

Isolation, Biochemical Characterization and DNA Identification of Yogurt Starters *Streptococcus thermophilus* & *Lactobacillus delbrueckii ssp. bulgaricus* in Gaza Strip

Kamal E. M. El Kahlout*, Ismail M. El Quqa, Mahmoud W. El Hindi, Tarek A. El Bashiti

Department of Biology & Biotechnology, Islamic University, Gaza, Palestine

Email: *kelkahlout@iugaza.edu.ps

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Abstract

Background: Milk is a healthy human nutrient, which is fermented to yogurt by lactic acid bacteria, mainly *Streptococcus salivarius ssp. thermophilus* and *Lactobacillus delbrueckii ssp. bulgaricus*. This study aimed to isolate thermophilic starter bacteria from homemade yogurt made in Gaza Strip. The study sought to define both the biochemical and molecular characteristics of the isolated bacteria. **Results:** Three of the isolates were identified as *Streptococcus thermophilus* while two isolates were recognized as *Lactobacillus delbrueckii ssp. bulgaricus*. All five identified isolates showed worthy acidification capacity. Based on these characters, strains were applied for making yogurt either as single pure cultures or as mixed cultures. When using mixed cultures from *S. thermophilus* and *L. bulgaricus*, all tested combinations showed positive results. **Conclusion:** Isolated strains showed remarkable biotechnological characters. The isolates are expected to stimulate and improve quality of the yogurt when they are used as mixed starter cultures.

Keywords

Thermophilic, Lactic Acid, Phenotypic, Genotypic, Yogurt

1. Introduction

Lactic acid bacteria are recognized as important industrial bacteria due to their fermentative capacity and their health and nutritious benefits. Yogurt is popular and familiar dairy product that is consumed in many countries around the world [1]. Mediterranean, Asian countries and in central Europe are regarded as the main areas where yogurt is produced and consumed [2]. Part of yogurt con-

sumed in Gaza Strip is manufactured by conventional methods where 1-day old yogurt is used as starter culture for fermenting the fresh milk. During the traditional homemade process, spontaneous uncontrolled fermentation conditions dominate. Such type of fermentation is considered as the primary method used to preserve milk.

Yogurt production mostly denotes the use of mixed starter (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) [3] [4]. Starters for yogurt production grow in a symbiotic association [5]. The bacteria play vital roles during fermentation, including lactose conversion to lactic acid, partial proteolysis of casein, development of aromatic compounds, developing characteristic viscosity and binding of water [1]. The high acidity of yogurt (0.85% - 0.95%) contributes to preservation of the product by inhibition growth of pathogenic or saprophytic bacteria [6]. A number of adopted manipulating processes offered to bring yogurt processing more attractive and give affordable product. Those processes include choice of competent starters that act to increase gelling capacity and bring reduction in syneresis. Due to variations in properties of lactic acid bacteria (LAB), it is important to make assessment of the strains from different origins to characterize efficient and effective strains having desirable properties relevant for yoghurt industry. Therefore, it is very important to screen, identify, classify and choose stains for upcoming applications in the industry [1]. For industrial applications, it is vital to identify the bacteria used for fermentation. Use of phenotypic and biochemical characterization is mostly unreliable for providing quality data about the bacteria and in some cases, the data and results are non-reproducible. These obstacles directed efforts to depend molecular approaches for characterization and subtyping (on strain level) of the isolated bacteria. Therefore, it is mandatory to create efficient and time-saving techniques to categorize the isolated bacteria regularly. Molecular methods are applicable as identification tools as they improve the efficiency of detection and enumeration [7] [8].

The present work aimed to isolate thermophilic yogurt bacterial strain's *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp *bulgaricus* from homemade traditional fermented milk and identify them. PCR-based techniques were employed together with the phenotypic categorization methods. Some relevant properties of the isolated bacteria were examined for biotechnological applications as candidate starters for production of yogurt.

2. Materials and Methods

Traditional homemade fermented milk samples: Six samples of cow's home-made traditional yoghurt were collected from three geographical areas of Gaza strip. Samples were collected in sterile cups and stored at 4°C until used. Ten grams of each sample homogenized and diluted by 90 ml sterile saline solution (0.85% NaCl) followed by 7-fold serial dilution for isolation [9].

Isolation of lactic acid bacteria strains

A 0.1ml taken from the dilutions and plated twice in M17 and MRS agar media used to detect cocci and bacilli, respectively. To prevent yeast contaminants, the media was complemented with 100 mg l⁻¹ of cycloheximide antibiotic. The cultured MRS and M17 agar media were incubated anaerobically by using the gas pack system to provide 42°C optimum temperature for *L. bulgaricus* and *S. thermophilus*, respectively. Colonies were randomly selected, then streaked on agar plates (MRS & M17) and incubated at 42°C to obtain pure cultures. For short-term preservation, isolated bacteria agar plates (MRS & M17) were kept at 4°C. For longer preservation, the isolates were stored in M17 and MRS broths supplemented by 20% glycerol as cryoprotectant [9].

