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Isolation and Characterization of Lactic Acid Bacteria from Fermented Goat Milk in Tajikistan

Gyu-Sung Cho[1,5], Claudia Cappello[2], Katrin Schrader[3], Olakunle Fagbemigun[4], Folarin A. Oguntoyinbo[4,7], Claudia Csovcsics[5], Niels Rösch[1], Jan Kabisch[1], Horst Neve[1], Wilhelm Bockelmann[1], Karlis Briviba[5], Monica Modesto[2], Elisabetta Cilli[6], Paola Mattarelli[2], and Charles M.A.P Franz[1]*

Max Rubner-Institut, Federal Research Institute for Nutrition and Food, Departments of [1]Microbiology and Biotechnology, Hermann-Weigmann-Str. 1, D-24103 Kiel, Germany
[2]Department of Agricultural Food Sciences, University of Bologna, Viale Fanin 42, I-40127 Bologna, Italy
[3]Department of Safety and Quality of Milk and Fish Products, Hermann-Weigmann-Str. 1, D-24103 Kiel, Germany
[4]Department of Microbiology, Faculty of Science, University of Lagos, Akoka, Lagos, Nigeria
[5]Physiology and Biochemistry of Nutrition, Haid-und-Neu-Str. 9, D-76131 Karlsruhe, Germany
[6]Department of Cultural Heritage, University of Bologna, Via degli Ariani 1, I-48121 Ravenna, Italy
[7]A.R. Smith Department of Chemistry and Fermentation Sciences, Appalachian State University, Boone, NC 28608

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**Corresponding author*
Phone: +49-(0)431-609-2340;
Fax: +49-(0)431-609-2306;
E-mail: Charles.Franz@mri.bund.de

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The lactobacilli associated with a fermented goat milk product from Tajikistan were isolated to characterize their technological properties and antibiotic resistances in order to assess their suitability for development as starter cultures. In this study, twenty three strains were identified by 16S rRNA sequencing as typical dairy-associated lactic acid bacterial strains, *i.e. L. plantarum*, *L. pentosus*, *L. delbrueckii*, *L. helveticus* and *L. paracasei*. These strains were generally susceptible to most antibiotics tested in this study and this allowed a selection of strains as safe starters. The draft genomes of four representative strains were sequenced and the number of contigs of the four assembled genomes ranged from 51 to 245 and the genome sizes ranged from 1.75 to 3.24 Mbp. These representative strains showed differences in their growth behavior and pH-reducing abilities in *in vitro* studies. The co-inoculation of these *Lactobacillus* spp. strains together with a yeast *Kluyveromyces marxianus* MBT-5698, or together with the yeast and an additional *Streptococcus thermophilus* MBT-2, led to a pH reduction to 3.4 after 48 h. Only in the case of fermentation inoculated with the co-culture, the viscosity of the milk increased noticeably. In contrast, fermentations with single strains did not lead to gelation of the milk or to a decrease in the pH after 24h. The results of this study provide a comprehensive understanding of the predominant lactobacilli related to Tajikistani fermented milk products.

Keywords: Lactic acid bacteria, fermentation, *Lactobacillus*, whole genome sequencing, milk

Introduction

Traditional fermentations are in many rural areas of the world still the main method for food processing and preservation. These fermentations are often done empirically, based on cultural knowledge, and they often involve using back-slopping to inoculate a new fermentation with a small portion of a previous successful batch [1]. In traditional fermentations, the biochemical changes of the product during fermentation are brought about by wild bacteria or yeasts, which originate from the raw materials [2, 3]. These are mainly lactic acid fermentations, in which lactic acid bacteria (LAB) such as *Lactobacillus* and *Leuconostoc* spp. predominate. However, in many traditional fermentations,

the fermentation may also involve mixed cultures of yeasts, bacteria and fungi [4] and some microorganisms may participate in parallel, while others act in a sequential manner with a changing dominant microbiota during the course of the fermentation. Which of these microorganisms occur is often not known for many of such products. There is still a lack of scientific knowledge on the course of many of these fermentations, as the preparation of many traditional fermented foods today remains a house art [3, 5].

