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ORIGINAL ARTICLE

Assessment of goat milk-derived potential probiotic *L. lactis* AMD17 and its application for preparation of dahi using honey

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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 pathogen adhesion to intestinal epithelium cell line Caco-2. The isolate significantly reduced the adherence of foodborne 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 that dahi prepared with *L. lactis* AMD17 and the addition of 3 % honey exhibited the highest score in taste and color. The texture characteristics were found to be 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 benefits and combats foodborne pathogens, possibly due to the antibacterial features of nisin peptide.

Keywords Goat milk · Probiotic · Sensory evaluation · Caco-2 · Nisin · Dahi

Introduction

Fermented foods play a significant role in diets since they contain enormous quantities of nutritious constituents with a wide diversity of aromas, flavors and textures (Shah and Prajapati 2014). Dahi is a popular fermented dairy product of South Asia. 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 (Singh 2007). For dahi production, a small portion of previously fermented product containing live culture is added to lukewarm milk as a starter culture. However, production of dahi with an individual culture of *Lactococcus lactis* (Yadav et al. 2006) or a combination of cultures containing lactobacilli and lactococci (Yadav et al. 2007) have been reported. Strains belonging to *Lactococcus* species have been well documented in the dairy industry for contributing typical taste and flavor to a variety of fermented dairy products (Whetstine et al. 2006). Unlike yoghurt 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* (Tong et al. 2014). Nisin was permitted as a safe food additive in over 50 countries around

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the world by their regulatory agencies, and is generally regarded as safe (Mitra et al. 2010). 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 (Xanthopoulos et al. 2012). Buffalo milk has several benefits and advantages in the manufacture of milk products, since it does not required fortification with milk powder or the addition of thickeners or stabilizers (Addeo et al. 2007). Different dairy products, such as soft and hard cheeses, butter oil (ghee), butter, ice cream and yoghurt, have long been prepared in Asian countries such as India and Pakistan using buffalo milk (both produce about 80 % of the world's buffalo milk) (Ahmad et al. 2008).

The major problem associated with dahi is rapid deterioration of its quality, lesser stability at low temperature and reduction of shelf life due to frequent microbial contamination. The incorporation of honey adds nutritional value and enhances the 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 on investigating the potential probiotic features of *L. lactis* AMD17 and on evaluating the stability and consumer acceptability of dahi produced using *L. lactis* AMD17 fortified with 1.0 % to 5.0 % (w/v) honey.

Materials and methods

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 up to 10^{-8} using Ringer solution. 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 the counting of colonies. Out of 114 LAB strains isolated from goat milk, *L. lactis* AMD17 was selected for further studies based on its potential in vitro probiotic properties, such as resistance to gastrointestinal stress, antimicrobial activity and inhibition of pathogen adhesion to intestinal epithelium cell line.

Strain identification and characterization of bacteriocin gene

Selected isolates were identified by 16S rRNA gene sequence analysis followed by phylogenetic studies. Universal primers 27 F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) were used for the amplification of 16S rRNA gene sequences. The PCR amplifications were performed in an Eppendorf thermocycler according to the method described by Manhar et al. 2015. The nisin gene was amplified using previously reported specific primers (nisRF 5′- CTATGAAGTTGCGACGCATCA- 3′ and nisRR 5′- CATGCCACTGATACCCAAGT- 3) by De la Fuente-Salcido et al. 2015. 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 s at 58 °C, elongation for 1 min at 72 °C, and final 10 min extension at 72 °C. PCR products were separated by electrophoresis in 1 % (*w/v*) agarose gel. The PCR products were purified and used for automated DNA sequencing using a 3130 Genetic Analyzer (Applied Biosystem, Switzerland). The sequences obtained were submitted to NCBI (National Center for Biotechnology information) Genbank. The deduced amino acid sequence obtained from the nucleotide sequence was subjected to homology searching in the NCBI database using the BLASTp program (http://www.ncbi.nlm.nih.gov). The phylogenetic tree was generated by the neighbor joining (NJ) method using MEGA 5.05 software (Kimura 1980; Felsenstein 1985; Tamura et al. 2011). 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 (Yang et al. 2015). Domain structure was prepared using DOG 1.0. (Ren et al. 2009).

