INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 9, No. 6, p. 415-430, 2016

RESEARCH PAPER OPEN ACCESS

Isolation, characterization and application of indigenous lactic acid bacteria in milk fermentation

Farah Nawaz[1], Sadia Mehmood[1], Shakira Ghazanfar[3], S.S. Tahir[2], Naseem Rauf[2], Muhammad Imran[1*]

[1]*Department of Microbiology,Quaid-i-Azam University, Islamabad, Pakistan*

[2]*Pakistan Council for Scientific and Industrial Research (PCSIR), Islamabad, Pakistan*

[3]*Institute of Microbial Culture Collection of Pakistan, National Agricultural Research Centre, Park Road, Islamabad-45500, Pakistan*

Key words: Indigenous starters, Milk, Lactic acid bacteria, Nutrition.

http://dx.doi.org/10.12692/ijb/9.6.415-430 Article published on December 31, 2016

Abstract

Lactic acid bacteria are essential part of milk fermentation impart characteristic attributes on product physio-chemistry, nutrition and sensory properties. The present study was designed to characterize the Lactic acid bacteria isolated from fermented milk product Dahi for potential application in milk fermentation. All isolates were Gram positive rods and cocci. Most of the isolates were heterofermentative shown carbon dioxide production. Isolates shown higher lipolytic, proteolytic and fair amylolytic activity. Physiological characterization revealed that cocci were showing optimum growth at 2 and 4% NaCl while rods at 6.5% followed by 4% salt. Agglomerative hierarchical clustering (AHC) was done based on growth rate and alteration in media pH at differenttemperatures. Identification of representative isolates from each group was confirmed by 16SrRNA gene partial sequencing.Selective strains were identified as *Lactobacillus delbrueckii* QAULb01(KT021869), *Streptococcus thermophilus*QauSt1(KT021870)and*Lactobacillus delbrueckii* Qaulbd16, and *Enterococcus mundtii* QAUEM02. Comparative fermentation experiments of commercial starter culture and indigenous isolates combination (QauSt1+Qaulb01) was done. The local strain combination shown more aroma as compare to commercial starter that was also confirmed by change in FTIR spectrum. Comparative physiochemical analysis shown that local cultures in term less syneresis, high solid content, stability in pH and acidity and higher viscosity.The nutrition of fermented milk produced by local strain in term mineral contents was comparable with significantly higher zinc and iron contents (K :720 mg/kg), Na: 642.5 mg/kg, Ca: 245.2 mg/kg, Fe: 2.7 mg/kg and 2 mg/kg of Zinc). Hence these strains can be successfully used to replace starter cultures at commercial scale for fermented milks.

* **Corresponding Author:** Muhammad Imran✉ m_imran766@hotmail.com

Introduction

Lactic acid bacteria (LAB) are generally recognized as safe (GRAS) microorganisms in food fermentations worldwide. In dairy and food industry, they are enormously used as a starter cultures because they are known to improve technological, nutritional, organoleptic, and shelf-life properties in diverse fermented foods and beverages(Wouters *et al.*, 2002;Capozzi *et al.*, 2012; Patel *et al.*, 2013;). These are categorized as gram positive cocci or rods, aero tolerant but anaerobic, produce energy and lactic acid by utilizing carbohydrates. The metabolic pathways include homofermentative in which two molecules of lactate are produced e.g. *Lactococcus* and *Streptococcus*. While in heterofermentation, lactic acid, ethanol and carbon dioxide are produced e.g. *Lactobacilli* and *Leuconostoc*(Mahmoudi *et al.*, 2013).

Now a day much focus is paid on the technological potential of LAB for the bio control of plant, food and soil born fungus and bacteria (Fhoula *et al.*, 2013). In dairy ecosystem LAB efficiently consume milk constituents, principally lactose and caseins, that's why they are used as starter cultures in the production of dairy products. Previous studies show affiliation between fast growth, rate of acid production and efficient milk casein degradation (Courtin, 2002). Amino acid and peptides are not freely available in milk for LAB (Zourari, 1992; Abu-Tarboush, 1996). LAB need an exogenous supply of amino acid and peptide which is provided by the proteolysis of milk casein. That's why they are called fastidious microorganism.Glucose containing mono and disaccharides that are normally present in natural food are readily available for amylolytic LAB (Guyot *et al.*, 2000).

Milk is enriched with indigenous lipoprotein lipase (LPL) and estrases. *Lactococcus* strains are the examples of producing intracellular Lipases (Casey, 1992; Fox *et al.*, 1996; Holland and Coolbear, 1996). During the lipolysis hydrolysis of triglycerides into short, intermediate fatty acid chains and glycerol is produced especially in cheese fermentation. Quality of cheese is directly influenced by the fats present in it, which affect the taste and texture of that cheese. Free fatty acids (FFA) formation occurs as a result of lipolysis affect the flavor compounds such as methyl ketones, alcohols and lactones present in cheese.

Co-inoculation of different species in food fermentations to improve the quality of existing products is widely applied now days. Also, industries are taking keen interest in using the molecular approaches for the survival and improvement of selected strains; by closer examination, namely the protocooperation between *S. thermophiles* and *L. bulgaricus*, phage resistance, utilization of prebiotic carbohydrates (Goin, 2010). Fermented foods in which the interactions, like protocooperation, mutualism and synergism, are important for the growth rate e.g. cultures consist of filamentous fungi, LAB and yeast (Sieuwerts *et al.*, 2008).

The present study was designed to Identify and characterize the lactic acid bacteria isolated from indigenous fermented milk product Dahi and evaluate the technological and physiochemical attributes of the LAB isolates, so that they could be successfully used for Milk fermentation.

Materials and methods

Isolation of lactic acid bacteria

Fifty Dahi samples were collected from different locations of Islamabad and Rawalpindi available at the retail under sterile conditions. Isolation of lactic acid bacteria from Dahi was done by Serial dilution method on MRS and M17 agar plates. The media plates were incubated at 37°C for 48 hrs. Isolates were maintained on MRS and M17 media at 4°C for further studies.

Morphological and biochemical identification

For morphological and biochemical characterization of the isolated strains several tests were performed: Gram staining, Oxidase test, Catalase test, and carbon dioxide (CO_2) production.For CO_2 production, MRS and M17 broth containing Durham tubes were used. Citrate excluding MRS and M17 broth were used

because citrate can produce CO_2. 100 µl of 24 hour old cultures were inoculated into 5ml citrate minus MRS and M17 broth and incubated for 72 hours at 37 °C.

Technological characteristics

Evaluation of enzymatic activity of lactic acid bacteria

For amylolytic activity,1 gram of starch was added in 1 gram of nutrient agar in 100 ml of distilled water. Surface dried plates of starch were streaked with 24 hours old culture of LAB and incubated at 37°C for 48 hours. These plates were then flooded with gram's iodine for 15 to 30 minutes. Then clear zone around the colonies were observed.

For proteolytic activity, Skim milk agar plate was prepared (10 g of skim milk and 2.5 gram of agar) (Gordon *et al.*, 1973). Surface dried plates of milk agar were streaked with 24 hours old culture LAB and incubated at 37°C for 48 hours. After incubation clear zone around the streak were observed.

Media used for the lipolytic activity was described by (Sierra, 1957) in this method tween 80 was used as a lipid substrate. Clear zone of hydrolysis was observed.

Physiological parameters

To check the survival of presumed Lactic acid bacteria isolates, different NaCl concentration 2%, 4 % for cocci and 4%, 6.5% for rods were used. To perform the experiment, 100 µl of overnight cultures were inoculated into 5ml of MRS with 2% 4 % and 6.5 % NaCl concentration.

Growth and pH of isolates was determined at three different temperatures 15°C, 30 °C and 45°C. 100µl of overnight activated cultures were inoculated into MRS broth for bacilli and M17 broth for cocci. Acidification potential was also determined at the same temperature by inserting probe of pH meter. Growth pattern was observed with the help of optical density at 600nm.

Identification of selected isolates by partial sequencing of 16SrDNA

For DNA extraction, bacterial isolates were inoculated in TSB broth and placed at 37 °C shaker for 24hr. DNA extraction of isolates was carried out using the CTAB method as described by Kate Wilson (Wilson, 1975). The gel was run at 100 volts, 400 milli-amperes current to visualize the extracted DNA quality.