While fermentations of milk in Europe predominantly rely on the use of LAB starter cultures and these bacteria are predominantly associated with European milk products, in African and Asian countries milk fermentations appear to be mixed fermentations with LAB and often involving *Lactococcus lactis*, *Streptococcus*(*S.*) *thermophilus* and *L. delbrueckii*, but yeasts such as *Saccharomyces cerevisiae* and *Candida* spp. also may play a role in the fermentation [3, 6, 7]. As yeasts in traditional fermentations can occur in high numbers, they may have a technological role and possibly also an input on the typical organoleptic properties of the products.

Goat milk is often fermented in small pastoral communities in northern Tajikistan by traditional methods. These sour milk fermentations mostly rely on spontaneous fermentation and are made from raw, unpasteurized milk without defined starter cultures. An example is fermented goat milk from the Yaghnob Valley in Tajikistan, which is traditionally produced by back-slopping, and has been previously studied with respect to the yeasts occurring in the product [8]. So far however, the LAB, especially the lactobacilli associated with the product, has not been identified. In this study, we report on the identification of the lactobacilli associated with Tajikistan's traditionally fermented milk products, their technological properties like acidifying and milk coagulating abilities, as well as their antibiotic resistances, in order to assess their suitability for development as starter cultures.

Materials and Methods

Bacterial Strains and Growth Conditions

Twenty three presumptive *Lactobacillus* strains were obtained from a previous study that investigated yeasts from fermented goat's milk in Tajikistan [8]. In this study, we focused on the *Lactobacillus* strains with regard to their functional and safety characteristics and their suitability as starter culture for goat's milk fermentation. The strains were cultured aerobically in MRS (de Man, Rogosa and Sharpe) (Roth, Germany) broth at 37°C for 18 h and plated out on MRS agar at least twice before use in further experiments. The *S. thermophilus* MBT-2 and *K. marxianus* MBT-5698 cultures were fermented milk isolates from our own culture collection. *S. thermophilus* was cultured in M17 (Merck, Darmstadt, Germany) broth at 42°C and *K. marxianus* was cultured aerobically in malt extract (Merck) broth at 25°C. Fresh overnight cultures were used to prepare stock solutions and these were kept at −80°C in MRS broth containing 20% (v/v) glycerol (Merck, Germany).

Phenotypic Characterization and Determination of Lactic Acid Configuration

The Gram-reaction was determined by the KOH method using 3% (w/v) aqueous KOH and visible amounts of bacterial colonies on glass slides according to Powers [9]. Growth at 10 and 45°C in MRS broth was evaluated after 24 h and 48 h of incubation, and the catalase reaction was tested with 3% (v/v) H_2O_2 as described by Mathara *et al*. [10]. The type and amount of D(-) and L(+) isomers of lactic acid produced from glucose were determined by the UV method using a commercial test kit (r-biopharm, Germany), following the manufacturer's instruction. The carbohydrate fermentation profiles were assessed using the API50 CH (BioMerieux, Germany) identification system.