Screening of probiotics properties

In vitro gastrointestinal stress tolerance test

The resistance of isolates to low pH conditions that mimic the human gastrointestinal (GI) tract environment was tested according to the method described by Maragkoudakis et al. (2006), with some modifications. Briefly, bacterial cells from an 18 h culture were harvested by centrifugation at 3000 x g for 5 min at 4 °C, washed once with phosphate buffered saline (PBS) (pH 7.4), and then resuspended (10^8 CFU mL^{-1}) in different PBS solutions with various pHs. In order to test simulated gastrointestinal transit tolerance, bacterial cells were resuspended in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). SGF was prepared by supplementing sterilized PBS pH 2, 3 and 4 (adjusted with 1 N 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 1 N NaOH/1 N 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.

Bile salt tolerance

The resistance to bile salts tested according to the slightly modified method described by Kaewnopparat et al. (2013). Briefly, bacterial cells were resuspended 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 °C. The resistance of *L. lactis* AMD17 in every condition assessed in terms of viable colony count on MRS agar plates after the treatment.

Antibacterial activity

The antimicrobial effects of the bacterial isolates were examined by an 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 7185, and *Salmonella enterica typhimurium* MTCC 1252) or Trypticase Soy Agar (for *Listeria monocytogenes* KF894986, local 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 the agar plates were loaded with 100 μl of cell-free supernatant . 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 (Ammor et al. 2006).

Cell aggregation

The aggregation assay was measured according to Del Re et al. 2000 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 intervals was mixed with 0.9 mL PBS and its absorbance at 600 nm was measured. Auto-aggregation 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.

Coaggregation assay

Equal volumes of cells 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 coaggregation (%) was calculated using the equation (Handley et al. 1987), $100 \times [(A_{pat} + A_{Lacto})/2) - (A_{mix})]/[(A_{pat} + A_{Lacto})/2]$, where A_{pat} and A_{Lacto} represent the A_{600} of control tubes and A_{mix} represents the A_{600} of the mixture of *L. lactis* AMD17 and pathogen strains at predetermined time intervals.

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 inhibition of foodborne pathogenic bacterial adhesion. The cell lines were routinely grown and maintained by following standard procedures (Bustos et al. 2012). 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 2 days, and Caco-2 monolayers at late post-confluence, i.e. after 14 d (80 % confluence), were used for the study. Media and reagents were purchased from Sigma Aldrich (USA) and Gibco® (Life Technologies).

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. 2014. 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 (Moorthy et al. 2010; Agaliya and Jeevaratnam 2012) 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 the adhesion assay, Caco-2 cell monolayers were inoculated with isolated strain (at a ratio of bacteria:eukaryotic cells of 10:1), and for the inhibition of pathogen adhesion, mixed inoculum containing isolates of each of the MTCC food spoilage indicator strains in MEM (ratio 5:1) were incubated for 1 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, the inhibition of pathogens adhesion to Caco-2 cell 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 to inhibit 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 pathogens 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 isolates were observed under bright field microscopy (100 x) after fixing with 3 % paraformaldehyde and gram staining.

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, pasteurized at 70 °C for 15 min, and cooled to room temperature. The anti-oxidant activities of the honey samples were measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay (Brand-Williams et al. 1995). The anti-oxidant activity of each sample was calculated as the percentage of RSA (radical scavenging activity) using the formula: $RSA = [(A_B\text{-}A_A)/A_B] \times 100$, 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 (Singleton and Rossi 1965). 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 mixed with 2400 μl water and 200 μl non-diluted Folin–Ciocalteu reagent and 600 μl sodium carbonate (20 % Na_2CO_3) was then added. After incubation at room temperature for 2 h, the absorbance of the 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-Grand et al. 1994. Briefly, honey samples were 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 (Meda et al. 2005). 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).