Phylogenetic analysis

After DNA extraction, isolated DNA was partially sequenced for 16S rRNA gene. Sequencing was carried out by Macrogen Commercial Seoul, South Korea. 16S rRNA gene sequences of the isolated strains and the most similar sequences from Gen Bank were identified through BLAST from NCBI. The alignments were then thoroughly analyzed and corrected. This was followed by construction of Phylogenetic trees for isolates with Bootstrap values using neighbor joining method.

Analysis of isolates for the milk fermentation

For the milk fermentation cultures were activated into the tryptone soya broth(TSB) broth for 24hours. Then inoculated into pasteurized milk for 24 hours. Fermentation was performed in two batches, one with starter culture that act as a control, was introduced into pasteurized milk, and other batch, with combination of (QauSt1+QauLb01) were inoculated.

Both of these combinations were examined for pH, titratable acidity, total solids, syneresis, and FTIR analysis at different intervals that described the biochemical changes occurred during the fermentation process. The experimental samples were also analysed for viscosity and Mineral content.

(A.O.A.C, 1990) method number 981.12 for the determination of pH was followed. (A.O.A.C, 1990) method number 967.16 was followed for determine the titratable acidity. Similarly, (A.O.A.C, 1990) method number 925.23 was used to calculate the total solid content present in yogurt. After separation of solid content, the resulting whey was used to determine percentage of syneresis.

Frontier transformed infrared spectroscopy was used in order to analyse the chemical changes taking place in yogurt fermentation. FTIR was done by Perkin ELMER spectrum 65FTIR spectroscopy equipped with ATR. For each sample the spectrum range was 650-4000c/m. In order to observe different peaks, an overlay was formed which showed the new peak. Changes occurred in the sample were compared to the control sample.

Measurement of viscosity

Viscosity was measured using a viscometer model LVDVE, DV-E Viscometer (Brookfield Engineering Laboratories Inc., Stoughton, MA) using spindle number 64 which was set clockwise at 100rpm. Every experiment was repeated 3 times to have some meaningful results after a statistical analysis. Viscosity was expressed as milli poises (m.p.s).

Determination of ash contents

The ash content was determined by dried ash method in a muffle furnace (Carbolite, Model No. CWF 1200) at 500˚C for 6 hours (overnight) as described by (Hernandez and Park, 2014). Ash contents were determined by the following formula:

$$\text{Ash \%} = \text{weight of crucible and Ash} - \text{weight of crucible} \times 100/\text{weight of sample}$$

Digestion of ash and mineral analysis

The digestion of ash samples was carried out to determine mineral concentration in Dahi. Ash was dissolved in 15-20 ml of nitric acid (1: 1 ratio) and heated on hot plate at 80°C till the colour of the fumes changed from brown to yellow; this process was performed in the fume hood. After dissolution 2M nitric acid was used to make the volume upto 25ml. minerals including Ca, Mg were determined by using the scientific Nov 300 Flame Atomic Absorption Spectrophotometer Fe, zinc concentration was determined by the Analytik Jena AAS vario 6 Atomic absorption spectrophotometer While Na and potassium were determined by AFP 100 flame photometer using their standards, and concentrations were noted.

Results and discussion

Morphological and biochemical identification

In current study, selective media such as MRS and M17 were used keeping in view the specific nutritionl requirements of LAB and twenty isolates were selected for identification and characterization from indigenous fermented milk product Dahi.

Table 1. Morphological and biochemical identification of the isolates.

Isolates	Colony Surface	Colony Margin	Colony colour	Gram staining	Cell Shape	Catalase Test	Oxidase test	CO_2 Production
QauLac 1	Pin point	Regular	Pale yellow	G (+)	Cocci	-	-	-
QauLeu3	Pin point	Regular	Pale yellow	G (+)	Cocci	-	-	+
QauLeu4	Pin point	Regular	Pale yellow	G (+)	Cocci	-	-	+
QauSt 9	Pin point	Regular	Pale yellow	G (+)	Cocci	-	-	+
QauEm02	Pin point	Regular	Pale yellow	G (+)	Cocci	-	-	+
QauLeu11	Pin point	Regular	Creamy	G (+)	Cocci	-	-	+
QauLac12	Pin point	Regular	Pale yellow	G (+)	Cocci	-	-	+
QauLeu14	Pin point	Regular	Pale yellow	G (+)	Cocci	-	-	+
QauSt1	Pin point	Regular	Pale yellow	G (+)	Cocci	-	-	+
QauLeu18	Pin point	Regular	Pale yellow	G (+)	Cocci	-	-	+
QauLbd16	Smooth	Irregular	Light grey	G (+)	Rods	-	-	+
QauLb01	Smooth	Irregular	Light grey	G (+)	Rods	-	-	+
QauLabf	Smooth	Irregular	transparent	G (+)	Rods	-	-	+
QauLabe	smooth	Irregular	Whitish	G (+)	Rods	+	-	-
QauLabf1	Smooth	Regular	Grey	G (+)	Rods	-	-	+
QauLabmo	Smooth	Irregular	Grey/white	G (+)	Rods	+	+	-
QauLabf3	Smooth	Irregular	transparent	G (+)	Rods	-	-	-
QauLabw2	Smooth	Regular	Transparent	G (+)	Rods	-	-	-
QauLabw3	Smooth	Regular	Transparent	G (+)	Rods	-	-	+
QauLabw5	Smooth	Irregular	Creamy	G (+)	Rods	+	+	-

The colonies were pinheaded shiny and transparent on MRS agar.

The results of gram staining showed the presence of purple rods individually and in chains which confirmed that they were gram positive. While on M17 agar the colonies were yellowish creamy and pin point in appearance. Gram staining indicated the presence of purple cocci.

Table 2. Evaluation of enzymatic potential of presumed Lactic acid bacteria.

Isolates	Lipolytic activity	Proteolytic activity	Amylolytic activity
QAULAC 1	+	+	-
QAULEU3	+	+	-
QAULEU4	+	-	+
QAU ST 9	+	-	-
QAUEM02	+	+	-
QAULEU11	+	+	+
QAULAC12	+	+	-
QAULEU14	-	-	-
QAUST1	+	+	-
QAULEU18	+	+	-
QAULAB16	+	+	+
QAULB01	+	+	-
QAULABF	+	+	+
QAULAB E	+	+	+
QAULABF1	+	+	+
QAULABMO	+	+	+
QAULABF3	+	+	+
QAULABW2	-	+	+
QAULABW3	+	+	+
QAULABW5	+	+	-

The dominance of catalase and oxidase negative gram positive rods and cocci among the isolates is consistent with the finding of (Naeem *et al.*, 2012) and (Maqsood *et al.*, 2013) who observed the presence of these bacteria from locally available dahi. Similar results were also presented by (Gad *et al.*, 2014) who confirmed the presence of these bacteria in dairy and pharmaceutical products.

Isolates were further analysed for the biochemical test. Results for CO_2 production test shown that six strains were homofermentative i.e. end product was lactic acid and fourteen were heterofermentative i.e. along with lactic acid other aromatic compounds and CO_2 were also formed. The results for morphological and biochemical identification are summarized in Table 1.

Technological characteristics

The results for enzymatic activity of isolates are summarized in the Table 2. Proteolytic activity (degradation of casein) was observed in seventeen isolates, when tested on skim milk agar. It is the most abundant protein in the milk as a main source of amino acids. Proteolytic properties are important for the development of organoleptic properties of different fermented products. During proteolysis, the amino acids which are produced, serve as a precursor for the development of flavouring compounds especially aldehydes in the product. Our study is in agreement with the earlier findings (Dagdemir and Ozdemir, 2008; Hassaïne, 2008).

Lipolytic activity was examined in eighteen isolates out of twenty. The isolates showed hollow zone

around the colonies which was an indication of lipid degradation while QauLeu14 and QauLabw2 showed negative results for lipolytic activity. It is predicted that such activities can be manifested in vivo for the reduction of cholesterol level in humans, as a starter and adjunct culture (Ramakrishnan 2012). Our study is contrary to the findings of (Bridget, 2011) where less lipolytic and amylolytic strains were screened in dairy industry these can be used in the hydrolysis of milk fat. Similar findings have been reported by (Fortune Akabanda, 2014).

Table 3. Physiological profiling of Lactic acid bacteria Isolates.