Identification of the isolated lactic acid bacteria: Genus of the isolated bacteria was determined by employing morphological, phenotypic and biochemical techniques. All isolates were subjected to gram staining, catalase test, gas production from glucose test, grown at 15°C & 45°C, grown at 2%, 4% and 6.5% NaCl concentrations [10].

Profile of sugar fermentation of the isolates:

The API 50 CH system (Biomérieux, France) was used to demonstrate the carbohydrate fermentation profiles of the isolated bacteria. The database of ABIS online for bacterial identification was employed for elucidations of the results obtained from the fermentation profile tests [11].

Molecular identification for isolated bacteria:

Identity of the species confirmed by using species-specific primers by PCR using 16S rDNA sequences [12].

DNA isolation:

Genomic DNA of the isolated bacteria was extracted by Genomic DNA Purification Kit Brand WISARD Promega® for isolation of gram-positive bacteria according to instructions of the manufacturer. Until being used as template for PCR, the extracted DNA was stored at -20°C.

Primers

cysmet2F (forward): 5' GGAACCTGAAGGCTCAAT 3'

cysmet2R (reverse): 5' GTCAACCACGGTAAAGGTC 3'

Primers for the methionine biosynthesis gene

9699–9700 are primers applied for amplifying of the 16S rRNA gene.

9699 5' ATCCGAGCTCAGAGTTTGATCCTGGC 3'

9700 5' TCAGGTCGACGCTACCTTGTTACGAC 3'

Primers for the amplifying of the 16S rRNA gene

PCR conditions

BioRad, (USA) thermal cycler used for amplification of PCR. For synthesis of

methionine gene, conditions of PCR and reaction of the mixture were as follows:

Step 1: 94 °C for 2 min (initial denaturation)	}	45 cycles
Step 2: 94 °C for 30sec (denaturation)		
Step 3: 54 °C for 40 sec (annealing)		
Step4: 72 °C for 45 sec (elongation)		
Step 1: 72 °C for 10 min (final annealing)		

Volume of PCR reaction was 30 µl while the volume of DNA was 1 µl.

For 16S rRNA gene, PCR conditions and reaction mixture were as follows:

Step 1: 95 °C, 2 min (initial denaturation)	}	35 cycles
Step 2: 95°C, 1min (denaturation)		
Step 3: 56 °C, 1min (annealing)		
Step4: 72 °C, 1min (elongation)		
Step 1: 72 °C, 10 min (final annealing)		

Restriction analysis of 16S rRNA gene product

The restriction reaction of the 16S rRNA gene product was achieved by endonuclease enzyme *Eco RI*. Mixture of the reaction composed from 1 X of the restriction enzyme buffer, 10 µl of PCR product and 0.5 µl *Eco RI* [13].

Separation of Amplification Products

For preparation of separation gel, 1.5% agarose was dissolved in 100 µl 1 X TAE buffer, temperature was lowered to 45°C and 15 µl of ethidium bromide solution added with stirring. The agarose mixture was poured in casting stand then combs were placed and left to form wells [14]. PCR products (5 µl) were mixed with gel loading dye (2 µl). Starting from the second well, the samples loaded. In the first well, 100 bp DNA ladder was loaded as marker for molecular weight of the separated DNA. Electrophoresis at 80 V for 1 hour applied for the PCR products and the marker. The gel documentation system (CSL-MICRODOC, UK) was used to visualize the migrated amplification products. Successful amplification was confirmed by presence 1500 - 2000 bp DNA fragments [15] [16].

Assessment of fermentation performance of the isolates:

Preparation of fermented milk

To make fermented milk, pasteurized mixtures of 60 g/L of powdered milk were dissolved in 5 liters of cow milk [17]. Pasteurization process was carried out by heating the mixture at 80°C for 15 min in water bath, homogenized and then rapidly cooled to 45°C [18]. Each identified isolated types (*Lactobacillus bulgaricus* & *Lactobacillus thermophilus*) was examined for fermentation performance separately and in combined manner. For separated performance test, 1% of activated inoculums (10^6 - 10^7 cfu/ml) of isolated yogurt starters were added to the mixture. Activated inoculums were prepared by incubation in 10%

(w/v) sterilized reconstituted skimmed milk and incubated at 37°C for 18 - 24 h before using pasteurized milk as the growth medium. For combined fermentation performance assessment, the milk mixture was inoculated with 5% (10^6 - 10^7 cfu/ml) of *S. thermophilus*, *L. bulgaricus* (1:1) with stirring after inoculation and incubated at 42°C in a shaker water-bath for 8 h. 50 ml of samples were taken in aseptic sterile cups for microbiological and biochemical tests every 1.5 - 2 hrs. Each experiment repeated three times [19] [20].

