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Potentialities of newly isolated *Bacillus subtilis* and *Lactobacillus sp* for curd preparation and a comparative study of its physico-chemical parameters with other marketed curds

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Two *Bacillus sp.* were isolated from the local fermented milk and identified on the basis 16S rRNA sequence profile as *Bacillus subtilis* AKL1 and by biochemical process as *Lactobacillus acidophilus* AKL2. These isolates were used as fresh inoculums for curd preparation individually and in combinations. Different physico-chemical and therapeutic properties of the newly prepared curd were examined and compared with marketed local (sweet and sour) and branded (Mother Dairy and Thackar) curds. The total hydrolyzed peptides, free amino acids, lactic acid were significantly higher, whereas, total solid, ash content, syneresis and free reducing sugar were lower in the curd prepared by a mixture of AKL1 and AKL2 (0.5:0.5, v/v). The antioxidant activity against $ABTS^{+}$, $DPPH^{\bullet}$, $OH^{\bullet}$ and Fe^{3+} were also higher in the newly formulated curd. Polyphenols (85.5µg/g), flavonoids (12.5µg/g) and free aromatic amino acids contents were also higher in AKL1+AKL2. All these components prevent excess protein oxidation that was revealed by SDS-PAGE. The curd also exhibited potent antimicrobial activity against some entero-pathogens like *Clostridium perfringens, Escherichia coli, Shigella dysentery, Vibrio cholerae* and *Staphylococcus aureus*. It can be concluded that the combination of these *Lactobacillus sp*. will be a fruitful inoculum for the preparation of curd having better health promoting effects.

Keywords: Antimicrobial, Antioxidant, Curd, Flavonoid, Lactic acid bacteria, Polyphenol

The art of lactic acid fermentation is one of the oldest technologies that preserve the milk in a wide diversity of flavours, aromas and texture[1]. At present, more than 400 traditional and industrialized fermented dairy products are available in the global market. Among them curd is very popular in part of globe including Indian subcontinent. About 9% of the total milk is converted into fermented dairy product in India[2]. Broadly two types of curds are available in the market namely unsweetened and sweetened. Curd is prepared by fermenting milk from cows, buffalos or goats with mesophilic lactic acid bacterial cultures and it is very easy to digest and enriched with vitamins, amino acids, pre-digested protein and bioactive peptides, etc[3]. With the changing lifestyle and dietary patterns, non-communicable diseases like obesity, diabetes, cardiovascular diseases and cancer have become major health problems worldwide. Antioxidant research has become a major scientific pursuit because of the evidence linking oxidative stress with many diseases and because of potential food preservative applications. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are activated forms of oxygen and nitrogen[4] and other non-radicals, arise from various endogenous and exogenous pathways. ROS and RNS have been implicated in the aetiology of numerous pathological conditions[5]. Oxidative stress results when the oxidant/antioxidant ratio tilts in favour of oxidant factors; it is involved in the aging process and also causes inflammation. Free radicals attack cellular components leading to the oxidation of lipids, proteins and DNA, thus causing structural and functional changes of these molecules[6]. Oxidation of food constituents is also a key event in food spoilage. The higher nutrition value of the curd and its consumption are health beneficial due to decrease in lactose intolerance, immuno-modulation, diarrhoea prevention, serum cholesterol reduction and adverse effect of drugs etc[7,8]. Besides several lactobacillus species enhance the gastrointestinal health acting as antiallergenic, antimicrobial, antihypertensive,

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antitumourigenic and antioxidant. It also helps to maintain the endogenous redox balance to prevent several patho-phsiological conditions[8].

However, the preparation of quality curd depends on the milk, fermentation temperature, potentiality of the starter culture, fermentation vessels and storage conditions. In the present study two bacterial strains were isolated and identified on the basis of biochemical and 16S rRNA sequencing. These bacterial strains were used as fresh inoculums individually and in combinations and the nutritional quality, physio-chemical, antioxidant and antimicrobial activity were compared with marketed local made (sweet and sour) and branded (Mother Dairy and Thackar) curds.

Materials and Methods

Isolation and selection of bacteria—The different unbranded curds were collected from various region of West Bengal, India and bacteria were isolated on MRS agar under anaerobic (5% CO_2) conditions at 37 °C for 48 h. The isolates were randomly picked, subcultured on fresh MRS broth and incubated under the previously stated conditions at agitation speed 120 rpm. The number of isolates was adjusted at 10^6 CFU/mL and the cultured broths were inoculated (1% v/v) in skimmed milk (pasteurized at 73 °C for 15 sec) and incubated at 37 °C for 24 h. After fermentation, 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity of the whey fractions were determined. The scavenging efficacy of eight isolates (AKL1-AKL8) were evaluated and potent two bacteria (AKL1, AKL2) having higher antioxidant activity were selected for the present study.