Isolates	Growth at different temperatures			pH at different temperatures			Growth at diff. NaCl concentrations OD at 600		
	15°C	30°C	45°C	15°C	30 °C	45°C	2%	4%	6.5%
QauLac1	0.89±0.48	3.67±0.37	3.70±0.93	7.33±0.49	6.58±0.43	6.91±0.16	0.34±0.04	1.69±0.05	-
QauLeu3	1.14±0.36	3.83±1.20	4.29±1.53	7.36±0.42	7.49±0.39	6.51±0.25	0.43±0.01	3.47±0.33	-
QauLeu4	1.17±0.35	5.32±1.88	3.66±1.06	7.09±0.27	7.11±0.27	6.63±0.32	0.34±0.03	2.78±0.13	-
QauSt 9	1.46±0.43	5.38±1.31	2.86±0.69	7.25±0.16	7.34±0.65	6.67±0.14	1.56±0.02	0.60±0.14	-
QauEm02	4.27±1.01	6.45±2.8	0.68±0.07	7.04±0.09	6.81±0.10	6.64±0.19	1.43±0.01	3.39±0.01	-
QauLeu11	0.97±0.18	3.14±0.69	3.78±1.54	7.24±0.23	7.55±0.72	6.76±0.40	0.51±0.01	2.28±0.09	-
QauLac12	1.31±0.57	4.13±2.61	4.93±1.54	7.36±0.44	7.47±0.44	6.81±0.34	2.28±0.04	0.29±0.20	-
QauLeu14	2.04±1.46	3.55±2.0	1.39±0.47	7.07±0.24	7.40±062	6.67±0.48	2.40±0.01	1.65±0.04	-
QauSt1	0.82±0.16	3.81±1.62	3.91±02.9	7.24±0.18	6.92±0.16	6.60±0.24	3.44±0.32	2.46±0.42	-
QauLeu18	1.44±0.16	4.45±1.50	4.64±1.23	6.81±0.18	6.89±1.07	6.45±0.25	2.43±0.22	0.57±0.05	-
QauLab16	1.03±0.2	2.41±0.40	2.62±0.46	5.42±0.55	5.55±0.46	4.83±0.15	-	4.39±0.23	6.96±0.12
QauLb01	0.98±0.04	4.58±2.87	4.59±2.34	6.18±1.17	6.11±0.98	4.89±0.12	-	0.80±0.22	3.31±0.19
QauLabf	1.19±0.41	3.23±1.67	1.02±0.64	5.86±0.50	5.16±0.53	4.83±0.15	-	2.67±0.15	2.58±0.10
QauLabe	0.87±0.15	3.90±1.49	5.15±0.78	7.07±0.42	6.94±0.86	4.88±0.49	-	5.41±0.14	3.36±0.35
QauLabf1	1.10±0.53	6.90±0.24	2.91±1.22	5.48±0.5	6.12±1.0	5.01±0.20	-	2.47±0.26	7.09±0.23
QauLabmo	0.83±0.47	4.48±3.95	3.31±2.15	7.27±0.37	6.21±0.21	6.56±1.7	-	5.08±0.26	6.62±0.10
QauLabf3	0.51±0.27	1.45±0.31	1.47±0.72	5.78±1.2	5.94±0.74	4.98±0.18	-	0.91±0.05	4.61±0.29
QauLabw2	0.54±0.43	3.43±1.88	3.83±1.88	6.06±0.21	6.63±0.3	5.07±0.68	-	1.16±0.01	4.22±0.19
QauLabw3	0.85±0.05	2.80±1.36	4.49±2.36	5.83±0.47	6.37±1.3	5.13±0.29	-	1.22±0.29	6.56±0.31
QauLabw5	2.84±1.18	3.73±1.85	5.53±2.0	5.43±0.60	6.55±1.6	4.82±0.24	-	1.10±0.15	1.49±0.10

Amylolytic *Lactobacillus* strains hydrolyse the starch granules into low molecular weight sugar causing the acidification of media (Giraud, 1994). This trait of the strains to convert directly the cheaply available starchy substances to lactic acid is quite economical for agriculture. Screening of amylolytic activity is in line with these reports (Reddy *et al.*, 2008; Tchekessi *et al.*, 2014; Vishnu, 2006). Ten Isolates had shown clear zone around the colonies which indicated degradation of starch. While other isolates showed negative results for amylolytic activity, this could be due to the absence of amylase enzyme (Table 2).

Physiological characteristics of isolates

Growth and pH at different temperatures indicated that six isolates were found to be mesophiles and showed maximum growth rate at 30°C and decline in pH at 30°C was observed only in one isolate (QauLabmo) as shown in (Table 3). Thermophilic isolates showed better logarithmic phase and increased acidification at 45°C. Some isolates showed increase in growth rate and rapid decrease in pH at two different temperatures i.e. at 30°C and 45°C. These represent meso/thermophiles. It is concluded that LAB had a growth survival better at 30°C and 45°C. Our finding is conclusive with the reports mentioned by the following (Mallesha SR, 2010; Nikita and Hemangi, 2012). Minor growth was observed at 15°C, however most of the rod shaped bacteria also shown a decline in pH at this

temperature. The obtained results revealed that the presumed *Lactobacilli* species were fast acid producers as compared to *Lactococci/Enterococci/Streptococci* species. pH of *Lactobacilli* decreased to 4 and remained 4 after the 4th day. However, heterofermentative bacteria are sensitive to low pH.

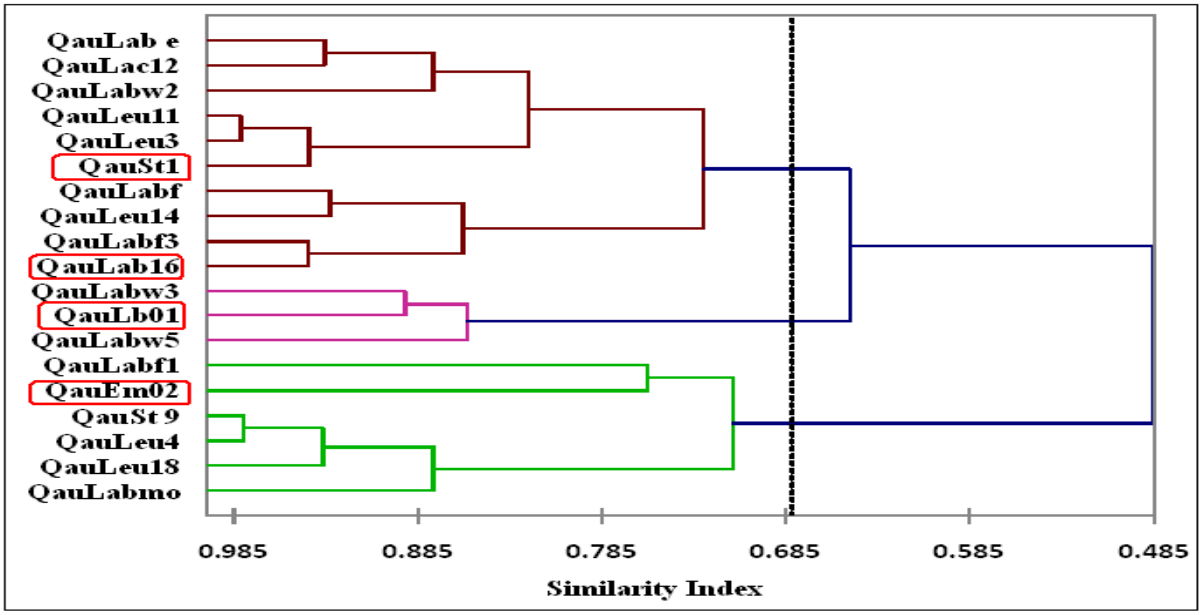

Fig. 1. Agglomerative hierarchical clustering (AHC) for the grouping of isolates based technological properties.