Measurement of pH and titration of acidity

The pH meter probes directly into a homogenized sample of the fermented milk to determine the pH. For determination of acidity (lactic acid %), 0.1 N NaOH was used to titrate 1 ml of homogenized fermented milk while phenolphthalein used as indicator (0.1%, 3 drops). Titration process was performed with continuous stirring until the color of the solution appeared clearly as pink color lasting for 30 seconds. Volume of NaOH recorded and acid concentration calculated by using the formula:

$$LA\% = \frac{10 * V \text{ NaOH} * 0.009 * 0.1}{W} * 100\%$$

where 10 is the dilution factor; W is weight of titrated sample; V NaOH is the volume of sodium hydroxide consumed for neutralizing the lactic acid and the 0.1 is normality of the sodium hydroxide [21].

3. Results

3.1. Isolation and Biochemical Identification of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*

Six samples of traditional homemade yoghurt prepared from cow's milk were collected and used to isolate and identify LAB. The isolates were incubated under anaerobic conditions at 42°C for 72 hours. As indicated previously, M17 agar medium used to isolate of *S. thermophilus* and MRS agar medium used to isolate *L. delbrueckii*. Observations of Gram-stained cells under a light microscope indicated that the isolated strains appeared in blue-purple color proving that the isolates are gram-positive bacteria. Two morphological structures were observed for isolates; one was cocci (isolates cultured on M17 medium) appearing predominantly as pairs or forming chains, while the isolates grown on MRS medium appeared as long bacilli with curved ends and mostly showing themselves as single cells or as chains of 3 - 4 cells (Figure 1).

The biochemical tests included catalase and gas production from glucose fermentation. Catalase test was negative (no babbles) for both isolates. Additionally, when isolates were examined for gas production by glucose fermentation, no gas accumulation seen in the tubes even after five days. With respect to the ability of isolates to grow at different concentrations of NaCl; cocci bacteria showed inability to grow even at 2% NaCl concentration. Bacilli isolates showed sensitivity to 4% and 6.5% NaCl concentration. Examining ability of isolates to grow at different temperatures was another criterion for classification. Cocci isolates

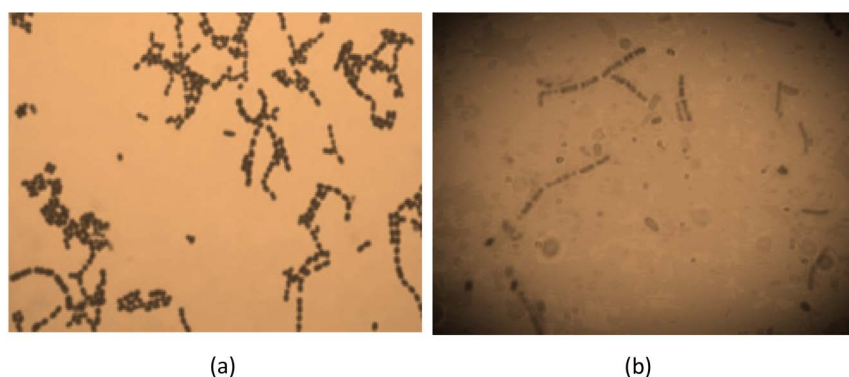


Figure 1. Microscopic appearance of bacterial isolates (a) Cocci and (b) Bacilli (40×).

showed inability to grow at 10°C and 45°C while the bacilli isolates were not able to grow at 10°C and 15°C, but they grew well at 45°C.

The well-intentioned test to identify the differences among strains is carbohydrate fermentation using API 50 CHL assays, which allows the fermentation of 49 different sugars as shown in **Table 1**. The profiles of the strains after the final reading were identified using ABIS online for bacterial identification (http://www.tgw1916.net/bacteria_logare_desktop.html). After processing of the obtained results with ABIS online software, the strains *Lactobacillus* and *Streptococcus* isolates identified as *Lactobacillus dekabueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* strains with high percentage of reliability (96% for *Lactobacillus* and 84% for *Streptococcus*, respectively). According to MRS isolates, glucose, fructose, lactose and mannose were affirmative with these isolates.

3.2. DNA Identification

Molecular approaches are applied to validate results obtained from the physiological and biochemical assays to identify and diagnose streptococci and lactobacilli isolates using PCR method and to achieve selective identification for proposed isolates. This is realized by methionine biosynthesis gene and primers (cysmet2F and cysmet2R) to identify the isolated strains of yoghurt samples. PCR method used to amplify these genes including partial amplification of the methionine gene giving 700 bp DNA products in all isolates (**Figure 2**).

Discrimination of lactobacilli from streptococci was achieved by ARDRA using endonucleases *Eco RI*. The amplified 16S rRNA genes showed nearly 1500 bp products in all isolates of lactobacilli and streptococci (**Figure 3**). When the entire amplified products were digested by *Eco RI* restriction enzyme, two sizes of lactobacilli around 700 and 850 bp were observed for the lactobacilli while for the streptococci the two sizes were around 650 bp and 850 bp as shown in **Figure 4(a)** & **Figure 4(b)**.

