenes* AMDK2 (L.m) and *S. enterica typhimurium* (S.t) to Caco-2 monolayer in the absence or presence of probiotic isolate.

3.4.3. Determination of antioxidant activity, total phenolic and flavonoid content of honey

Honey can prevent deteriorative oxidation reactions in foods, such as lipid oxidation in meat and enzymatic browning of fruits and vegetables. Honey has therefore great potential to serve as a natural food antioxidant[41]. Antioxidant potential of honey is due to the presence of different types of phenolic compounds, flavonoids and vitamins[42, 43].

The antioxidant activity, total phenolic content and flavonoid content of three different types of honey was evaluated and it was found that the honey obtained from the place Goroimari, Tezpur, Assam was the superior type of honey in terms of antioxidant activity (50.86 %), total phenolic content (92.2 mg GAE/100g honey) and flavonoid content (9.6 mg Quercetin/100 g honey) that differs significantly ($p<0.05$) from the other two types of honey (Table 3.3). The antioxidant activities of different honey samples were previously described and reported that it differs from location to location even if the floral source might be the same[44]. The honey for Goroimari was used for further studies.

Table 3.3 Total phenolic content, flavonoid content and antioxidant activity of honey samples

Location	Total phenolic content (mg GAE/100g honey)	Flavonoids (mg Quercetin/100 g honey)	RSA (%)
Goroimari	92.2 ± 1.87^{a}	9.6 ± 2.66^{c}	50.86 ± 5.22^{e}
Borghat	78.4 ± 3.4^{b}	4.3 ± 2.15^{d}	34.57 ± 3.56^{f}
Napaam	84.3 ± 4.8^{b}	5.8 ± 1.8^{dc}	42.23 ± 6.04^{fe}

Data are expressed as mean ± Standard deviation. Significant differences are determined by Tukey's multiple comparison test at $p<0.05$. Values on the same column with different letters are significantly different.

3.4.4. Survivability of *L. lactis* AMD17 in honey-enriched dahi

Fig. 3.4 illustrates the changes in the viable colony count of *L. lactis* AMD17 in storage conditions with or without the supplementation of honey. It was observed that in case of control the viability of the bacteria decreases significantly with increase in the storage time. Conversely the addition of honey retains its viability since the

viability did not change significantly as compared to the initial viability. It is essential to maintain the sufficient viability and survival of cells throughout the projected shelf life of a product for imparting proper health benefit[45]. The maximum viability was observed with 3% (w/v) honey followed by 4% and 5% (w/v) honey. It was suggested that probiotic products should contain lactic acid bacteria count of at least 10^7 CFU/ml[46]. Our findings are in agreement with this suggestion. Similar result was reported in probiotic goat milk yogurt produced by Wang et al[47]. During first week, the increase in cell count was observed in control whereas dahi enriched with honey showed no significant growth in cell viablity. After two weeks, the decrease in cell viability was obsserved in control experiment whereas, dahi fortified with honey showed lesser reduction in cell count. Antioxdant activity exhibited by honey may enhance the shelf life of probiotic *L. lactis* AMD17[48].