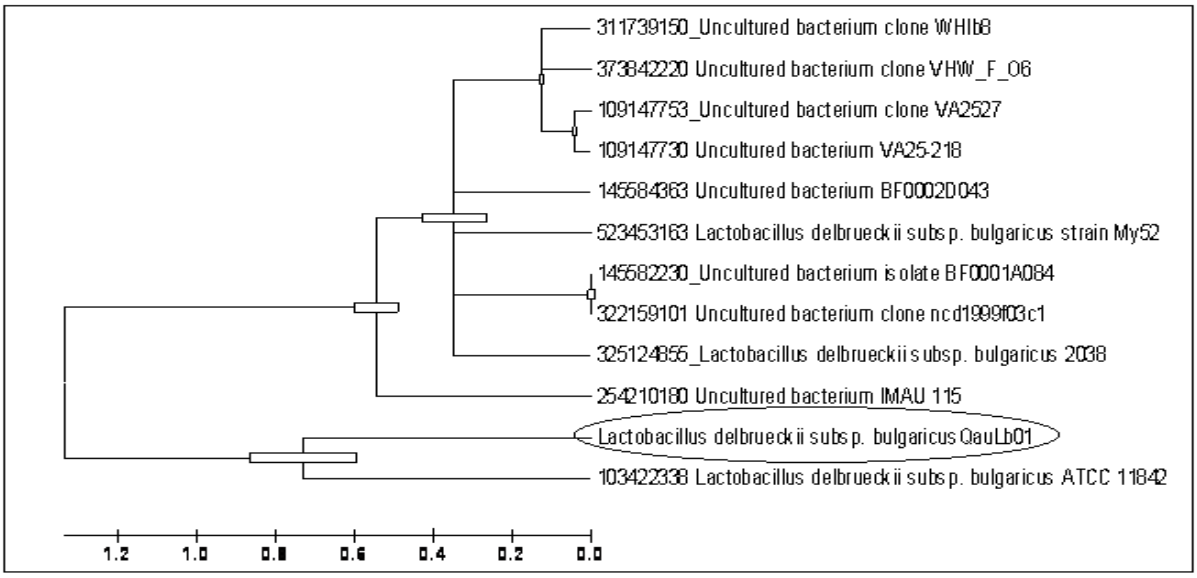

Fig. 2. Dendeogram of phylogenetic analysis of *Lactobacillus bulgaricus* subsp. *Delbrueckii.*

This study is in agreement with the finding of (Abd El Gawad *et al.*, 2010; Hassaïne, 2008; Khedid, 2009). Rapid acidification is the good candidate for the development of starter culture. Slow acid producers can be used as adjunct starter i.e. in the production of flavour compounds, playing the role of nonstarter culture in order to inhibit other strains as well as accelerate the ripening process (Herreros, 2003).

Strains were tested for their growth at different NaCl concentrations i.e. 2%, 4% and 6.5%. For cocci 2 and 4% NaCl was used, for rods 4 and 6.5% NaCl concentrations were used. Mostly *Lactobacilli* isolates showed better survival at 6.5%. rather than 4% of

NaCl concentration. Survival of *Lactococci* isolates in 2% and 4% NaCl concentrations was of equal ratio. When LAB are confronted with the decreased water activity i.e. during drying over the long period of time, salt tolerant LAB can accumulate betaine and carnitine. These solutes help in the survival in harsh environment. Such strains are important in food and feed industry where dried frozen starter cultures are used (Edwin *et al.*, 1996).

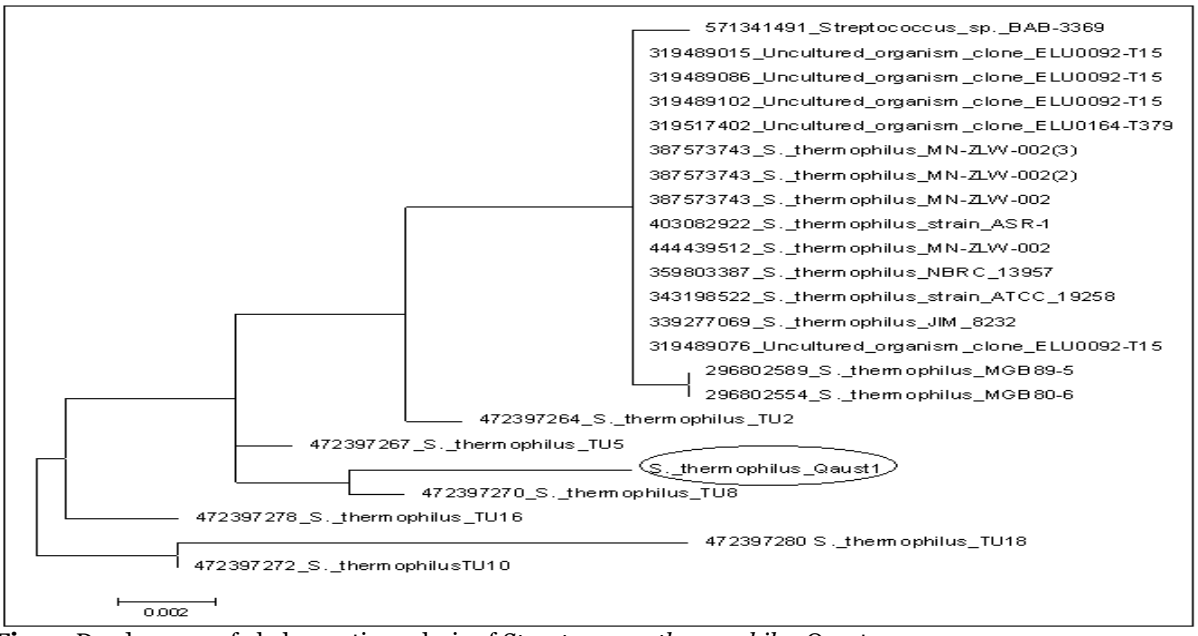

Fig. 3. Dendrogram of phylogenetic analysis of *Streptococcus thermophilus* Qaust1.

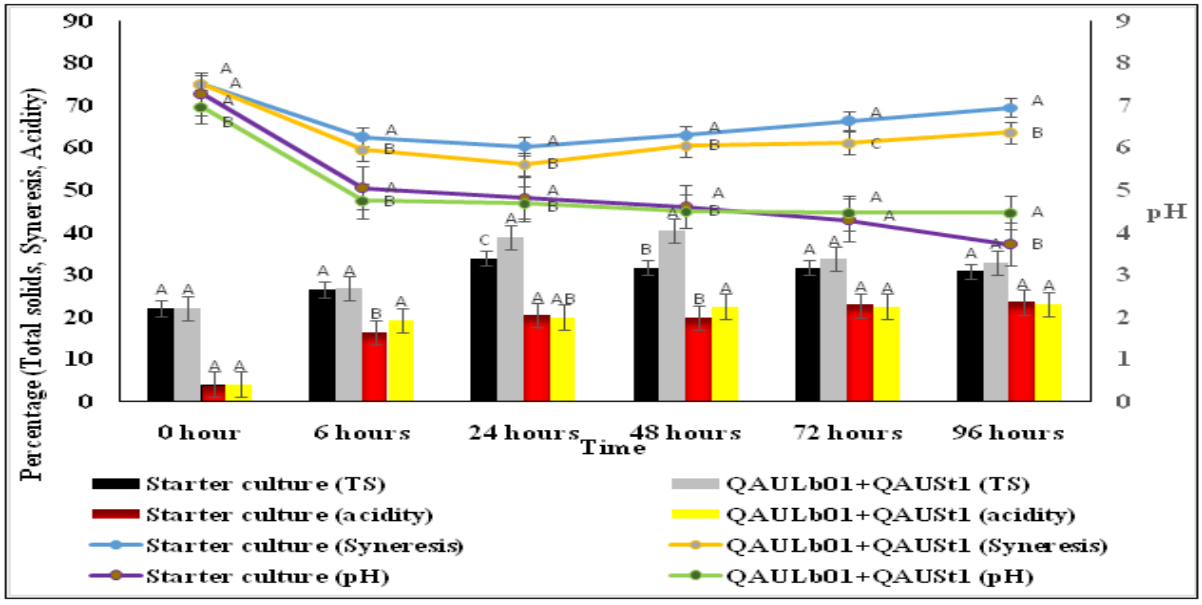

Fig. 4. Graph showing the change in pH, Acidity, Total Solid content, Syneresis after fermentation for 96 hr.

Selection of isolates for phylogenetic identification based on their growth rate and pH

Agglomerative hierarchical clustering (AHC)was used to group the isolates into different groups based on their growth rate and pH.