Identification of bacteria—Both AKL1 and AKL2 were identified based on Gram staining reaction, cellular morphology, biochemical tests and carbohydrate utilization with the Hicarbo kit (Hi-Media, India) according to the manufacturer's instructions and with reference to Bergey's manual. Identification of AKL1 was confirmed by 16S rDNA gene by sequencing reaction of PCR amplicon generated by 8F (5′-AGAGTTTGATCCT GGCTCAG-3′) and 1492R (5′-ACGGCTACCTTGT TACGACTT-3′) primer. Consensus sequence of 16S rDNA gene was generated from forward and reverse sequence data using BLAST with nr database of NCBI GenBank using MEGABLAST algorithm. Based on the maximum identity score, the first ten sequences were selected and aligned using multiple sequence alignment software program ClustalW. The evolutionary history was inferred using the Neighbor-Joiningmethod (Saitou and Nei 1987) and distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA4 software.

Preparation of curds and whey fraction collection—Cow milk (3% fat) was heated to 90 °C for 15 min and then cooled to 37 °C. The isolated two bacteria were cultured as stated previously and the populations were adjusted at 10^6 CFU/mL by haemocytometric counting. Thereafter, these were inoculated (1% vol) in 100 mL cow milk individually and in combination 1:1 (0.5% v/v of each isolate) and allowed for fermentation at 37 °C for 18 h. The prepared curds designed as AKL1, AKL2 and AKL1+AKL2 and nonhydrolyzed casein was removed by centrifugation at 10, 000 *g* for 10 min. The collected whey was filtrated on 0.45 µm filter and different physiochemical parameters were compared with the whey obtained from local sweet (Lsw), local sour (Lso), Mother Dairy (MDR), Thacker Dairy (THR) curds which were collected from different regions of Midnapore, India. Fresh pasteurized milk without bacterial fermentation was used as control.

Analysis of whey made from different curds—The *p*H of whey fractions were measured by using *p*H meter and titratable acidity was measured by titrating 5mL of whey with 0.05 *N* NaOH using phenolphthalein as indicator[9]. The tritrable acidity was expressed as lactic acid level in the curd. The ash content (g/kg) of the curds was evaluated by oven drying and incineration method at 600 °C. The drainage method was adopted to evaluate syneresis of curds and value was expressed as percent whey in mL separated after 1 h[10]. The hydrolysable peptides, free sugar and free amino acids were measured and expressed in 100 mL of whey [11-13].

Antioxidant activity

DPPH radical scavenging assay—Antioxidant capacity of the whey was determined 2,2-dipheny l-1-picrylhydrazyl (DPPH) method with some modifications[14]. Briefly, 100 µL of whey was added to 1.9 mL of 0.1 mM DPPH in methanol up to completing 2 mL. After 10 min incubation free radical

scavenging capacity was evaluated by measuring the decrease of absorbance at 517 nm. Antioxidant capacity was expressed as mM of TROLOX equivalent (TE) /L of whey, using the calibration curve varying the concentration 20-100 µg/mL.

$$\text{Scavenging activity (\%)} = (1 - A_{sample}/A_{control}) \times 100$$

where A_{sample} was the absorbance in the presence of the sample or reference material and $A_{control}$ was the absorbance of the control containing all the reaction reagents except the sample or reference material. All determinations were performed in triplicate.

$ABTS^+$ scavenging assay—Antioxidant activity of the fermented extracts was estimated by the ABTS radical decolorization method[15]. Stock solutions of ABTS (7 mM) and potassium peroxodisulfate (140 mM) in water were prepared, and mixed together to a final concentration of 2.45 mM potassium peroxodisulfate. The mixture was left to react overnight (12-16 h) in the dark, at room temperature. On the day of analysis, the ABTS radical solution was diluted with ethanol to adjust the absorbance (0.70±0.02) at 734 nm. All measurements were performed as follows: 100 µL of whey was added to 1.9 mL of the ABTS radical solution, and the absorbance was taken after exactly 6 min against the appropriate reagent blank of 100 µL of ethanol instead of the sample. The results, obtained from triplicate analyses, were expressed as mM of TE/L of whey varying concentration 20-200 µg/mL.