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 Napam, Tezpur and heated for 15 min at 90 °C (Vijayendra and Gupta 2012) with intermittent stirring 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 to maintain its potential activity. It was inoculated at 7.4 log CFU/mL and incubated at 37 °C until dahi formation (14 h). By this time, the milk curdled and became semi-solid and the preparations were 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 pots 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.

Sensory evaluation

Sensory evaluations were privately conducted after 1 day. 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 facial 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 yoghurt was offered and then the remaining five samples were served in a random order.

Texture profile of probiotic dahi during one month storage

Texture evaluation of the extrudates was performed weekly with a 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.

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 were determined by Tukey's Multiple comparison test at $p < 0.05$.

Results and discussion

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 *L. lactis* strain AMD17 (KF113841). The amplified Nisin gene was 610 bp (Fig. 1a). The deduced amino acids of the Nisin gene showed (Fig. 1b) strong similarities (100 % identity) to the nisin biosynthesis regulatory protein NisR (Q07597.1), followed by the 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. 1c). The motif analysis using motif search (http://www.genome.jp/tools/motif/) reveals that the Nisin gene contains a response regulator receiver domain at the N terminal and transcriptional regulatory protein at the C terminal (Fig. 1d). Previous studies reported that NisR can activate the transcription of *nisABTCIP* and *nisFEG* after a phosphoryl group is transferred to an aspartate residue on the regulator protein (Li and O'Sullivan 2002).