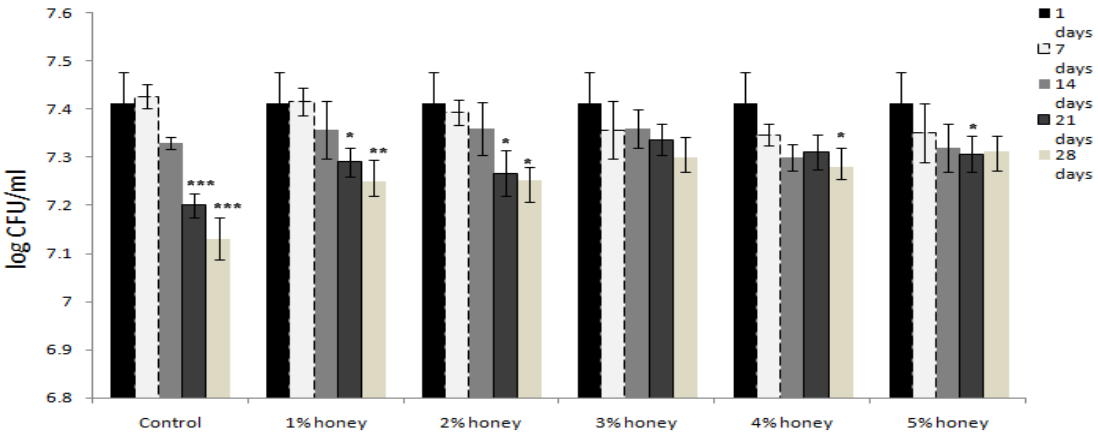


Fig. 3.4. Survivability of *L. lactis* AMD17 in honey-enriched dahi under low temperature. Viable counts (log CFU/ml) of each treatment were estimated after 1, 7, 14, 21 and 28 days. ***p<0.001, ** p<0 .01, and *p< 0.05 indicates significant differences using Tukey's Multiple comparison test. n = 3.

3.4.5. Sensory evaluation

Increase in the amount of added honey (1%-5%) contributed to the increase in sweetness of all samples. In addition, honey has the ability to decrease the sourness of solutions and hence can serve to increase consumer acceptability[49]. The fermentation required for buffalo yoghurt takes longer respect to bovine yoghurt[50]. Honey, a prebiotic source may recompense extended fermentation time and the taste of the

dahi. The tastes of dahi were found to increase significantly with 3-5% honey as compared to control. Lisak et al[51] had also reported better taste score for yoghurt with added sweetener at highest concentration (5%). Sensory scores for color and texture were found to be different for honey incorporated samples as compared to control. Scores for overall acceptability of dahi ranged between 5.75 and 7.66 (Table 3.4). The overall scores showed that the best evaluated samples were those with added honey (3-5%).

Table 3.4. Sensory evaluation scores of curd formulations with different concentration of honey (1%- 5%)

Attributes	Control	1% Honey	2% Honey	3% Honey	4% Honey	5%Honey
Taste	3.50 ± 0.925^{b}	4.37 ± 1.026^{b}	4.75 ± 1.133^{b}	7.30 ± 1.33^{a}	6.80 ± 1.131^{a}	7.30 ± 0.744^{a}
Colour	4.75 ± 0.755^{bc}	6.50 ± 1.414^{ac}	6.93 ± 0.776^{a}	7.31 ± 0.593^{a}	7.25 ± 0.707^{a}	7.37 ± 1.060^{a}
Texture	3.25 ± 1.035^{b}	6.12 ± 1.827^{a}	7.0 ± 1.603^{a}	7.56 ± 0.979^{a}	6.81 ± 1.307^{a}	7.25 ± 1.069^{a}
Overall acceptability	5.75 ± 1.195^{bc}	6.51 ± 1.626^{ac}	6.97 ± 0.928^{ac}	7.66 ± 0.843^{a}	7.37 ± 0.942^{a}	7.43 ± 0.821^{a}

Results are expressed as mean±S.D (n=9). a-c Different superscript letters in a row denote significant differences between trials ($p < 0.05$)

3.4.6. Texture Profile

The texture profile of dahi during refrigerated storage is shown in Fig. 3.5. Comparing days 1 to 28 for all dahi formulations during storage, the addition of increased concentration of honey (1 % - 5 %) resulted in firmer and gummy products ($P \leq 0.05$) and had no effect on the cohesiveness and springiness of the product as compared to first day formulation. Interaction between the ingredients present in dahi formulation continued to occur during refrigerated storage, which could explain the gradual increase in hardness for these products throughout the storage period. Earlier reports suggested that exopolysaccharides (EPS) produced by probiotic cultures could increase the viscosity, water retention and interaction with other ingredients of milk lead to firmness of the casein matrix in the final product[52]. The augmented firmness is interrelated to an improvement of the texture since firm dahi is less susceptible to rearrangements within its network and hence less susceptible to shrinkage and serum expulsion[53, 54]. Besides the storage period, the presence of honey might also have contributed to the significant changes in the texture profile of the products.