3.3. Assessment of Fermentation Performance of Strains

The viable counts of starter cultures during fermentation

Fermentation performance of the isolated bacteria carried out by using single

Table 1. Biochemical test results of the isolates by using identification system API50 CHL.

#	0	1	2	3	4	5	6	7	8	9
SUGER	NC	GLY	ERY	DARA	LARA	RIB	DXYL	LXYL	ADO	MDX
<i>BACILLUS A1</i>	–	–	–	–	–	–	–	–	–	–
<i>BACILLUS A2</i>	–	–	–	–	–	–	–	–	–	–
<i>COCCUS B1</i>	–	–	–	–	–	–	–	–	–	–
<i>COCCUS B2</i>	–	–	–	–	–	–	–	–	–	–
<i>COCCUS B3</i>	–	–	–	–	–	–	–	–	–	–
#	10	11	12	13	14	15	16	17	18	19
SUGER	GAL	GLU	FRU	MNE	SBE	RHA	DUL	INO	MAN	SOB
<i>BACILLUS A1</i>	–	+	+	+	–	–	–	–	–	–
<i>BACILLUS A2</i>	–	+	+	+	–	–	–	–	–	–
<i>COCCUS B1</i>	+	+	–	–	–	–	–	–	–	–
<i>COCCUS B2</i>	–	+	–	–	–	–	–	–	–	–
<i>COCCUS B3</i>	+	+	+	–	–	–	–	–	–	–
#	20	21	22	23	24	25	26	27	28	29
SUGER	MDM	MDG	NAG	AMY	ARB	ESC	SAL	CEL	MAL	LAC
<i>BACILLUS A1</i>	–	–	+	–	–	–	–	–	–	+
<i>BACILLUS A2</i>	–	–	+	–	–	–	–	–	–	+
<i>COCCUS B1</i>	–	–	+	–	–	–	–	–	–	+
<i>COCCUS B2</i>	–	–	–	–	–	–	–	–	–	+
<i>COCCUS B3</i>	–	–	–	–	–	–	–	–	–	+
#	30	31	32	33	34	35	36	37	38	39
SUGER	MEL	SAC	TRE	INU	MLZ	RAF	AMD	GLYG	XLT	GEN
<i>BACILLUS A1</i>	–	–	–	–	–	–	–	–	–	–
<i>BACILLUS A2</i>	–	–	–	–	–	–	–	–	–	–
<i>COCCUS B1</i>	–	+	–	–	–	–	–	–	–	–
<i>COCCUS B2</i>	–	+	–	–	–	–	–	–	–	–
<i>COCCUS B3</i>	–	+	–	–	–	–	–	–	–	–
#	40	41	42	43	44	45	46	47	48	49
SUGER	TUR	LYX	TAG	DFUC	LFUC	DARL	LARL	GNT	2 KG	5 KG
<i>BACILLUS A1</i>	–	–	–	–	–	–	–	–	–	–
<i>BACILLUS A2</i>	–	–	–	–	–	–	–	–	–	–
<i>COCCUS B1</i>	–	–	–	–	–	–	–	–	–	–
<i>COCCUS B2</i>	–	–	–	–	–	–	–	–	–	–
<i>COCCUS B3</i>	–	–	–	–	–	–	–	–	–	–

NC: NEGATIVE CONTROL, GLY: GLYCEROL, ERY: ERYTHRIOL, DARA: D-ARABINOSE, LARA: L-ARABINOSE, RIB: RIBOSE, DXYL: D-XYLOSE, LXYL: L-XYLOSE, ADO: ADONITOL, MDX: β -METIL-D-XYLOSIDE, GAL: GALACTOSE, GLU: D-GLUCOSE, FRU: D-FRUCTOSE, MNE: D-MANNOSE, SBE: L-SORBOSE, RHA: RHAMNOSE, DUL: DULCITOL, INO: INOSITOL, MAN: MANNITOL, SOB: SORBITOL, MDM: α -METHYL-D-MANNOSIDE, MDG: α -METHYL-D-GLUCOSIDE, NAG: N-ACETYL-GLUCOSAMINE, AMY: AMIGDALIN, ARB: ARBUTIN, ESC: ESCULIN, SAL: SALICIN, CEL: CELLOBIOSE, MAL: MALTOSE, LAC: LACTOSE, MEL: MELIBIOSE, SAC: SACCAHROSE, TRE: L-TREHALOSE, INU: INULIN, MLZ: MELEZITOSE, RAF: D-RAFFINOSE, AMD: AMIDON, GLYG: β -GLYCOPEN, XLT: XYLITOL, GEN: β -GENTIOBIOSE, TUR: D-TURANOSE, LYX: D-LYXOSE, TAG: TAGAROSE, DFUC: D-FUCCOSE, LFUC: L-FUCCOSE, DARL: D-ARABITOL, LARL: L-ARABITOL, GNT: GLUCONATE, 2 KG: 2-KETO-GLUCONATE, 5 KG: 5-KETO-GLUCONATE.