16S rRNA Gene Sequencing and Strain Genotyping by RAPD-PCR

The total genomic DNA of the 23 strains was isolated from 4 ml of fresh overnight cultures grown at 37°C in MRS broth using the method of Pitcher *et al*. [11] as modified by Björkroth and Korkeala [12]. The 16S rRNA gene was amplified using PCR. The PCR products were amplified in 50 µl volumes containing 100 ng template DNA, 1 × *Taq* DNA polymerase buffer (GE Healthcare), 125 µM of each dNTP (Peqlab, Erlangen, Germany), 25 pM of each forward and reverse primer (16S fw 5′-AGA GTT TGA TCM TGG CTC AG-3′ and 16S rev 5′-TAC GGY TAC CTT GTT ACG ACT-3′) and 1.5 U *Taq* DNA polymerase (GE Healthcare). The PCR reaction was done using an initial denaturation step at 94°C for 3 min, followed by 32 cycles of 94°C for 30 sec, 55°C primer annealing for 30 sec, 72°C extension for 1 min 30 sec, followed by a final extension step at 72°C for 5 min. All PCR products were purified using PCR cleaning columns (Qiagen, Hilden, Germany) and subsequently sequenced at GATC Biotech (Cologne, Germany). The sequences of 16S rRNA gene PCR products were compared to those present in the EzTaxon database [13].

The randomly amplified polymorphic DNA (RAPD) PCR reaction was performed in a 50 µl volume using 100 ng chromosomal DNA, 1 × *Taq* DNA polymerase buffer (GE Healthcare), 125 µM of each dNTP's (Peqlab), 50 pM of primer M13 (5′-GAG GGT GGC GGT TCT-3′) 3 mM $MgCl_2$ and 1.5 U *Taq* DNA polymerase (GE Healthcare) and methods as described before [14]. The PCR products were subjected to electrophoresis in 1 × TBE buffer for 16.5 h at 48 V on 1.8% (w/v) agarose (Peqlab) gels. Gels were stained with ethidium bromide (Roth) and were visualized using a

Fluorchem Imager 5500 system (Alpha Innotech, USA). The profile of RAPD band patterns was analyzed using the BioNumerics software packages (V 7.1 Applied Math, St-Martens-Latem, Belgium). The fingerprints were compared using the unweighted pair group method with arithmetic averages (UPGMA) clustering method.

Whole Genome Sequence

The total genomic DNA of selected lactobacilli was isolated using the peqGOLD Bacterial DNA Kit (Peqlab, Erlangen, Germany). For paired-end sequencing, the library of genomic DNA was prepared with an Illumina Nextera XT Library Prep Kit (Illumina, USA) and sequencing was done on the MiSeq sequencer with 2 × 250 cycles. The raw paired-end sequencing data containing adapter sequences were trimmed using the Trimmomatic (v. 0.32) pipeline [15] and then *de novo* assembled with SPAdes (v. 3.11.1) [16]. The qualities of the obtained draft genome sequences were evaluated with the QUAST tool [17] and all contigs that were longer than 500 bp were used for annotation by the RAST server [18]. *In silico* analyses to identify acquired antibiotic resistance genes were done using the Resfinder pipeline [19], while plasmid related sequences were detected using the Plasmidfinder pipeline [20].

Fermentation of Goat's Milk with *Lactobacillus* Strains

To determine the growth and acidification ability of potential starter strains, commercial, pasteurized goat's milk was obtained from a local supermarket in Germany and was used in fermentation experiments. The pasteurized goat's milk was inoculated with the *Lactobacillus* isolates identified in this study as *L. plantarum* TJA 26B, *L. delbrueckii* TJA 31, *L. paracasei* TJB 4 and *L. helveticus* TJA 10. The strains were inoculated singly at 1×10^7 CFU /ml, or all four strains were inoculated together at this inoculation level. In addition, in one fermentation, the four selected strains were co-inoculated with *S. thermophilus* MBT-2 (1×10^7 CFU /ml) and *Kluyveromyces marxianus* MBT-5698 (5×10^6 CFU /ml), while in another fermentation the 4 selected strains were co-inoculated with only the yeast *K. marxianus* (5×10^6 CFU /ml). The yeast was chosen as *K. marxianus* was previously identified as a major component of the yeast population of this fermented product [8].