$$\text{Scavenging activity (\%)} = (1 - A_{sample}/A_{control}) \times 100$$

Hydroxyl radical scavenging assay—The hydroxyl radical scavenging activity was assayed based on quantification of the degradation product of 2-deoxy ribose and condensation with thiobarbituric acid[16]. Hydroxyl radical was generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system (Fenton reaction) contained 2-deoxy-2-ribose (2.8 mM), KH_2PO_4 buffer (20 mM, *p*H-7.4), $FeCl_3$ (100 µM), EDTA (100 µM), H_2O_2 (1 mM), ascorbic acid (100 µM)[20]. The reaction mixture (1 mL) was mixed with 100 µL of whey and allowed for 1 h incubation at 37 °C. After that 1 mL 5% trichloroacetic acid and 1 mL 1% thiobarturic acid were added and were bolied for 10 min to develop the colour. It was centrifuged at 5000 rpm, and absorbance of supernatant was measured at 532 nm against an appropriate blank. Mannitol was considered as a positive control and hydroxyl radicals scavenging activity was expressed mg of mannitol equivalent (ME)/L of whey.

$$\text{Scavenging activity (\%)} = 1 - A_{sample}/A_{control} \times 100$$

Fe^{3+} to Fe^{2+} reducing activity—The reducing power was estimated in which 0.5 mL of whey was mixed with 0.5 mL of phosphate buffer (0.2 mol/L, *p*H 6.6), and the reaction mixture was incubated with 0.5 mL of a 0.1% aqueous potassium ferricyanide solution at 50 °C for 30 min[17]. The reaction was terminated by adding 0.5 mL of 5% TCA solution and the mixture was centrifuged at 5000 rpm for 10 min. A volume of 2.5 mL of the supernatant was mixed with 0.5 mL of 0.1% (V/W) aqueous ferric chloride solution, and the absorbance was taken at 700 nm. Reducing activity was expressed mM of ascorbic aicd equivalent (AE)/L of whey.

Determination of total phenolics and flavonoids—The amount of total phenolics in whey was determined using the Folin-Ciocalteu method with some modifications in which 50µL whey was added to 1 mL of diluted (1:2) Folin-Ciocalteu reagent[18]. After 10 min incubation at 37 °C, 4.0 mL of 20% (w/v) Na_2CO_3 solution was added. It was boiled for 5 min in a boiling water bath and after cooling absorbance was taken at 765 nm. The result was expressed as mg of gallic acid equivalent (GAE)/100 mL of whey. The total flavonoid was assayed in which 0.5 mL of aqueous solution was added to the 0.5 mL of distilled water followed by 0.75 mL of 5% (w/v) $NaNO_2$. After 5 min incubation 0.150 mL of 10% (w/v) $AlCl_3$ was added and incubated for 20 min. Then 0.2 mL of 1 *M* NaOH was added and absorbance was taken at 510 nm. The flavonoids content was expressed as mg of quercetin equivalent (QUE)/100 mL of whey[19].

Thin layer chromatography (TLC) analysis—For the analysis of phenolics and flavonoids in the whey on TLC plate by using n-butanol: methanol: 16% aqueous ammonia solution (5:4:3) was used as a solvent system[20]. After running TLC plate was dried and the compound were visualized by spraying with 0.5 (N) Folin-Ciocalteu with 20% Na_2CO_3 (Folin-Ciocalteu reagent) and 10% $AlCl_3$, 5% $NaNO_2$ with 1 (N) NaOH (alumminium tetrachloride reagent). DPPH scavenging activity of the phenolics was visualized by spraying 50 mM DPPH solution in methanol. Free amino acids were analyzed by using n butanol: water: acetic acid

(4:5:1) as a mobile phase and TLC plate was developed by spraying 0.5% ninhydrin solution in acetone.

Oxidative protein damage—The bovine serum albumin (BSA) 100 µL at concentration of 1mg/mL was induced for oxidation by 100 mM of 2, 2´-azo (2-amidinopropane) dihydrochloride (AAPH) and inhibited by addition of 200 µL different curd whey. Trolox was used as a standard antioxidant varying concentration 0.5 mg/mL to 2.5 mg/mL. After incubation for 24 h at 37 °C, 0.02% butylated hydroxy toluine (BHT) was added to the reaction mixture to prevent the further the formation of further peroxyl radicals and the degree oxidation was assayed by SDS-PAGE[21].

Antibacterial activity of curd—The antibacterial activity of different whey was evaluated by agar well diffusion method using *Salmonella typhi E1590, Shigella dysenteriae 4717, Vibrio cholera K1510, Staphyllococcus aureus ATCC6538,Clostridium perfringen ATCC13124* and *Escherichia coli ATCC25922* as reference organism. The active bacterial cultures were spreaded over mullar hinton agar (Hi-Media, India) and 6 mm in diameter were made on each plate using a sterile glass borer. For the preparation of control 2% lactic acid was added to the sterilized cow milk (3% fat), then centrifuged for 20 min at 3,000 rpm; the *p*H of the supernatant was adjusted to the *p*H of respective curds. The whey and control supernatant of 100 µL was dispensed into different wells and incubated at 37 °C for 24 h. The zone of inhibition was measured using Vernier calipers and expressed in millimetres (mm).