Partial sequencing 16SrRNA Gene and phylogenetic analysis of selected lactic acid bacteria

Molecular identification of selected isolates was carried out to confirm the identity of isolated strains through 16S DNA sequencing. The result of 16S

sequencing indicated that isolated strains were *Lactobacillus bulgaricus* subsp. *delbrueckii* and *Streptococcusthermophilus*, *Enterococcus mundtii* etc.

Phylogenetic analysis of 16S rRNA gene showed that the selected isolate belonged to genus *Lactobacillus***.**Our isolate is more homologous to the ATCC strain 11842 dairy products; Bulgarian yogurt. The sequence for *Lactobacillus delbrueckii* QAULB01is submitted with accession no KT021869 (Fig. 2).

Phylogenetic Analysis showed thatour isolate was more homologous to the *S. thermophilus TUS5* isolated from dairy ecosystem in Taif Saudi Arabia. The sequence for*Streptococcus thermophilus* Qaust01 is submitted in the gene bank under the accession no. KT021870in NCBI (Fig. 3).

Comparative fermentation ability of isolated strainswith commercial starter culture

On the basis of biochemical and technological characterization two strains were selected and inoculated in the milk in combination (*Lactobacillus delbrueckii* QauLb01+ *Streptococcus thermophilus* QauSt1). There affect was studied in comparaison withcommercial starter (Clerici-Sacco, Italy). Milk after fermentation was subjected to following Rheological parameters: pH, Solid content, Syneresis, titratable acidity, FTIR analysis, Sensory evaluation, Viscosity, Proteolysis and Mineral analysis etc.

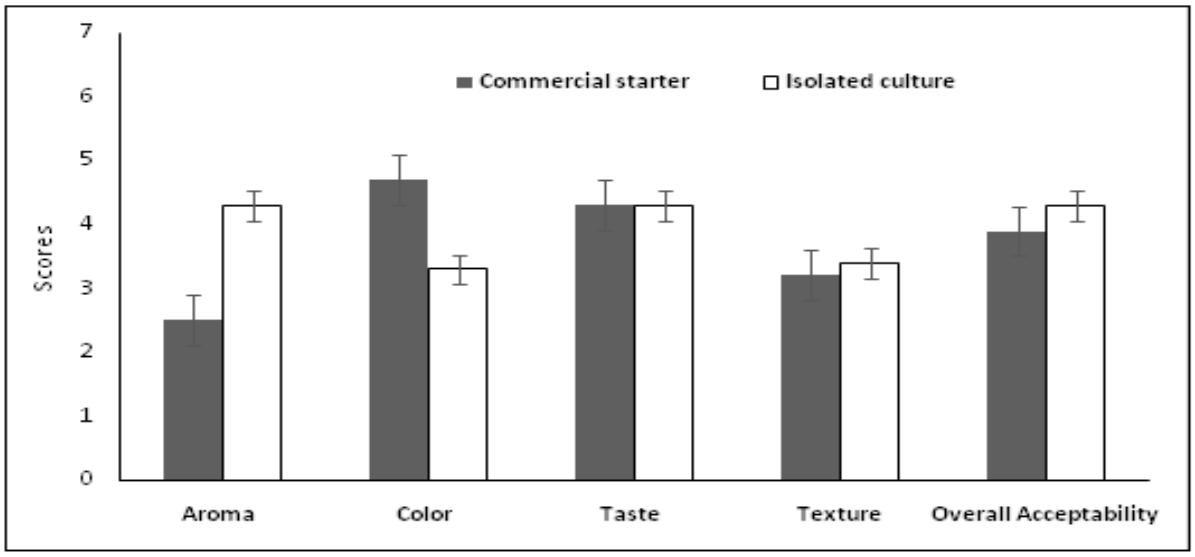

Fig. 5. Graph showing the sensory evaluation of commercial starter and Experiment 2 (QauSt1+QauLb01).

(Experiment 1= commercial starter), (Experiment 2= (QauSt1+QauLb01).

Effect of Isolates on The pH and titratable acidity of Fermented Milk with time

pH of Fermented Milk was continuously measured with the help of pH meter. Readings were noted after every 24 hours for 4 days. Experiment 2(QauSt1+QauLb01)showed rapid decrease in pH (6.98) from 0hr even greater than starter culture (control) pH (7.8), upto the 4th day where the pH (4.46) for Experiment 2became stable however for Experiment 1pH shown continuous decline (3.72). For the analysis of variance, Annova (Tukey) treatment was done. There was a significant difference between Experiment 1 and Experiment 2 at 0hr, as Experiment 1 shown higher mean value as indicated by A in the figure (Fig. 4). However after 96 hrs. Experimnet 2 (A) shown higher mean value with stability in pH and it was significantly different from Experiment 1(B).

Titratable acidity was measured with the equivalent weight of Lactic acid present in the sample by titrating it against the 0.1N NaOH used. Results showed that in Experiment 2(QauSt1+QauLb01)the

acidity was significantly increased after 6 hours and 48 hours but on 4th day the acidity was same as compared to Experiment 1 (starter culture). Annova shown that there was no statistically significant difference between the acidity of both experiments (Fig. 4). With the help of titratable acidity increase in acid production was also conformed. The presence of lactic acid is responsible for the sour taste, safety and improves microbial stability.

Solid content was assessed by the centrifugation followed by successive drying of yogurt sample in dry oven. The results shown that there was no statistically significant difference between solid content of both experiments for 6hrs as shown by (A). After 6 hrs. there was a significant difference between solid content of Experiment 2 (QauSt1+QauLb01). (A) (Fig. 4). However, at 96hrs although mean value for Experiment 2 was high than Experiment 1 but there was less significant difference between Experiment 2 and 1. The increase in solid content led to more thick consistency in milk fermented with isolated strains as compared to control sample. Increase in solid content could be related to the increase in proteolytic activity that contribute towards the taste, texture and flavoured compounds formation (Akabanda *et al.*, 2013).