The milk was fermented in 50 ml volumes at 30°C for 48 h and the pH and numbers of bacteria were determined after 0 h (immediately after inoculation), 24 h and 48 h. For enumeration, 1 ml of milk was removed and diluted in quarter-strength Ringer's solution in a ten-fold dilution series. Appropriate aliquots from appropriate dilutions were plated out onto MRS agar (Merck, Darmstadt, Germany) plates for determining the *Lactobacillus* counts and on M17 (Merck) agar for the *S. thermophilus* counts (only for the fermentation that contained *S. thermophilus* MBT-2. The yeast count was determined by plating onto YGC-agar (Merck) to which 0.2% of a 10% tartaric acid solution was added after autoclaving, to adjust the pH to 4.6. Both MRS (anaerobic) and M17 plates (aerobic) were incubated at 30°C for 48 to 72 h, while YGC agar plates were incubated at 25°C for 72 h.

Determination of Rheological Properties

For the measurement of the viscosity, a rheometer MCR 302 (Anton Paar, Ostfildern, Germany) was used. Aliqouts of 0.7 ml of the fermented product were placed into the cone and plate measuring system (diameter 50 mm) using a syringe without needle. A flow curve was recorded at a temperature of 20°C using a logarithmic ramp of the shear rate (10 to 1,000 s^{-1}). These measurements were performed after 24 h and 48 h of fermentation.

Antibiotic Resistance Profile

The susceptibilities of the strains towards antibiotics were determined using the LAB susceptibility test medium (LSM) [21]. The minimum inhibitory concentrations (MICs) of ampicillin, gentamicin, tetracycline, erythromycin, streptomycin, vancomycin and chloramphenicol (Sigma, Germany) were determined. In order to do this, the overnight fresh cultures were inoculated at a concentration of 1×10^6 CFU /ml in 100 µl of LSM using a 96-well plate (Merck, Darmstadt, Germany), which contained a two-fold dilution series of each of the antibiotics. The MIC breakpoint values for each antibiotic were adopted from EFSA [22].

Results

Identification of the Lactic Acid Bacteria Strains and RAPD Strain Typing

Twenty three predominant *Lactobacillus* strains were isolated from fermented milk from Tajikistan using MRS agar plates. All strains were gram positive, catalase-negative, produced lactate as an end product of metabolism and had rod-shaped morphology, and could therefore be characterized as presumptive lactobacilli. In order to identify the strains further to species and strain level, bacterial growth at different temperatures, the enantiomers of lactate produced, sequencing of the 16S rRNA gene and RAPD PCR strain typing were done.

The lactobacilli could be identified as belonging to one of five species, *i.e. L. paracasei, L. pentosus, L. delbrueckii*, *L. helveticus* or *L. plantarum* (Table 1) by uploading the 16S rRNA gene sequence into the EzTaxon database and searching for the nearest relatively. The relatedness scores for the EzTaxon analyses and the corresponding strain identifications are shown in Table 1. The cluster analysis of RAPD-PCR fingerprints of lactobacilli from Tajikistani fermented milk obtained with primer M13 is shown in Fig. 1. Three major subgroups (I, II, and III) clustering at a correlation value of r = 43.3% could be discriminated. Most of the isolates clustered in subgroup I at r = 67.9% and all of these, except for one (TJB 4) which was identified as *L. paracasei* in 16S rRNA gene sequencing, showed at least 2 common bands (Fig. 1). All isolates from subgroup I except

Table 1. Differential characteristics of *Lactobacillus* isolates and 16S rRNA sequences.