Statistical analysis—Collected data are presented as the arithmetic mean of three replicas (mean±SD). The variations in antioxidant activity and antimicrobial activity were examined by ANOVA (Kruskal-Wallis).

Results

Isolation and identification—Preliminary screening of the whey fraction of skimmed milk fermented for 24 h with the eight isolates obtained from the fermented curd using the DPPH radical scavenging assay showed that two isolates had potent antioxidant activity. Both AKL1 and AKL2 were identified based on Gram stain reaction, cellular morphology, biochemical tests and carbohydrate utilization. From the above test it was observed that AKL2 was Gram+ve bacilli and it fermented sucrose, maltose, lactose, glucose, glactose and fructose to produce acid. From these characters the isolate was identified as *Lactobacillus acidophilus* AKL2. The nucleotide homology and phylogenetic analysis of another isolate revealed that it was most similar with *Bacillus subtilis* strain Q45 (GenBank accession no. JX515568.1) (Fig. 1) and it was identified as *B. subtilis* AKL1 (Accession no. KC428745.1).

Analysis of whey made from different curds—The physical parameters like ash, total solid, syneresis were significantly lower in curds prepared in combination of *L. acidophilus* AKL2 and *B. Subtilis* (Table 1).

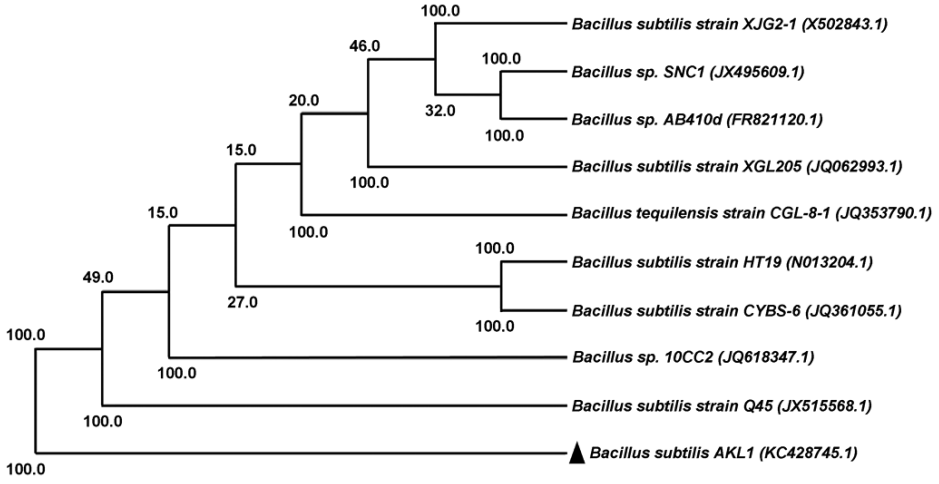

Fig. 1—Phylogenetic tree of 11 taxa generated by comparing 16S rDNA homology in MEGA4 showing the location of strain *Bacillus subtilis* AKL1. Numbers of the branches represent percentage (C50%) of 500 bootstrap replications. A bar represents the evolutionary distance of 0.05. The GenBank accession numbers are shown in parentheses.

Antioxidant activity—The $DPPH^{\bullet}$ and $ABTS^{+}$ radicals were scavenged by antioxidants by single electron and hydrogen-atom transfer mechanisms and transformed into a yellov or colorless product. The scavenging activity of whey against these radicals was expressed as TE/L of whey. DDPH scavenging activity of the control milk, AKL1 and AKL2 were respectively 0.28, 0.71 and 1.25 mM of TE/L. But it was 1.84 mM of TE /L in AKL1+AKL2 that was significantly (P<0.05) higher than other prepared and marketed local and branded curds (Table 2). The $ABTS^{+}$ reducing activity of the control milk was 0.41 mM of TE/L whereas the curd whey of AKL1+AKL2 was 2.52 mM of TE/L that was significantly (P<0.05) higher than others. It was also noted that $ABTS^{+}$ scavenging activity was higher than $DPPH^{\bullet}$ radicals (Table 2). The $OH^{\bullet}$ and Fe^{3+} to Fe^{2+} reducing activity were evaluated and expressed as ME and AE/L of whey. The $OH^{\bullet}$ and Fe^{3+} to Fe^{2+} reducing activity of the curds in combination were 3.47 mg of ME/L and 0.90 mM of AE/L and these values were also statistically different (P<0.05) than other curd whey fraction (Table 2).