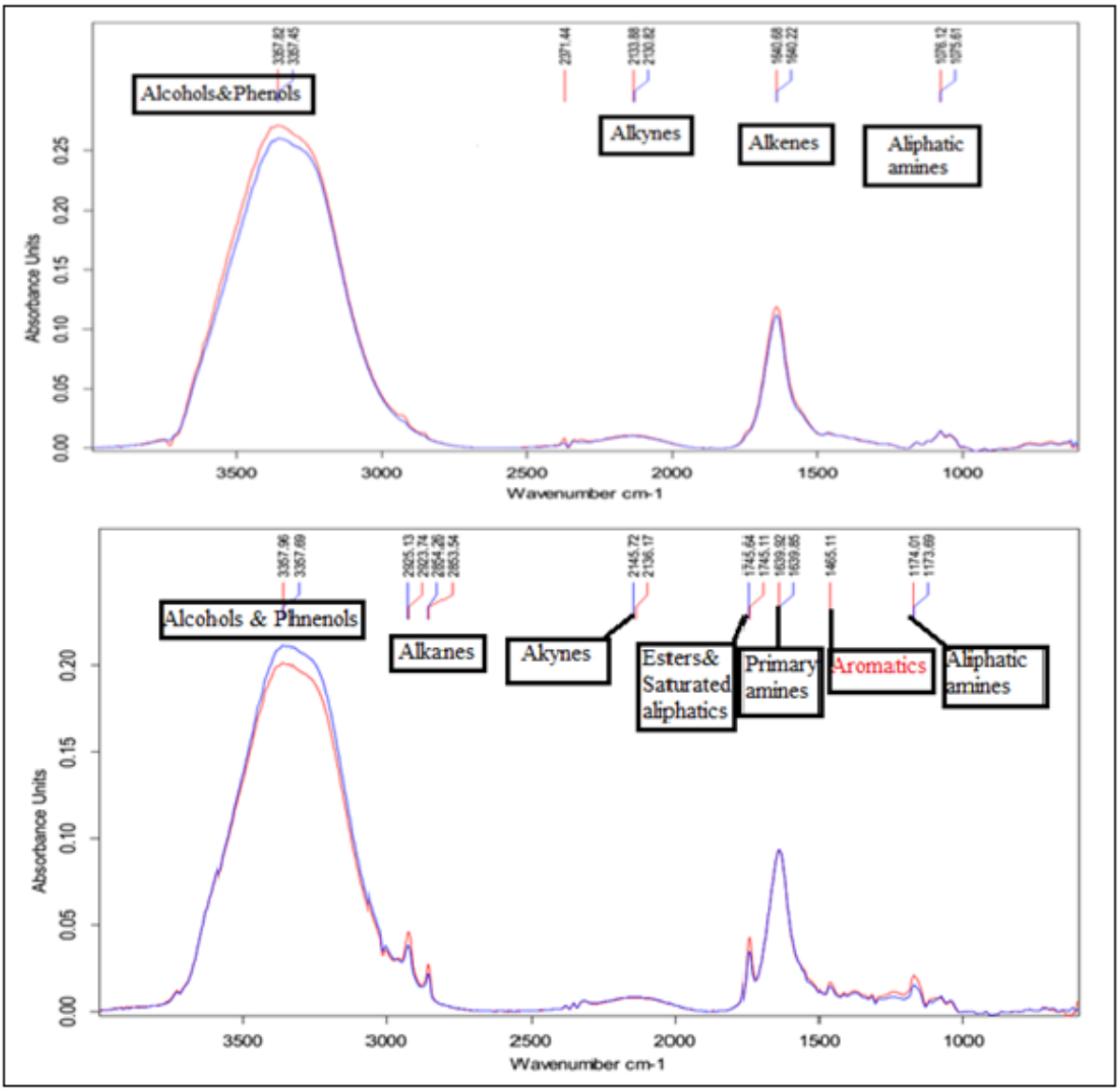

Fig. 6. FTIR spectrum of Commercial starter (blue line) and Experiment 2 as (QauSt1 + QauLb01; Red line); A: After inoculation at start of fermentation. B: after 6hrs of fermentation.

Liquid content that is present during the yogurt formation is called Syneresis. Syneresis is calculated after every 24 hours for about 4days. Syneresis was less in Experiment 2 as compared to the Experiment 1 and remained less until the 4th day.

There was statistically significant difference between syneresis of Experiment 1, as indicated by its higher mean value (A) and experiment 2 with less mean value (B) until 96 hrs. (Fig. 4). Study revealed that syneresis and whey separation is the result of instability of weak yogurt gel network. In this way protein molecule is unable to trap the water in three dimensional networks. Our finding is in agreement with the results that syneresis usually increases with decrease in pH (Olson and Aryana, 2008).

Sensory evaluation

The end products of Experiment 1 and Experiment 2 were tasted by trained Judges to check the organoleptic characteristics of fermented milk of both treatments. Though there was a huge significant difference in taste as compared to commercial starter. Relatively higher scores given to aroma, texture and taste of the product fermented by isolated cultures as compared to starter culture. In colour, more score was given to the stater culture (Fig. 5). The white colour of yogurt sample in Experiment 1 is caused by the light scattering of fat globules and casein micelles. While the yellowish colour of the product was due to the presence of riboflavin in the Experiment 2 (Walstra, 2006). Our results were contrary to the finding of (Aryana and McGrew, 2007).

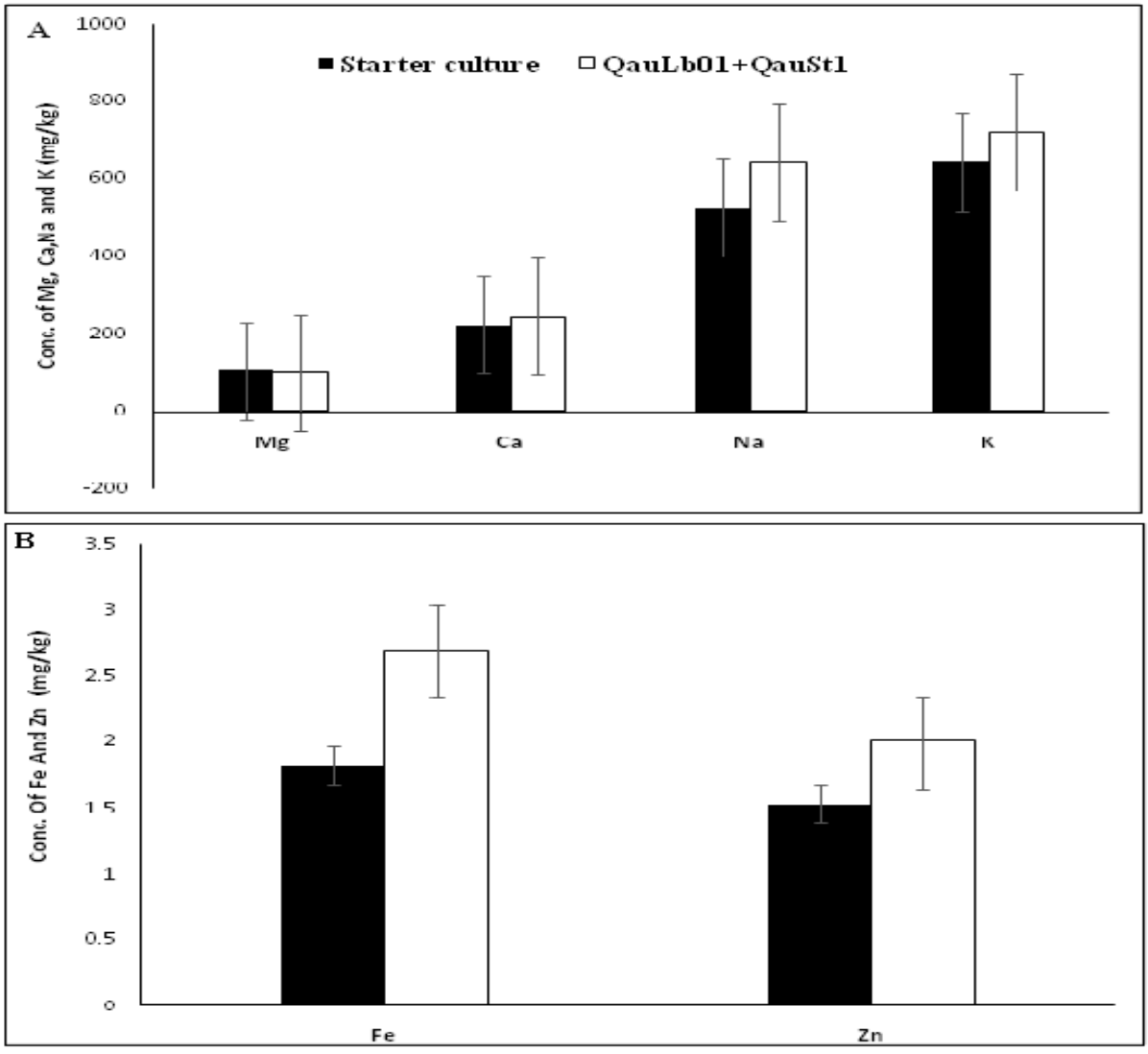

Fig. 7. Concentration of macronutrient (A) and oligo nutrients (B) in fermented milk produced by commercial starter culture and isolated cultures.

Viscosity

Viscosity of yogurt samples was calculated at sixth hour of incubation. Starter culture showed 400 to final viscosity: 200 milli poises**.** Experiment 2 (QauSt1 + QauLb01), 550 to final viscosity: 400 milli poiseswas observed. Significant increase in sample was quite evidentwhich means that Experiment2must had higher stabilityas compared to Starter culture. Increase in apparent viscosity might be due to rearrangement of the fat globules, more proteins (mainly caseins) from the serum get attached. As a result, the number of structure building components increase (Robinson, 2006). Increase in protein matrix as well as the formation of bigger number of bonds that are resistant to flow.

Estimation of biochemical changes during fermentation by FTIR

ATR-FTIR of Experiment 1 (commercial starter culture) and Experiment 2 (QauSt1 + QauLb01), were carried out at time of inoculation. Two main stretches and one in the minor stretch fingerprinting region were observed. The first wave number obtained is 3357cm^{-1} indicated the presence of alcohols and phenols. Peak at 1640 cm^{-1} indicated the presence primary amines or alkenes. Peak at 1076 cm^{-1} represented aliphatic amines (Fig. 6A).