Strain	Gas production	Growth temperature		Lactate enantiomer	Related Taxa	% Similarity of 16S Sequences [13]
		15°C	45°C			
TJA 1	-	-	-	D	*L. delbrueckii* subsp *bulgaricus*	99.72%
TJA 2	-	-	+	D	*L. delbrueckii* subsp *bulgaricus*	99.65%
TJA 6A	-	+	+	DL	*L. helveticus*	99.65%
TJA 7A	-	+	+	DL	*L. plantarum*	99.93%
TJA 9	-	-	+	D	*L. plantarum*	99.93%
TJA 10	-	-	+	DL	*L. helveticus*	100%
TJA 11B	-	+	-	DL	*L. helveticus*	100%
TJA 11S	-	-	+	DL	*L. helveticus*	99.79%
TJA 12	-	-	+	D	*L. delbrueckii* subsp *bulgaricus*	99.71%
TJA 16	-	-	+	DL	*L. helveticus*	100%
TJA 17	-	-	+	D	*L. delbrueckii* subsp *bulgaricus*	99.58%
TJA 19	-	-	+	D	*L. delbrueckii* subsp *bulgaricus*	99.72%
TJA 20	-	-	+	DL	*L. helveticus*	99.93%
TJA 21	-	-	+	D	*L. delbrueckii*subsp *bulgaricus*	99.72%
TJA 24A	-	-	+	DL	*L. helveticus*	100%
TJA 26B	-	+	-	DL	*L. plantarum*	99.79%
TJA 26S	-	+	-	DL	*L. pentosus*	99.81%
TJA 27	-	-	+	DL	*L. helveticus*	100%
TJA 28	-	-	+	D	*L. delbrueckii* subsp *bulgaricus*	99.79%
TJA 29	-	-	+	D	*L. delbrueckii* subsp *lactics*	99.71%
TJA 31	-	-	+	D	*L. delbrueckii* subsp *bulgaricus*	99.79%
TJA 32	-	-	+	DL	*L. helveticus*	99.79%
TJB 4	-	+	-	L	*L. paracasei*	99.93%

Production of D-, L-, and DL lactic acid as an end product.
+: Positive growth; -: negative growth

TJB 4 were identified by 16S rRNA gene sequencing as *L. delbrueckii* isolates. These strains all produced the D-lactate enantiomer, which is typical for the *L. delbrueckii* species. Most *L. delbrueckii* strains are, in accordance with

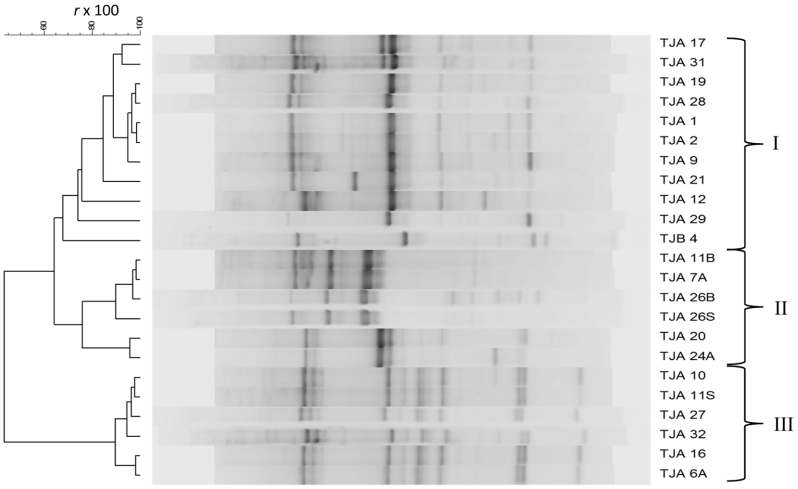

Fig. 1. Dendrogram obtained by UPGMA of correlation value *r* of RAPD-PCR fingerprint patterns with primer M13 of strains isolated from fermented milk of Tajikistan.

their usual habitat of milk, able to ferment lactose (Table 2) and accordingly all these strains in this study were able to utilize lactose.

RAPD fingerprinting furthermore showed that isolates in subgroup II clustered at r = 75.9% and 4 isolates (TJA 11B, 7A, 26B, and 26S) clustered very close at r = 89.8% (Fig. 1). A comparison in the EzTaxon database identified three of these isolates in subgroup II (TJA 11B, 7A, and 26B) as *L. plantarum* strains and one isolate (TJA 26S) as *L. pentosus*.