The total phenolics and flavonoids—The total phenolics and flavonoids content were 2.1 mg of GAE/100 mL and 1.5 mg of QUE/100 mL in control milk. But these levels were increased in each prepared (AKL1, AKL2, AKL1+AKL2) curds. The levels of phenolics and flavonoids were also higher in marketed curds (MDR, THR, Lso, Lsw) whey than control milk. Fermentation of milk in combination with AKL1+AKL2 level of free phenolics and flavonoids were 85.5 mg of GAE /100 mL and 12.5 mg of QUE/100 mL of whey that was higher than others prepared and marketed curds whey (Fig. 2).

TLC analysis—The diversity of phenolics and flavonoids in different whey were qualitatively

Table 1—Analysis of different physiochemical parameters of prepared and locally marketed curds and its whey
[Value are mean ± SD of 3 replicates]

Parameters	Types of curds						
	AKL1	AKL2	AKL1+AKL2	Lsw	Lso	MDR	THR
Ash	12.1±0.5	10.3±0.2	8.3±0.4	12.3±0.5	11.5±0.2	9.6±0.5	9.8±0.3
Total solid	17.3±1.3	18.6±1.0	15.4±1.5	30.2±1.3	18.3±1.1	17.5±1.4	16.7±1.4
Syneresis	30.5±0.7	35.8±1.0	27.3±0.97	23.6±0.8	25.7±1.3	26.2±0.9	29.4±0.9
*p*H	4.3±0.1	3.9±0.2	3.7±0.1	5.1±0.3	3.9±0.1	4.3±0.2	4.2±0.1
Titratble acidity (%)	1.65±0.4	1.27±0.3	1.95±0.8	0.96±0.15	1.66±0.78	1.57±0.8	1.52±0.5
Free reducing sugar (%)	4.44±1.5	2.12±1.2	1.63±1.0	3.78±1.5	1.94±0.70	3.17±0.95	3.23±0.94
Hydrolysable protein (%)	2.65±0.9	1.38±0.2	3.72±0.5	1.25±0.44	1.42±0.7	2.75±0.65	2.58±0.76
Free amino acids (%)	1.1±0.22	0.75±0.1	1.35±0.42	0.54±0.11	0.96±0.32	1.10±0.54	1.03±0.88

Table 2–Antioxidant activity of whey collected from different prepared and marketed curds against various free radicals
[Values are mean ± SD of 3 replicates]

Types of curd	Antioxidant activity			
	$DPPH^{\bullet}$ mmol of TROLOX equivalent/l	$ABTS^{+}$ mmol of TROLOX equivalent/l	$OH^{\bullet}$ mg of mannitol equivalent/l	Fe^{3+} to Fe^{2+} mmol of ascorbic acid equivalent/l
Control milk	0.28±0.030[a]	0.41±0.0208[a]	0.71±0.0265[a]	0.12±0.020[a]
AKL1	0.71±0.0208[b]	1.05±0.0200[b]	1.56±0.0777[b]	0.36±0.0458[b]
AKL2	1.25±0.0200[c]	1.76±0.0503[c]	2.32±0.0321[c]	0.45±0.0603[c]
AKL1+ AKL2	1.84±0.0200[d]	2.52±0.0800[d]	3.47±0.0651[d]	0.90±0.0493[d]
Lsw	0.86±0.0473[e]	0.92±0.0252[e]	2.38±0.0351[c]	0.27±0.0252[e]
Lso	1.11±0.0503[f]	1.23±0.0351[f]	2.54±0.0503[e]	0.58±0.0252[bf]
MDR	1.32±0.0303[g]	1.65±0.0500[e]	2.73±0.0569[f]	0.59±0.0351[f]
THR	1.05±0.0432[h]	1.37±0.0603[g]	2.69±0.0503[f]	0.53±0.0404[f]

Different superscripts (a,b,c,d,e,f,g) within a column are significantly different at P<0.05.

analysed by TLC and it was observed that the types of free phenolics milk was higher than others marketed curds whey. Two types of free phenolics were present in AKL1 whereas three in AKL2. But in AKL1+AKL2 five types of phenolics were released as free form into whey and among these three belong to flavonoids. But the others marketed curds contained only 2-3 types of phenolics and 1-2 types of flavonoids. The profile of free phenolics and flavonoids in different whey were shown in Fig. 3a and b. After spraying DPPH, the yellow spot on TLC indicated that both phenolics and flavonoids were potent DPPH radicals' scavenger (Fig. 3c). TLC analysis revealed that three free amino acids were