However, after 6th hour of yogurt formation, peak at 1745.11cm^{-1} indicated the presence of esters. Similar ester groups within 1740-1750 cm-1 were also observed (Naumann, 2000). Ethyl esters, originated from the enzymatic or chemical esterification of acids with ethanol possess sweet and fruity notes and contribute to the aroma of dairy products (Molimard and Spinnler, 1996). Peaks at 2923.74 cm^{-1} and 12853.54cm^{-1} indicated the presence of alkanes group. Spectral region between 2850 and 3000cm^{-1} was dominated by $-CH_3$>CH_2 and >CH groups usually present in fatty acid components (Naumann, 2000; Nicolaou *et al.*, 2010; Sakhamuri, 2004). Peak at 1455.11cm^{-1} was only evident in Experiment 2 (Fig. 6B) represents the aromatics group as compared to commercial starter. These aroma compounds could be the result of microbial, enzymatic, or chemical transformations of lactose, lipids, citric acid, and proteins/amino acids present in milk. One major pathwayis through lipolysis or oxidation of fatty acids in milk fat, while another is microbiological transformations of lactose by lactic acid bacteria whichproduce flavour e.g. lactic acid, acetaldehyde, diacetyl, acetoin, and ethanol (Adriana *et al.*, 2014).

Impact of starter on Mineral contents

The average % age of ash for Experiment 2 was (0.765±0.035) which was higher than control (Experiment 1) (0.478 ± 0.072). The ash content determines the degree of mineralization indirectly that's why the digested ash was analyzed further by flame photometry and Atomic absorption spectrometer.

The digested ash samples were subjected to Flame Photometer and Atomic absorption spectrometer to analyze the Na, K and Ca, Mg, Fe, Zn respectively. In the present study, Experiment 2 shown the highest concentration of K720 mg/kg as compared to the Experiment 1 (starter culture) 645 mg/kg followed by other macronutrients i.e. Na, Ca and Mg. Also Experiment 2 (QauSt1 + QauLb01), showed higher concentration of all these nutrients in contrast to Experiment 1 (starter culture), except for Mg which was slightly higher for starter culture (Fig. 17A). These results are in accordance with the study of (Amellal-Chibane and Benamara, 2011) on natural yogurt in Algeria.

They reported that the potassium, calcium, sodium and magnesium concentrations were up to the mean values of 540.58 ± 38.33, 1950.41 ± 67.90, 684.72 ± 8.92 and 132.16 ±16.36 mg/L respectively while the highest concentration of these minerals was reported by (Bilandžić *et al.*, 2015) in Croatia. The oligo nutrients containing Fe and Zn content were also measured within a range of 2.7 mg/L and 2 mg/L respectively in the Experiment 2 greater than Experiment 1 (Fig. 7B) and these results again confirmed the findings of (González-Martín *et al.*, 2011; Amellal-Chibane and Benamara, 2011; Chekri *et al.*, 2012).

Conclusion

The study revealed that different genera of Lactic acid bacteria like *Lactobacillus delbrueckii*, *Streptococcus thermophilus, Enterococcus faecium* and *Enterococcus mundtii* are quite predominant in indigenous fermented milk product Dahi. Their presence imparts unique taste and good therapeutic features to the product due to their physiological and technological characteristics. Moreover, the study is unique as it signifies the isolation of wild Lactic acid bacteria (*Lactobacillus delbrueckii, Streptococcus thermophilus* etc.)from indigenous milk product Dahi, and their application in milk fermentation against international starter culture to reproduce the comparable results. The combination of these strains shown high solid content, less rate of syneresis, and high concentration of nutrients i.e. K, Na, Ca, Fe and Zn then the starter culture on fermented milk. Additionally, the application of these characterized strains for milk fermentation at pilot scale needs to be done as it will cut down the expenses on the import of foreign starter cultures for milk fermentation.

Acknowledgement

This work was supported by Higher Education commission of Pakistan.

References

AOAC. 1990. In Helrich K. (Ed.)Official methods of analysis .(15th ed.). Arlington, VA, USA.

Abd El Gawad IA, Abd El Fatah AM, Al Rubayyi KA. 2010. Identification and characterization of dominant Lactic acid bacteria isolated from traditional rayeb milk in Egypt. Journal of American Science **610,** 728-735.

Abu-Tarboush H. 1996. Comparison of associative growth and proteolytic activity of yogurt starters in whole milk from camels and cows. Journal of Dairy Science **793,** 366-371.
https://doi.org/10.3168/jds.s0022-0302(96)76373-7

Adriana P, Vodnar D, Carmen S, Simona M. 2014. Monitoring the Evolution of Major Chemical Compound in Dairy Products During Shelf-Life by FTIR. Food Science and and Technology **71,** 3-5.
https://doi.org/10.15835/buasvmcn-fst:10777

Akabanda F, Owusu-Kwarteng J, Tano-Debrah K, Glover RL, Nielsen DS, Jespersen L. 2013. Taxonomic and molecular characterization of lactic acid bacteria and yeasts in nunu, a Ghanaian fermented milk product. Food microbiology **342,** 277-283.
https://doi.org/10.1016/j.fm.2012.09.025

Amellal-Chibane H, Benamara S. 2011. Total contents of major minerals in the nature yoghurt and in the yoghurts with the date powder of three dry varieties.American Journal of Food and Nutrition **1(2),** 74-78.
https://doi.org/10.5251/abjna.2011.1.2.74.78

Aryana KJ, McGrew P. 2007. Quality attributes of yogurt with Lactobacillus casei and various prebiotics. LWT - Food Science and Technology **40,** 1808-1814.
https://doi.org/10.1016/j.lwt.2007.01.008

Bilandžić N, Sedak M, Đokić M, Božić, Đ, Solomun-Kolanović B, Varenina I. 2015. Differences in macro-and microelement contents in milk and yoghurt. Archives of Biological Sciences **67,** 1391-1397.
https://doi.org/10.2298/abs140312117b

Bridget OO, Enyioha LC. 2011. Phenotypic identification and technological properties of lactic acid bacteria isolated from selected commercial Nigerian bottled yoghurt.African Journal of Food Science **5(6),** 340 - 348.
http://www.academicjournals.org/ajfs.

Capozzi V, Russo P, Dueñas MT, López P, Spano G. 2012. Lactic acid bacteria producing B-group vitamins: a great potential for functional cereals products. Applied microbiology and biotechnology **966,** 1383-1394.
https://doi.org/10.1007/s00253-012-4440-2

Casey LS, Lichtman AH, Boothby M. 1992. IL4 induces IL-2 receptor ~75 B-chain gene expression and IL-2dependent proliferation in mouse T lymphocytes. Journal of Immunology. **148,** 3418-3428.

Chekri R, Noël L, Millour S, Vastel C, Kadar A, Sirot V, Leblanc JC, Guérin T. 2012. Calcium, magnesium, sodium and potassium levels in foodstuffs from the second French Total Diet Study. Journal of Food Composition and Analysis **252,** 97-107.
https://doi.org/10.1016/j.jfca.2011.10.005

Courtin CM, Delcour JAl. 2002. Arabinoxylans and endoxylanases in wheat flour bread-making. Journal of cereal science **35(3),** 225-243.
https://doi.org/10.1006/jcrs.2001.0433

Dagdemir E, Ozdemir S. 2008. Technological characterization of the natural lactic acid bacteria of artisanal Turkish White Pickled cheese. Interbational Journal of Dairy Technology. **61(2),** 133-140.
ttps://doi.org/10.1111/j.1471-0307.2008.00394.x

Edwin PWK, Teunissen PJM, DBJAM. 1996. Effect of Compatible Solutes on Survival of Lactic Acid Bacteria Subjected to Drying . Applied and environmental microbiology. **62(1),** 259-261.

Fhoula I, Najjari A, Turki Y, Jaballah S, Boudabous A, Ouzari H. 2013. Diversity and antimicrobial properties of lactic acid bacteria isolated from rhizosphere of olive trees and desert truffles of Tunisia. BioMed research international **2013,** 1-14
https://doi.org/10.1155/2013/405708

Fortune Akabanda JOK, Kwaku Tano-Debrah, Charles Parkouda 3, Lene Jespersen. 2014. The Use of Lactic Acid Bacteria Starter Culture in the Production of Nunu, a Spontaneously Fermented Milk Product in Ghana. International Journal of Food Science., **2014,** 1-11.
https://doi.org/10.1155/2014/721067

Fox P, Wallace J, Morgan S, Lynch C, Niland, E, Tobin J. 1996. Acceleration of cheese ripening. Antonie van Leeuwenhoek **702(4),** 271-297.
https://doi.org/10.1007/bf00395937

Gad GFM, Abdel-Hamid AM, Farag ZSH. 2014. Antibiotic resistance in lactic acid bacteria isolated from some pharmaceutical and dairy products. Brazilian Journal of Microbiology **45(1),** 25-33.
https://doi.org/10.1590/s151783822014000100005

Giraud E, Champailler A, Raimbault. 1994. Degradation of raw starch by a wild amylolytic strain of Lactobacillus plantarum.Applied and Environmental Microbiology **60,** 4319 - 4323.