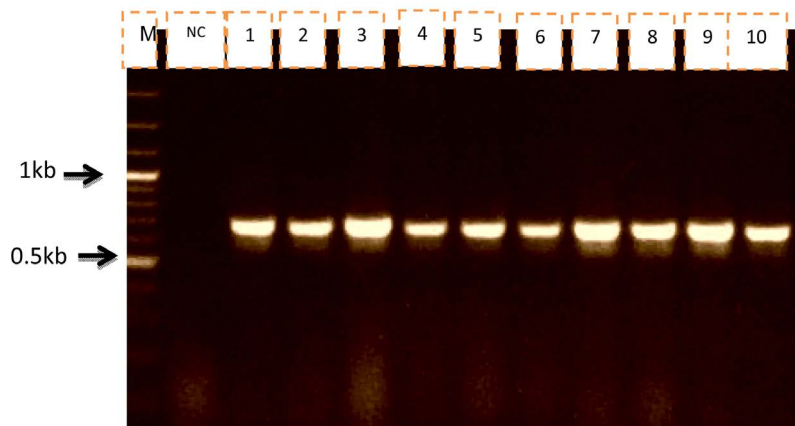


Figure 2. Partial amplification of the methionine biosynthesis gene in traditional home-made yoghurt isolates. Lanes 1, 2: *Coccus B1*, lanes 3, 4: *Coccus B2*, Lanes 5, 6: *Coccus B3*, Lanes 7, 8: *Bacillus A1*, Lanes 9, 10: *Bacillus A2*, NC: Negative control (no DNA control), M: 100 bp DNA ladder.



Figure 3. Amplification of 16S rRNA gene from isolates that amplified methionine biosynthesis gene product. Lanes 1 - 4: *Lactobacilli A1, A2* respectively, lanes 5 - 10: *Streptococci B1, B2, B3* respectively, M: 100 bp DNA ladder.

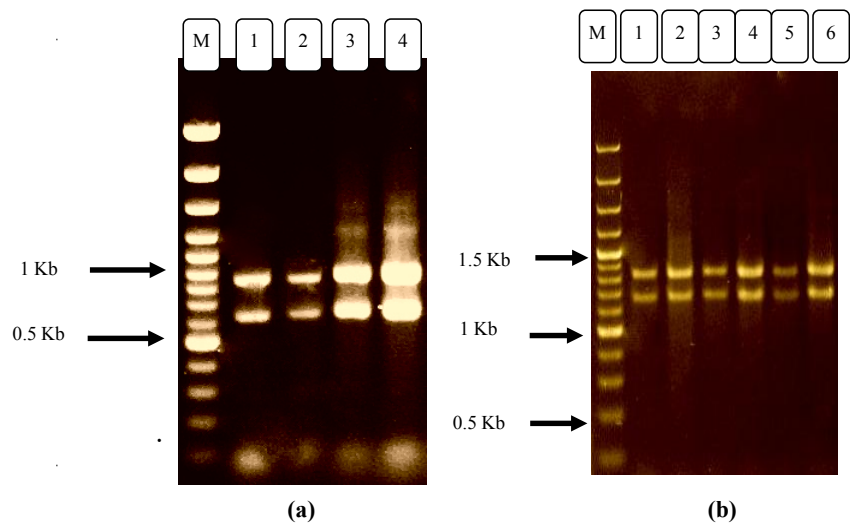


Figure 4. Restriction analysis of 16S rRNA gene with *EcoRI*. (a) Restriction analysis of 16S rRNA gene with *EcoRI* for *Lactobacilli A1, A2* respectively (b) Restriction analysis of 16S rRNA gene with *EcoRI* for *Streptococci B1, B2, B3* respectively, M: 100 bp DNA ladder.

and mixed cultures from two *L. bulgaricus* isolates (A1 & A2), and three *S. thermophilus* isolates (B1, B2 & B3) (see **Table 2** & **Figure 5**). Results of single fermentation processes showed that initial total counts of bacterial population were approximately similar with minor increases after 2 h of incubation. The populations of starters for all cultures during fermentation course showed a steady increase in number. *L. bulgaricus* A2 isolate showed the fastest growing pattern among all single cultures. This conclusion is supported by the higher counts of *L. bulgaricus* A2 than any other single strains at any time interval while *S. thermophilus* B1 isolate exhibited the least count number. When performing mixed fermentation processes by blending *L. Bulgaricus* & *S. thermophilus* (A1:B3) for 8 hours.

3.4. Biochemical Analysis

Changes in the total acidity and pH

Table 2 and **Figure 6** showed pH change and total acidity changes during fermentation period while **Table 2** & **Figure 7** showed changes in total acidity. Results pointed that pH decrease was connected with increase of lactic acid produced during fermentation time. The results showed fermentation performed by mixed inoculums of *L. bulgaricus* & *S. thermophilus* (1:1) gave higher acidity and lower pH compared with fermentation performed by single isolates.