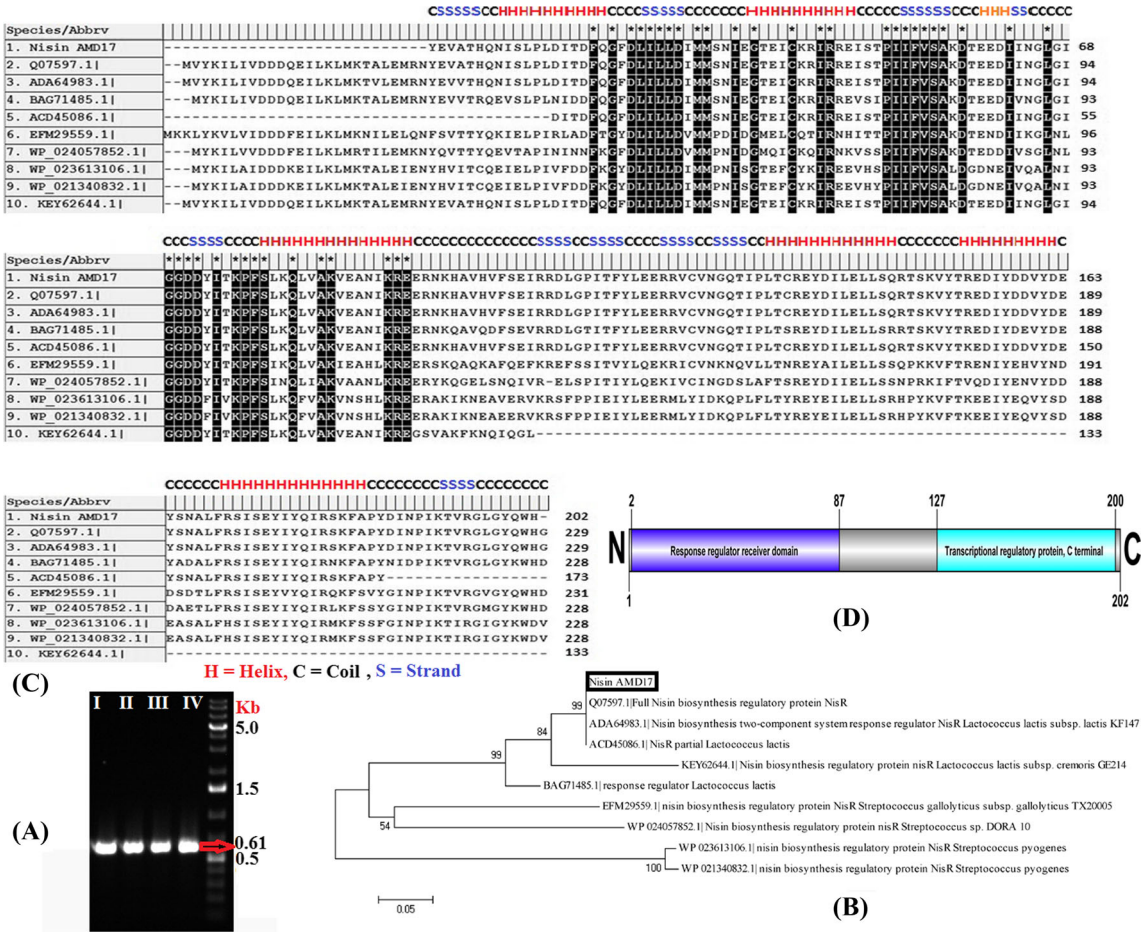

Fig. 1 Sequence alignment, domain organization and phylogeny of *Lactococcus lactis* AMD17 Nisin gene. (**a**) Multiple sequence alignment of *Lactococcus lactis* AMD17 Nisin gene with non-redundant protein sequence obtained from databases at the NCBI using the BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Conserved amino acids are indicated in black (100 %). Secondary structure were predicted at the top of the sequence using I-TASSER Suite. (**b**) Domain structure was prepared using DOG 1.0. (**c**) Agarose gel electrophoresis (1 %) of Nisin gene. Lanes I–IV, showing amplicon PCR products (~6 10 bp). (d) 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

Screening of probiotics

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 the human gastrointestinal tract. The tolerance patterns of *L. lactis* AMD17 are depicted in Fig. 2. The viability was decreased to approximately 4 log units upon exposure to pH 2.0 and pH 3.0 after 3 h. However, isolates showed better survivability at pH 4.0 and the cell populations remained over 6 log CFU/mL after 3 h (Fig. 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. 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. The survivability of *L. lactis* AMD17 was not inhibited to a greater extent and showed considerable resistant to the artificial gastrointestinal stress conditions, although its viability decreased from log 8.5 to about log 4.2 CFU/ml. This is in accordance with the findings of Faye et al. (2012), who also reported a reduction in the viability of *L. lactis* at lower pH . *L. lactis* has been widely used in fermented milk products, though it shows relatively less viability than *Lactobacillus* spp. to gastrointestinal stress condition (Lee et al. 2015).