Goin CD. 2010. Growth of Lactic Acid Bacteria: Infuence of Protocooperation, Bacteriophage Infection, and Prebiotic Carbohydrates.

González-Martín I, Hernández-Hierro J, Revilla I, Vivar-Quintana A, Ortega IL. 2011. The mineral composition (Ca, P, Mg, K, Na) in cheeses (cow's, ewe's and goat's) with different ripening times using near infrared spectroscopy with a fibre-optic probe. Food chemistry **127(1),** 147-152.
https://doi.org/10.1016/j.foodchem.2010.12.114

Gordon RE, Haynes WC, Pang CHN. 1973. The genus bacillus. Agricultural Research Service, US Department of Agriculture.

Guyot JP, Calderon M, Morlon-Guyot J. 2000. Effect of pH control on lactic acid fermentation of starch by *Lactobacillus manihotivorans* LMG 18010T. Journal of Applied Microbiology **88,** 176–182.

Hassaïne O, Zadi-Karam H, Karam NE. 2008. Phenotypic identification and technological properties of lactic acid bacteria isolated from three breeds dromedary raw milks in south Algeria. Journal of Food and Agriculture **20(1),** 46-59.

http://dx.doi.org/10.9755/ejfa.v12i1.5180

Hernandez K, Park YW. 2014. Evaluation of 20 Macro and Trace Mineral Concentrations in Commercial Goat Milk Yogurt and Its Cow Milk Counterpart. Food and Nutrition Sciences **5(10),** 889-895.
http://dx.doi.org/10.4236/fns.2014.510098

Herreros MA, Fresno JM, Gonzalez Prieto M. J, Tornadijo ME. 2003. Technological characterization of lactic acid bacteria isolated from Armada cheese (a Spanish goats' milk cheese). International Dairy Journal **13(6),** 469-479.
http://dx.doi.org/10.1016/s0958-6946(03)00054-2

Holland R, Coolbear T. 1996. Purification of tributyrin esterase from Lactococcus lactis subsp. cremoris E8. Journal of Dairy Research **630(1),** 131-140.
http://dx.doi.org/10.1017/s0022029900031605

Khedid K, Faid M, Mokhtari A, Soulaymani A, Zinedine A. 2009. Characterization of lactic acid bacteria isolated from the one humped camel milk produced in Morocco. Microbiological Research. **164(1),** 81-91.
http://dx.doi.org/10.1016/j.micres.2006.10.008

Mahmoudi F, Hadadji M, Guessas B, Kihal M. 2013. Identification and Physiological Properties of Bifidobactérium Strains Isolated from Different Origin. Journal of food Science and Engineering **3,** 196-206.

Mallesha SR, SD, Jagannath JH. 2010. Isolation and identification of lactic acid bacteria from raw and fermented products and their antibacterial activity. Recent Research in Science and Technology **2(6),** 42-46.

Maqsood S, Hasan F, Masud T. 2013. Characterization of lactic acid bacteria isolated from indigenous dahi samples for potential source of starter culture. African journal of Biotechnology **12(33),** 5226-5231
http://dx.doi.org/10.5897/ajb09.1172

Molimard P, Spinnler H. 1996. Review: Compounds involved in the flavor of surface mold-ripened cheeses: Origins and properties. Journal of Dairy Science **792,** 169-184.
http://dx.doi.org/10.3168/jds.s00220302(96)76348-8

Naeem M, Ilyas M, Haider S, Baig S, Saleem M. 2012. Isolation characterization and identification of lactic acid bacteria from fruit juices and their efficacy against antibiotics. Pakistan Journal of Botany **44,** 323-328.

Naumann K. 2000. Influence of chlorine substituents on biological activity of chemicals: a review. Pest management science **56(1),** 3-21
http://dx.doi.org/10.1002/(sici)15264998(200001)56:1<3::aid-ps107>3.3.co;2-g

Nicolaou SA, Gaida SM, Papoutsakis ET. 2010. A comparative view of metabolite and substrate stress and tolerance in microbial bioprocessing: from biofuels and chemicals, to biocatalysis and bioremediation. Metabolic engineering **12(4),** 307-331.
http://dx.doi.org/10.1016/j.ymben.2010.03.004

Nikita C, Hemangi D. 2012. Isolation, identification and characterization of lactic acid bacteria from dairy sludge sample. Journal of Environmental Research And Development Vol **7(1A).**

Olson D, Aryana K. 2008. An excessively high Lactobacillus acidophilus inoculation level in yogurt lowers product quality during storage. LWT-Food Science and Technology **415,** 911-918.

Patel A, Shah N, Prajapati J. 2013. Biosynthesis of vitamins and enzymes in fermented foods by lactic acid bacteria and related genera-A promising approach. Croatian Journal of Food Science and

Technology **52,** 85-91.

Ramakrishnan V, Balakrishnan B, Rai AK, Narayan B, Halami PM. 2012. Concomitant production of lipase, protease and enterocin by Enterococcus faecium NCIM5363 and Enterococcus durans NCIM5427 isolated from fish processing waste. . International Aquatic Research. 4, 14.

Reddy G, Altaf M, Naveena B, Venkateshwar M, Kumar EV. 2008. Amylolytic bacterial lactic acid fermentation—a review. Biotechnology advances **26(1),** 22-34.
http://dx.doi.org/10.1016/j.biotechadv.2007.07.004

Robinson BH. 2006. Lactic acidemia and mitochondrial disease. Molecular genetics and metabolism **89(1-2),** 3-13.
http://dx.doi.org/10.1016/j.ymgme.2006.05.015

Sakhamuri SB, Jamie Irudayaraj, Joseph Demirci Ali Z. 2004. Simultaneous determination of multiple components in nisin fermentation using FTIR spectroscopy.

Sierra G. 1957. A simple method for the detection of lipolytic activity of micro-organisms and some observations on the influence of the contact between cells and fatty substrates. Antonie van Leeuwenhoek **23(1),** 15-22.
http://dx.doi.org/10.1007/bf02545855

Sieuwerts S, De Bok FA, Hugenholtz J, Van Hylckama Vlieg JE. 2008. Unraveling microbial interactions in food fermentations: from classical to genomics approaches. Applied and Environmental Microbiology **7416,** 4997-5007.

Tchekessi CKC, IYB, Azokpota P, Agbangla C, Daube G, Scippo ML, Korsak N, Gotcheva V, Blagoeva G, Angelov A. 2014. Isolation and Quantification of Lactic Acid Bacteria from Traditional Fermented Products in Benin. International Journal of Current Microbiology and Applied Sciences **11(1-8),** 2319-7706.
ISSN: 2319-7706.
http://www.ijcmas.com

Vishnu C, Naveena BJ, Altaf MD, Venkateshwar M, Reddy G. 2006. Amylopullulanase: a novel enzyme of L. amylophilusGV6 indirect fermentation of starch to L(+) lactic acid. Enzyme and Microbial Technology **38(3-4),** 545-550.
http://dx.doi.org/10.1016/j.enzmictec.2005.07.010

Walstra P, Wouters JTM, Geurts TJ. 2006. Dairy Science and Technology. Taylor and Francis, Abingdon, Oxford, UK.
http://dx.doi.org/10.1016/j.fm.2007.02.003

Wouters JTM, Ayad EHE, Hugenholtz J, Smit G. 2002. Microbes from raw milk for fermented dairy products. International Dairy Journal **12(2-3),** 91-109.
http://dx.doi.org/10.1016/s0958-6946(01)001510

Zourari AAJP, Desmazeaud MJ. 1992. Metabolism and biochemical characteristics of yogurt bacteria. A review. Le lait, Dairy Science and Technology **72(1),** 1-34.
http://dx.doi.org/10.1051/lait:199211