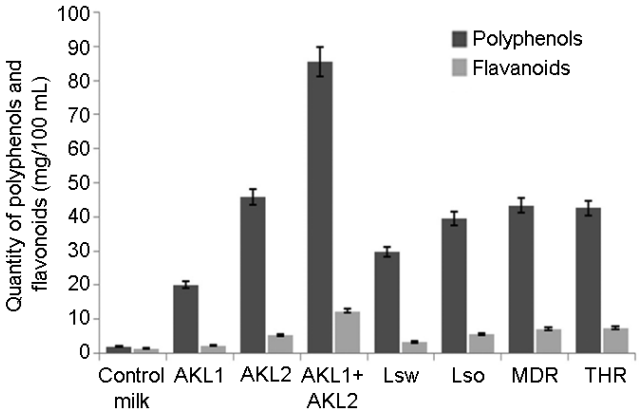

Fig. 2—The quantitative analysis of polyphenol and flavonoids (mg of GAE and QUE /100 mL) in the whey of different curds.

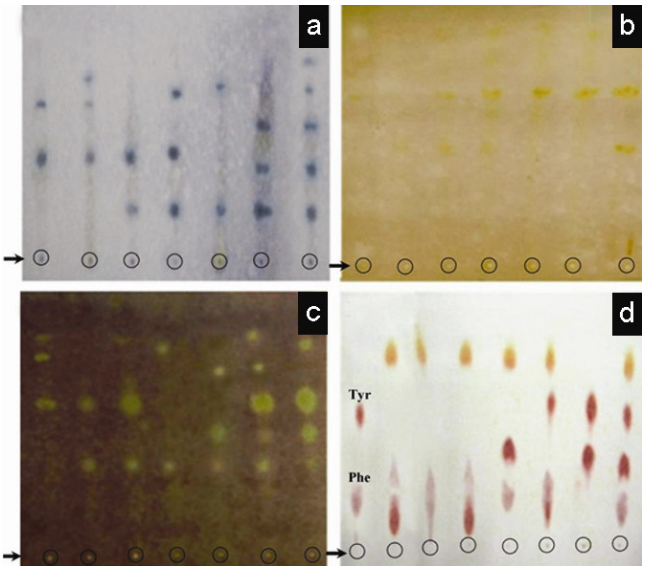

Fig. 3—TLC analysis of (a) Polyphenols, (b) flavonoids, (c) DPPH scavenging activity and (d) free amino acids in the whey of different curd samples [Arrow indicate the spoting sequence of local sweat, local sour, Mother Dairy, Thacker, AKL1, AKL2 and AKL1+AKL2 reopctively].

produced in AKL1 whereas two in AKL2 but the fermentation in combination of *B. subtilis* AKL1 and *L. acidophilus* AKL2 released four amino acids into whey including tyrosine and phenylalanine (Fig. 3d). But, other marketed curds contained more or less 3 types of amino acids including phenylalanine but absence of tyrosine.

Oxidative protein damage—During oxidative stress, different reactive species were generated and underlying cause of senescence-associated deleterious alterations. The standard antioxidant, TROLOX inhibited AAPH induced BSA oxidation with increase of concentration and at 2.5 mg/mL it completely inhibited the degradation (Fig. 4a). It was also observed that 200 µL of curd whey prepared in combination of *B. subtilis* AKL1 and *L. acidophilus* AKL2 exhibited higher protective effect against oxidation of BSA (Fig. 4B) that was equivalent to 2 mg/mL of TROLOX.

Antimicrobial activity of whey—All the curd whey exhibited a variable degree of antibacterial activity against six food and entero-pathogens. From the results, it was evident that curds prepared with culture combination *B. subtilis* AKL1 and *L. acidophilus* AKL2 showed significant (P<0.05) antimicrobial activity than the control milk as well as others marketed local and branded curds (Table 3). The curd whey prepared with culture combination exhibited higher inhibition against *Escherichia coli ATCC25922* with inhibition zones greater (>16 mm) than the curds whey prepared with single isolate and other local and branded curds (9.12- 12.30 mm). Subsequently, antimicrobial activity was exhibited respectively against *Shigella dysenteriae 4717, Vibrio cholera K1510, Staphyllococcus aureus ATCC6 538, Salmonella typhi E1590, Clostridium perfringen*