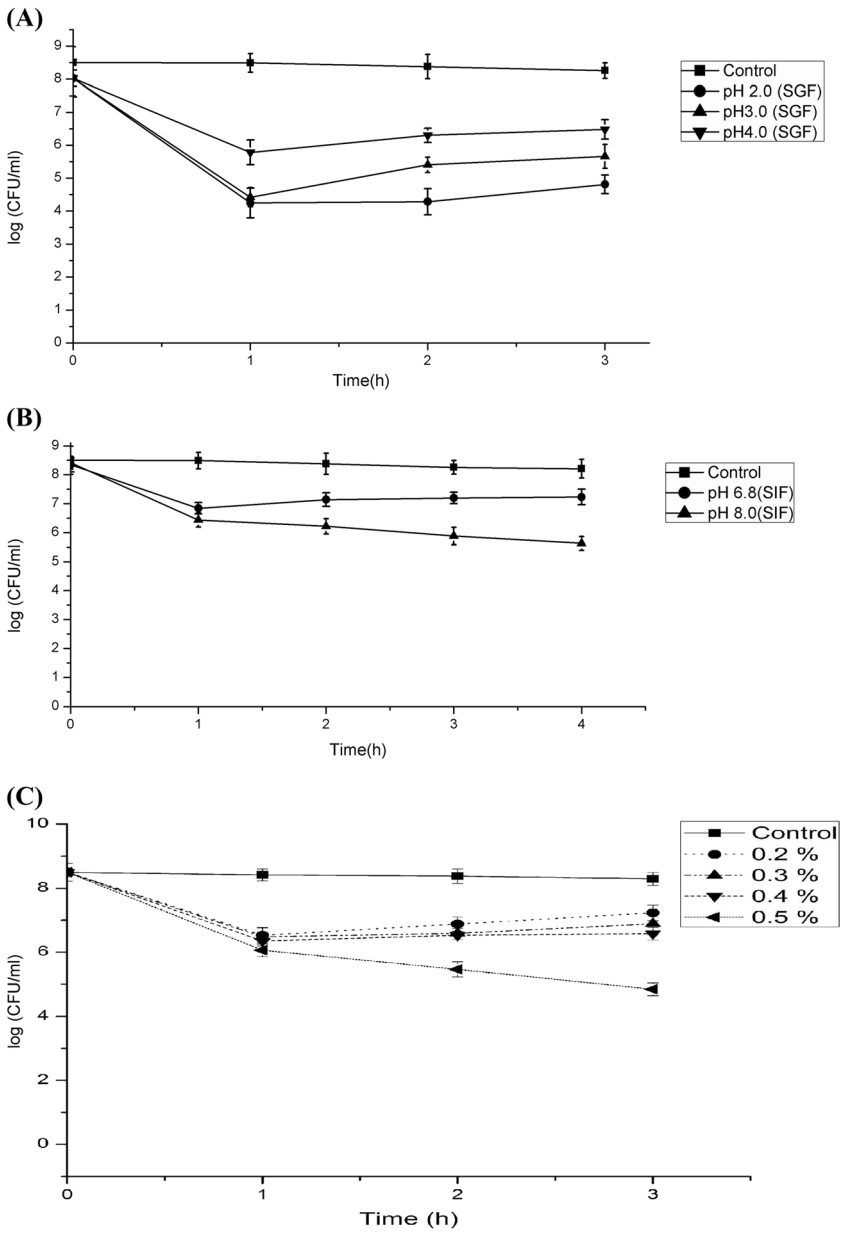

Fig. 2 Survival of *Lactococcus lactis* AMD17 in simulated human GI tract conditions: (**a**) simulated gastric fluid (SGF) pH 2.0, (SGF) pH 3.0 and (SGF) pH 4.0; (**b**) simulated intestinal fluid (SIF) pH 6.8 and (SIF) pH 8.0; (**c**) different concentration of bile (Oxgall)

The *L. lactis* AMD17 showed relatively better survivability under different concentrations of bile salts treatment; the usual bile salt concentration present in the human stomach is 0.3 % (Kaushik et al. 2009). The survival rates of the isolate at up to 0.4 % bile did not change significantly after exposure for 3 h and the cell populations remained over 6 log CFU/mL (Fig. 2c). However, the isolate showed lesser survivability (4.8 log CFU/mL) at 0.5 % bile salt concentration after exposure for 3 h. Our results were in accordance with the earlier report that showed high bile salts tolerance at 0.3 % (Floros et al. 2012).

Antibacterial activity

L. lactis AMD17 showed antagonistic effect against indicator strains tested. The isolate showed the highest zone of inhibition against *Listeria monocytogenes* AMDK2 (13 ± 1.0 m,m) followed by *Bacillus cereus* MTCC 430 (Table 1). The antimicrobial substance is a bacteriocin-like peptide produced by our isolate, since cell-free culture supernatant after treatment with proteinase K, pepsin and trypsin showed no zone of inhibition, which corroborates the findings of Kelly et al. 1996. These observations are evidence that *L. lactis* AMD17 is a nisin-producing strain. Thus, *L. lactis* AMD17 can reduce the number of undesired microorganisms in fermented dairy product and enhance the safety of a food product for human consumption.

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 a prerequisite for colonization and infection of the gastrointestinal tract by pathogens through adhesive ability (Del Re et al. 2000); 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 (Dunne et al. 2001). It was found that auto-aggregation ability increases significantly ($p < 0.05$) with increase in incubation ti,me and was observed as 75.5 ± 3.0 % (Table 2) after 24 h. *L. lactis* AMD17 was able to co-aggregate with the tested enteropathogens, and maximum co-aggregation was observed with *S. enterica typhimurium* (55.84 ± 1.2 %) in 24 h.