4. Discussion

The biochemical tests included catalase and gas production from glucose fermentation. Catalase test was negative (no babbles) for both the isolates. Additionally, when the isolates were examined for gas production test from glucose

Table 2. Effect of culture composition on total acidity, acidification characteristics and viable counts of pure & mixed cultures grown on milk at 42°C.

(h) Time Isolated Strain	Viable counts of the starter culture strains (log10cfu-ml)					Changes in the Total acidity						Total acidity				
	0	2	4	6	8	0	2	4	6	8	24	0	1.5	3	4.5	6
<i>Lb. bulgaricus</i> A1	3.95	4.04	5.41	6.30	7.61	6.39	6.28	5.90	5.44	4.98	4.88	0.17	0.44	0.48	0.70	1.20
<i>Lb. bulgaricus</i> A2	4.54	4.61	5.65	6.90	8.03	6.39	6.24	5.86	4.73	4.71	4.67	0.17	0.40	0.50	1.18	1.38
<i>S. thermophilus</i> B1	4.39	4.66	5.05	5.80	6.71	6.39	6.33	5.73	5.17	4.88	4.80	0.18	0.19	0.23	0.31	0.51
<i>S. thermophilus</i> B2	4.61	4.82	5.32	6.43	7.26	6.40	6.38	6.11	5.29	5.01	4.98	0.18	0.22	0.27	0.36	0.47
<i>S. thermophilus</i> B3	4.11	4.48	5.51	6.68	7.52	6.38	6.35	6.05	5.15	4.99	4.85	0.18	0.24	0.31	0.44	0.63
Mix (A1:B1)	4.70	4.95	6.05	7.48	8.20	6.39	5.99	5.45	4.88	4.68	4.64	0.17	0.44	0.54	0.84	1.66
Mix (A1:B2)	4.63	4.91	6.39	7.64	7.91	6.39	6.20	4.90	4.71	4.63	4.57	0.17	0.54	0.60	1.20	1.88
Mix (A1:B3)	4.76	5.02	6.18	7.61	8.33	6.40	6.21	5.16	4.83	4.67	4.61	0.17	0.54	0.78	1.36	1.76
Mix (A2:B1)	3.93	4.33	6.30	7.07	7.61	6.40	6.12	5.02	4.83	4.67	4.40	0.18	0.52	0.74	1.32	1.90
Mix (A2:B2)	3.98	4.53	6.54	7.34	8.05	6.41	6.14	5.10	4.61	4.58	4.50	0.18	0.54	0.66	1.46	1.84
Mix (A2:B3)	4.84	4.91	5.57	7.12	8.02	6.39	6.17	4.93	4.41	4.38	4.20	0.17	0.48	1.16	1.80	2.80

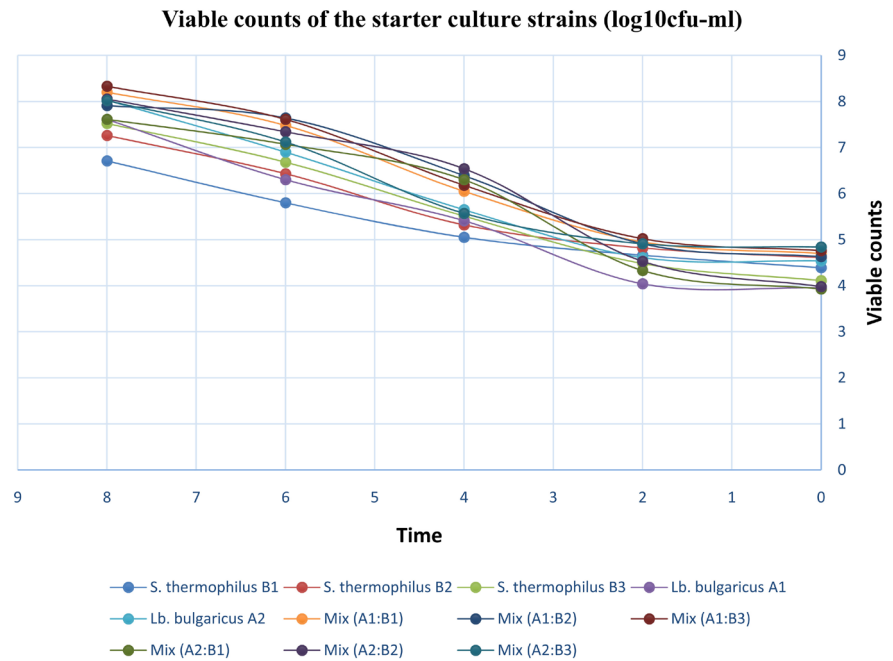


Figure 5. Changes in the viable counts of the isolated bacteria during fermentation of raw milk for 8 h at 42°C.