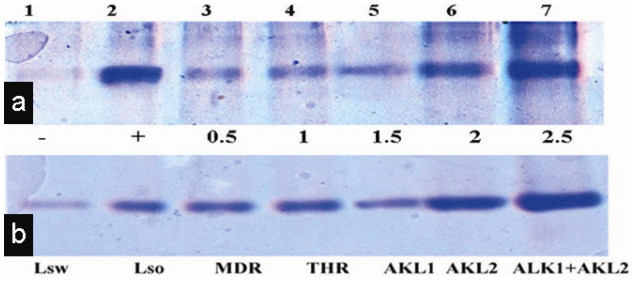

Fig. 4—The inhibition of oxidative damage of BSA by whey and standard antioxidant, TROLOX at various concentrations [Lane 1: 100 mM AAPH+BSA, Lane 2: BSA Oxidative damage intibition by TROLOX lanes 3-7 (BSA + 100 mM AAPH + 0.5-2.5 mg/mL of TROLOX. (b) Lanes 1-7: inhibition of BSA fragmantaton by 200 µL of different whey]

Table 3—Antimicrobial activity of whey collected from different curds against some enteric pathogens

[Values are mean ± SD of 3 observations each]

Types of curd	Inhibition zone (mm)					
	Clostridium perfringens	*Escherichia coli*	*Shigella dysentery*	*Vibrio cholerae*	*Staphylococcus aureus*	*Salmonella typhi*
Control milk	8.00±0.36^{a}	8.10±0.15^{a}	8.20±0.20^{a}	8.1±0.22^{a}	8.3±0.2^{a}	8.0±0.25^{a}
AKL1	8.6±0.208^{b}	10.50±0.20^{b}	10.06±0.13^{b}	9.80±0.09^{b}	10.2±0.1^{NS}	8.70±0.15^{b}
AKL2	8.00±0.050^{NS}	9.44±0.10^{c}	8.85±0.18^{c}	8.35±0.08^{a}	8.5±0.12^{b}	8.02±0.26^{NS}
AKL1+ AKL2	12.37±0.026^{c}	16.80±0.10^{d}	15.75±0.15^{d}	14.58±0.19^{c}	14.4±0.15^{c}	13.66±0.25^{c}
Lsw	8.00±0.096^{NS}	9.12±0.20^{c}	8.60±0.20^{c}	8.42±0.05a^{d}	9.2±0.37^{d}	8.02±0.37^{NS}
Lso	9.25±0.0964^{d}	12.30±0.20^{e}	10.60±0.35^{e}	12.77±0.08^{e}	11.6±0.15^{e}	10.53±0.14^{d}
MDR	10.22±0.080^{e}	11.05±0.19^{f}	11.08±0.16^{f}	10.54±0.06^{f}	10.8±0.25^{NS}	9.87±0.16^{e}
THR	10.35±0.076^{e}	11.48±0.13^{f}	11.35±0.20^{f}	10.05±0.07^{g}	10.0±0.30^{NS}	9.06±0.18b^{f}

Different superscripts (a, b, c, d) within a column were significantly different at $P<0.05$.

ATCC13124 and its difference ($P<0.05$) with others products is shown in Table 3.

Discussion

The different physiochemical analysis of the different curds prepared by *B. subtilis* AKL1 and *L. acidophilus* AKL2 individu ly and in comb nation revealed that cumulative actions of two isolates were much more superior for the quality curd preparation than others. The single activity of *B. subtilis* AKL1 was highly effective to release free peptides and amino acid in the whey but unable to form hardness of the curds that was reflected by synersesis value. But the *L. acidophilus* AKL2 in which level of free reducing sugar was significantly reduced than fermented by *B. subtilis* AKL1 and in combination conferred the superior quality with lower syneresis that attributed to entrapment of water in the gel. The proteolytic activity of *B. subtilis* AKL1 majorly responsible for higher amino acid accumulation[22] and released of higher amount of peptides by reducing the level of solid milk protein and lactic acid fermentation by *L. acidophilus* AKL2 decreased the free reducing sugar than other marketed products. This quality of the curds may reduced the lactose intolerance and pre-digested free nutrients like peptides and amino acids attributed better health promoting effect [2,10].