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). The results suggested that *L. lactis* AMD17 (14.20 ± 2.74 %) adhered relatively better than *L. rhamnosus* MTCC 1408 (10.08 ± 0.51 %). 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 (Monteagudo-Mera et al. 2012). Microscopic observation of adhesion between Caco-2 cell culture and probiotic strain was performed to support the data obtained from quantitative plate assay. Probiotics are also known to reduce adherence of pathogens to epithelial cell lines (Collado et al. 2007). Our study suggested that *L. lactis* AMD17 significantly ($p < 0.05$) reduced the adherence of *Listeria monocytogenes* AMDK2 (KF894986, local isolate) to Caco-2 cells by 47.46 ± 0.17, % whereas *S. enterica typhimurium* (MTCC 1252) showed relatively lower adherence (17.21 ± 1.19) %. *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 (Todoriki et al. 2001). This may explain the reduction of adhesion

Table 1 Antimicrobial activity of *L. lactis* AMD 17 cell-free culture supernatant against different indicator strains

Microorganisms	Zones of inhibition (mm)
Bacillus cereus MTCC 430	12.66 ± 1.1
Staphylococcus aureus MTCC 3160	12.33 ± 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

Table 2 Auto-aggregation and co-aggregation

	Auto-aggregation	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

ability of pathogens to Caco-2 cells. Thus, nisin-producing *L. lactis* AMD17 could be helpful to reduce colonization of the human gut by foodborne pathogens.

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 (Gheldof et al. 2002). Antioxidant potential of honey is due to the presence of different types of phenolic compounds, flavonoids and vitamins. (Ferreres et al. 1994; Andrade et al. 1997).

The antioxidant activity, total phenolic content and flavonoid content of three different types of honey were evaluated, and it was found that the honey obtained from the place of Goroimari, Tezpur, Assam was the superior type of honey in terms of antioxidant activity (50.86 %), total phenolic content (92.2 mg GAE/100 g honey) and flavonoid content (9.6 mg Quercetin/100 g honey), differing significantly ($p < 0.05$) from the other two types of honey (Table 3). The antioxidant activities of different honey samples were previously described, and it was reported that the activity differs from location to location even if the floral source might be the same (Al-Mamary et al. 2002). The honey from Goroimari was used for further studies.

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

Figure 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 the case of the control, the viability of the bacteria decreases