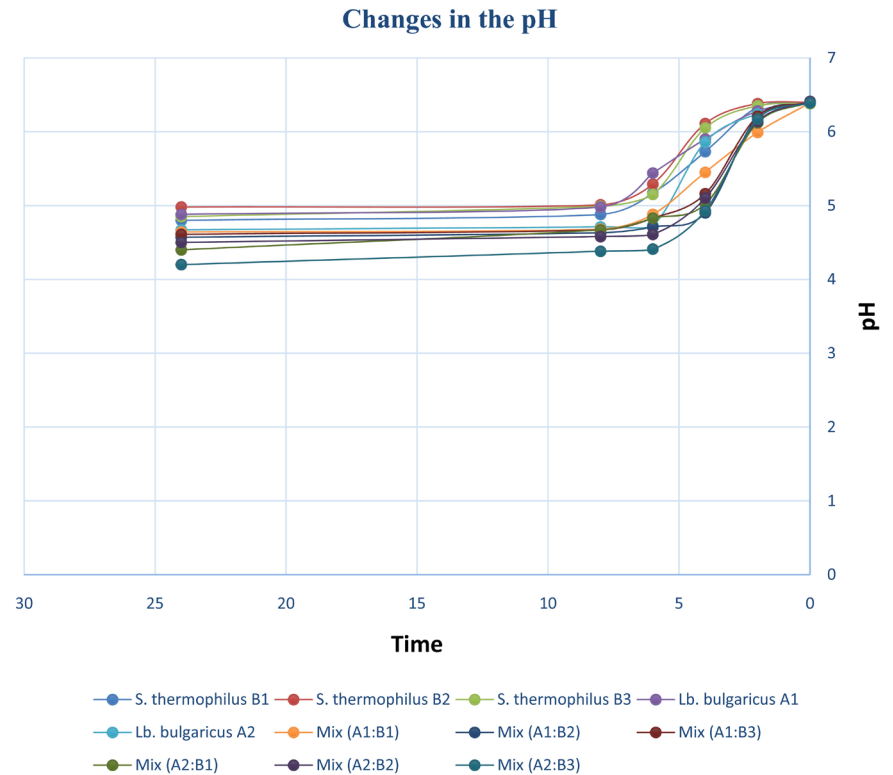


Figure 6. Changes in the pH of the isolated bacteria during fermentation of raw milk at different times on 42°C.

fermentation, there was no gas accumulation in test tubes. This means that the isolates behave as homofermenters of glucose and this result agreed with those

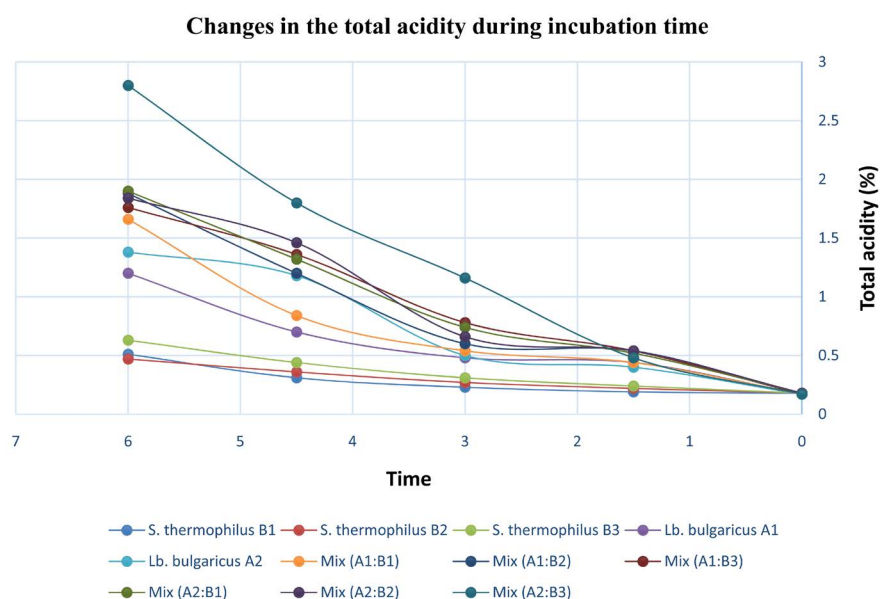


Figure 7. Changes in total titratable acidity of the isolated bacteria during fermentation of raw milk for 6 h at 42°C.