Natural defence mechanisms eliminate negative effects of the activity of free radicals. However, they are not always adequate to totally neutralize all endogenous and exogenous free radicals[6]. Thus scavenging properties of free radicals by food grade cultures can be useful in food manufacturing and can provide additional dietary sources of health enhancing antioxidants[5,7]. In the present study, the antioxidant activity of the control milk and these fermented with *L. acidophilus* AKL1 and *B. subtilis* AKL2 isolated from some indigenous curds was assessed and compared with marketed curds. Indigenous fermented milk products are not usually heat treated and fermented milk products are more generally acce ‘able. In addit ˙n, a recent st ‘y has reported the development of antioxidant activity in whey during milk fermentation with lactic acid bacteria. But the present study clearly indicated the higher antioxidant activity in combination (1:1) of isolated *L. acidophilus* AKL1 and *B. subtilis* AKL2 were superior to other curds whey prepared by single isolate and marketed branded and local products. These entire prepared and marketed products can scavenge the $DPPH^{\bullet}$, $ABTS^{+}$, $OH^{\bullet}$ radicals and Fe^{3+} to Fe^{2+} reducing activity with varying potentialities. Although these may exhibit antioxidant activity in varying ways but it was difficult to distinguish only one mechanism or compound behind this activity[14,17]. *In vitro* TLC analysis revealed that the presence of higher level of free phenolics and flavonoids contributed to the scavenging activity against DPPH free radicals that was correlated with their qualities. In addition to higher quantities of phenolics and flavonoids in AKL1+AKL2 were more active against $ABTS^{+}$ radicals than $DPPH^{\bullet}$ indicated the degree of solubility and hydrophobicity in the whey that depend on their interaction with milk solids. Generally, there are four potential types of interactions that can occur between phenolic molecules and proteins, including hydrogen bonding, hydrophobic, ionic, and covalent interactions[23]. There are a number of factors that affect the interactions between phenolic molecules and protein including *p*H, temperature, phenolic

structure, and molecular weight and amino acid composition. The lower *p*H and cumulative actions of enzymes of *L. acidophilus* AKL1 and *B. subtilis* AKL2 during fermentation not only released polyphenols but also some hydrolysable peptides and free aromatic amino acids like phenylalanine and tyrosine those may strongly linked to $OH^{\bullet}$ radicals and Fe^{3+} to Fe^{2+} reducing activity having hydrogen donating and electron extorting capability[15,17]. During some pathophysiological state like hypoxic acclimatization, irritable bowel disease, chorrn disease, diabetes, and different drugs consumption alter the indigenous flora and initiate the oxidative stress that severely affect the intestinal epithelial cell[1,5,6]. The increase in protein carbonyl content is indicative of both oxidative damage as well as chemical modification. Overwhelmed level of this reactive carbonyls may decrease in the activities of different detoxify intestinal enzymes like glyoxalases I and II and aldose reductase. In such stressful state bacterial methylglyoxal synthase increased them carbonyl derivatives like methylglyoxal that delayed bacterial clearance and consequent alteration intestinal microbial imprint. The higher protein oxidation inhibition activity of curd whey, prepared in combination of two new isolates may attribute to better gastrointestinal promoting agent than other marketed and local curds. Aside by the whey were highly effective against *E. coli* followed by *Shigella dysenteriae 4717, Vibrio cholera K1510, Staphyllococcus aureus ATCC6538, Salmonella typhi E1590, Clostridium perfringen ATCC13124.* The curd prepared by *L. acidophilus* and *Bacillus subtilis* individually showed lower activity than in combination against these enteropathogens. However weak inhibitory activity of market curd samples against *E. coli* and *Staphylococcus aureus*, whereas no inhibition was observed against *Salmonella typhi*; curd samples prepared using *Lactococcus lactis ssp. lactis* C10 had a weak antibacterial activity against *Salmonella typhi*[31]. The antibacterial activity of *Streptococcus lactis ssp. diacetylactis* strains DRC1 and DRC2 against *Staphylococcus aureus* S6 and *Pseudomonas fragi* is reported[24]. Similarly, pre-feeding with probiotic curd containing *L. acidophilus* and *L. casei* ameliorated *Salmonella enteritidis* infection by stimulating specific and non-specific immune response, and lowered colonization of the gastrointestinal tract as well as leading to translocation of *Salmonella enteritidis* and a modulated immune response in experimental mice[6,25]. It was reported that the *Bacillus subtilis* secreted different antimicrobial and antihypertensive peptides into soya products during fermentation. Potentialities of *L. acidophilus* strains having antimicrobial activity against enteropathogenic *E. coli* than market curd samples have indicated that the antimicrobial activity of *L. acidophilus* isolates against Gram positive bacteria like *B. cereus* and *S. aureus* and Gram negative bacteria like *P. aeruginosa* and *K. pneumoniae* may be due to the production of bacteriocin-like substance[6,24]. In the present study enhanced antimicrobial activity in combination of *L. acidophilus* AKL1 and *B. subtilis* AKL2 than control milk as well as others products indicated the accumulation of higher antimicrobial components in the whey other than the organic acid.

Conclusion

The curds prepared in combination of two new isolates *L. acidophilus* AKL2 and *B. subtilis* AKL1 enhanced the antioxidant potentialities as well as antimicrobial activity against some food and entero-pathogens with higher nutritional value and superior quality than other marketed and branded curds. Therefore it is proposed that these bacterial strains in combination will be a fruitful starter culture for the preparation and consumption having better health promoting effects.

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