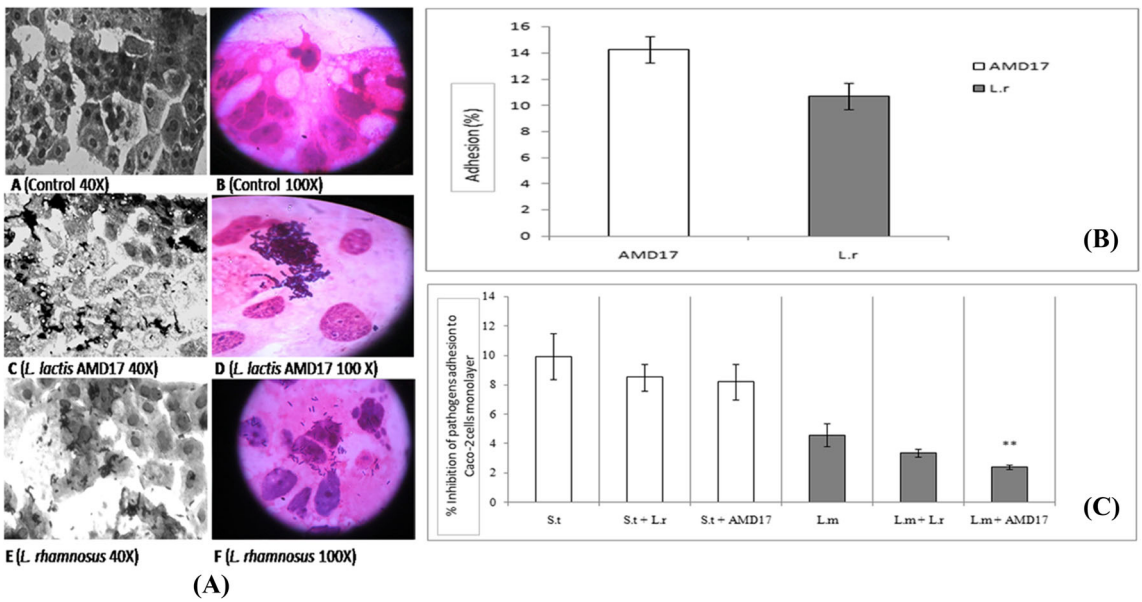

Fig. 3 (**a**) Adhesion to Caco-2 cell line observed under inverted microscope at 40 x and by Gram Stain using bright field microscopy at 100 x. (**b**) Adherence (%) of *L. lactis* AMD17 and *L. rhamnosus* MTCC 1408 (L.r) to Caco-2 monolayer. (**c**) Percentage inhibition of *Listeria monocytogenes* AMDK2 (L.m) and *S. enterica typhimurium* (S.t) adhesion to Caco-2 monolayer by *L. lactis* AMD17 and *L. rhamnosus* MTCC 1408 (L.r)

Table 3 Total phenolic content, flavonoid content and antioxidant activity of honey samples

Location	Total phenolic content (mg GAE/100 g 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}
Napam	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

significantly with increase in storage time. Conversely, with the addition of honey, it retains its viability, since the viability did not change significantly as compared to the initial viability. It is essential to maintain sufficient viability and survival of cells throughout the projected shelf life of a product to impart proper health benefits (Shah 2000). 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 a lactic acid bacteria count of at least 10^7 CFU/mL (Ishibashi and Shimamura 1993). Our findings are in agreement with this suggestion. A similar result was reported in probiotic goat milk yoghurt produced by Wang et al. 2012. During the first week, an increase in cell count was observed in the control, whereas dahi enriched with honey showed no significant growth in cell viability. After 2 weeks, the decrease in cell viability was observed in the 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* (Duda-Chodak et al. 2008)

Sensory evaluation

An 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 (Varga 2006). The fermentation required for buffalo yoghurt takes longer with respect to that of bovine yoghurt (Nguyen et al. 2014). 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. (2012) 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 4). The overall scores showed that the best evaluated samples were those with added honey (3–5 %).

Texture profile

The texture profile of dahi during refrigerated storage is shown in Fig. 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

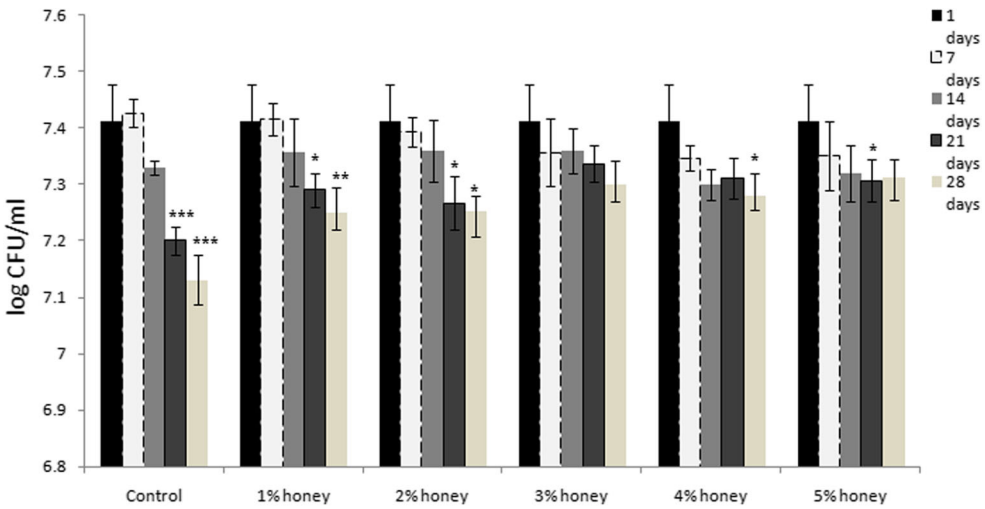

Fig. 4 Survivability of *L. lactis* AMD17 in honey-enriched dahi under low temperature. Viable counts (log CFU/ml) of each treatment were estimated after 1, 7, 14, 21 and 28 days. ***$p < 0.001$, ** $p < 0.01$, and *$p < 0.05$ indicates significant differences using Tukey's Multiple comparison test. $n = 3$