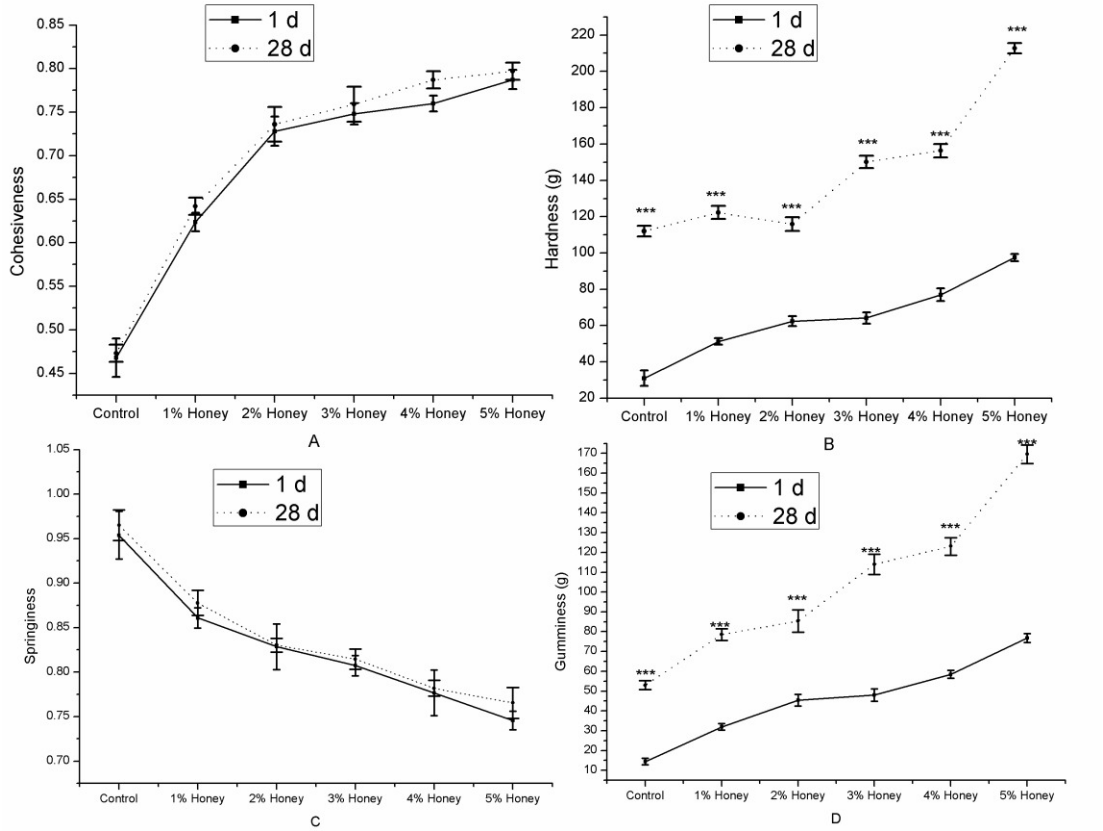


Fig. 3.5. Texture profile analysis of dahi enriched with different concentration of honey (1-5 %) after 1, 7, 14, 21 and 28 days .

With the increased levels of firmness during storage, gumminess (multiplication of firmness and cohesiveness) also increased. The cohesiveness and springiness during storage did not differ significantly to which gel could be deformed while eating the product.

3.5. Conclusions

Since there is no standard culture for dahi, this nisin producing isolate *L. lactis* AMD17 could be considered as promising starter culture candidate for dahi making. Our isolate producing nisin in situ and fermenting milk to dahi will certainly be of great advantage to control the growth of undesirable bacteria in the dahi. The data showed that the reported bacterial strain and honey work synergistically to improve storage time and sensory qualities. In addition honey significantly (3 - 5 %) reduces the decrease in cell viability.

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