obtained by authors [22] [23] [24]. The cocci isolate show no growth at 2% NaCl while bacilli isolates grew at that concentration. This showed that bacilli isolates were sensitive to 4% and 6.5% concentration and this is in agreement with results obtained by others [25] [26] [27]. Cocci isolates were unable to grow 10°C while they grow very well at 45°C and this result agreed with those in literature [28] [29]. Bacilli isolates did not grow at 15°C but grew 45°C. According to the results obtained from online ABIS software, API 50 CH fermentation tests showed that the cocci isolates belong to *S. thermophilus* and the bacilli isolates belong to *L. delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* strains with high percentage of reliability. This study applied quick and selective identification method for characterization of both *L. bulgaricus* and *S. thermophilus* from mixed cultures using a specific PCR based method of methionine biosynthesis gene and restriction analysis method of 16S rRNA gene using *Eco RI* as previously reported [30]. Molecular diagnosis of the isolates showed that lactobacilli produced a different profile than that of streptococci when performing restriction analysis (Figure 4(a) & Figure 4(b)) and this result agreed with a previous study [13]. The present work seeks to identify yoghurt starters isolated from traditional homemade yoghurt in Gaza Strip. As stated in previous studies, the amplicons of the methionine biosynthesis genes are present in *L. lactis* and *L. helveticus* thus, it is applicable for discrimination of these cells. The discrimination of *L. bulgaricus*, *L. lactis* and *L. helveticus*, attained by ARDRA as devised previously [13]. From our findings *L. bulgaricus* A2 isolate showed the fastest growth pattern among all single cultures. Abu-Tarboush (1996) contradicted this finding by stating that *lactobacilli* were always less numerous than the *streptococci* during fermentation of camel milk for 4 h at 42°C. However, our results agree with Lore *et al.* 2005, who found that the total LAB counts in *suusac*

(a Kenyan traditional fermented camel-milk) were $6.8 \log_{10}\text{cfu/ml}$ and the prominent genus was *Lactobacillus* ssp. Likewise, Abdel Moneim *et al.* 2006, stated the main genus was *Lactobacillus* in garris product (Sudanese traditional fermented camel milk). Our results showed that the final pH after 24 h fermentation of the mixed cultures were lower than pH of single cultures. This was supported by Carrasco *et al.* (2005) who stated that the pH of blend cultures were much lower than pH of the pure cultures. Also when Abu-Tarboush (1996) studied the action of various strains of commercial cultures in whole camel milk, he displayed that the last pH of the blends *L. bulgaricus* with the other isolates of *S. thermophilus* gave lower values comparing with isolates alone at 42°C for 4h. The results obtained from this work showed that the pH of the fermented raw milk by the isolated starters ranged from 4.20 to 4.98 which is compatible with (Lore *et al.*, 2005) results that described for suusac with pH around 4.30, but were higher than the results described in garris (pH 3.25 - 3.40) which is Sudanese traditional fermented camel milk.

5. Conclusion

Two isolates of *L. delbrueckii* ssp. *Bulgaricus* and *S. thermophilus* were selected to produce starters. These starters are low in cost and are easily affordable for production of yoghurt at small and large scale. The whole information obtained from the study proved the need to conjoint phenotypic, biochemical and molecular techniques for identifying screened *L. bulgaricus* and *S. thermophilus* from natural sources for approving them as starters for manufacturing of yogurt as they were mostly imprecisely identified by phenotypic and biochemical tests only. PCR-ARDRA was found to be beneficial tool for identification of homofermentative and thermophilic dairy bacterial species or subspecies. Distinguishing between isolated cultures by PCR-ARDRA is recommended for identification of lactic acid bacteria in combination with API 50 CHL. After testing and assessing the isolated strains on yogurt production, the data indicated that certain microbial and biochemical changes happen during fermentation of milk. The results showed that microorganisms differed in growth, acid production and titration of acidity. Generally, mixed yogurt culture manifests superior growth, acid production and titrable acidity than single starter cultures. Finally, identifying the starters of yogurt is the first step for using these starters for production in the dairy industry. It is important also to screen all parameters of the isolated LAB concerning their suitability as starter cultures, including acidifying activity, aroma compounds production, particularly acetaldehyde and diacetyl, exopolysaccharides production and resistance toward bacteriophage invasion. Determination of such characteristics would be helpful for industrial applications in future. Analysis of yoghurt starters is a complicated process and involves various techniques, especially phenotypic, biochemical, molecular-based and even genomic techniques.

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Authors' Contributions

Dr. Kamal El Kahlout and Dr. Tarek El Bashiti designed the study, analyzed and interpreted the data, supervised all experiments, identified lactobacilli using 16S-ARDRA and drafted and reviewed the manuscript. Ismail El Quqa was in charge of isolating the lactobacilli and their identification by Biochemical tests, isolated DNA from lactobacilli and was responsible for 16S rDNA. Mahmoud El Hindi revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Sampling process was part of the master duties of the student and no legal or ethical permissions found needed from the ethics commission for such type of sampling work.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

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Abbreviations

LAB: Lactic Acid Bacteria, TAE: Tris Acetate EDTA, Cfu: Colony for unit, LA: lactic acid, NaOH: sodium hydroxide, MRS: de-man, Rogosa and sharpe medium, NaCl: sodium chloride, CHL assays: Carbohydrate fermentation kit, PCR: polymerase chain reaction, ARDRA: Amplified Ribosomal DNA Restriction Analysis, Eco RI, rRNA: ribosomal ribonucleic acid, EPS: Exopolysaccharide.