Table 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	3.5 ± 0.925^{b}	4.37 ± 1.026^{b}	4.75 ± 1.133^{b}	7.3 ± 1.33^{a}	6.8 ± 1.131^{a}	7.3 ± 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}	6.125 ± 1.827^{a}	7.0 ± 1.603^{a}	7.5625 ± 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}	7.375 ± 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 ($p < 0.05$)

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 (Duboc and Mollet 2001). 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 (Brennan and Tudorica 2008; Oliveira et al. 2011). Besides the storage period, the presence of honey might also have contributed to the significant changes in the texture profile of the products. 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 as to which gel could be deformed while eating the product.

Conclusions

Since there is no standard culture for dahi, the nisin-producing isolate *L. lactis* AMD17 could be considered as promising starter culture candidate for dahi making. Our isolate for 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 show 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.

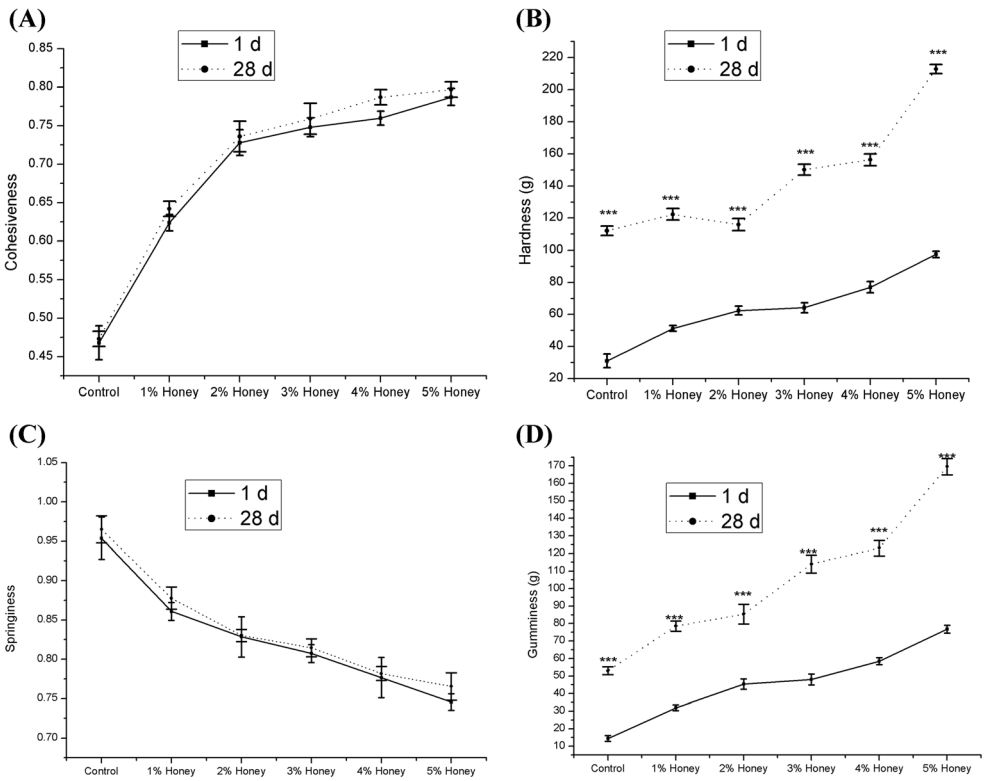

Fig. 5 Texture profile analysis of dahi enriched with different concentration of honey (1–5 %) after 1, 7, 14, 21 and 28 days

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