The six strains in subgroup III showed a very similar band pattern, clustered together very closely at r = 89.7%, and were identified as *L. helveticus* strains by 16S rRNA gene sequencing. Interestingly, the two strains TJA 20 and TJA 24A clustering together with the *L. plantarum* strains in subgroup II were also identified as *L. helveticus* strains by 16S rRNA gene sequencing. All strains identified by 16S rRNA gene sequencing as *L. helveticus,* and which clustered together in subgroup III and subgroup II in RAPD fingerprinting, grew well at 45°C, and they all fermented glucose, mannose, and lactose, which is typical for these bacteria.

Table 2. Carbohydrate fermentation of *Lactobacillus* strains.

No. of strains / Carbohydrate fermentation	*L. plantarum* (3) *L. pentosus* (1)	*L. helveticus* (8)	*L. delbrueckii* (10)	*L. paracasei* (1)
LARA	+	-	-	-
RIB	-	-	-	-
GAL	+	+ (75%)	-	+
GLU	+	+	+ (80%)	+
FRU	+	+ (37.5%)	+	+
MNE	+	+	+	+
RHA	+	-	-	-
MAN	+	-	-	+
MDM	+	-	-	-
MDG	-	-	-	-
NAG	+	+ (75%)	-	+
AMY	+	-	-	-
ARB	+	-	-	+
SAL	+	+ (25%)	-	+
CEL	+	-	-	+
MAL	+	-	-	+
LAC	+	+	+	+
MEL	+	-	-	-
SAC	+	-	-	+
TRE	+	+ (50%)	-	+
MLZ	+	-	-	+
RAF	+	-	-	-
GEN	+	-	-	+
TUR	+	-	-	+
DARL	+	-	-	-
GNT	+	-	-	+

LARA L-arabinose, RIB D-Ribose, GAL D-galactose, GLU D-glucose, FRU D-fructose, MNE D-mannose, RHA L-rhamnose, MAN D-mannitol, MDM methyl-α-D-mannopyranoside, MDG methyl-α-D-glucopyranoside, NAG N-acetylglucosamine, AMY amygdalin, ARB arbutin, SAL salicin, CEL D-cellobiose, MAL D-Maltose, LAC D-lactose, MEL D-melibiose, SAC D-saccharose, TRE D-trehalose, MLZ D-melezitose, RAF D-raffinose, GEN gentiobiose, TUR D-turanose, DARA D-arabitol, GNT potassium gluconate

+ : Positive fermentation; percentage was calculated like positive number divided by total number of species, - : negative fermentation.

Whole Genome Data Analysis of Potential Starter Cultures

The genome sequences of all major technologically important LAB are available, which has given new insight into functional genomics of LAB associated with food fermentations [23]. In our study, the genomes of four strains selected as representative of each species were sequenced and analyzed for typical functions related to fermentation activities and for the absence of transferable antibiotic resistance genes. Briefly, the contigs of the four assembled genomes ranged from 51 to 245 and the genome sizes ranged from 1.75 to 3.24 Mbp (Table 3). The largest N_{50} value was 131,900 for *L. plantarum* TJA 26B and the lowest N_{50} value was 21,570 for *L. helveticus* TJA 10. No plasmid replication-related sequences were detected in the genome sequences of these strains. Two strains each possessed at least one bacteriocin gene, which may be important for inhibiting the growth of closely related bacterial strains. Thus, the presence of bacteriocin genes for helveticin J and plantaricins EF, JK and N could be determined for *L. helveticus* TJA 10 and *L. plantarum* TJA 26B, respectively. Furthermore, the *L. helveticus* TJA 10, *L. plantarum* TJA 26B and the *L. paracasei* TJB 4 strains contained genes encoding a citrate lyase involved in citrate metabolism and an acetolactate synthase gene which is responsible for production of the diacetyl precursor α-acetolactate (Table 3).

Characterization of Goat's Milk Fermentation

One strain of each species, *i.e. L. plantarum* strain TJA 26B, *L. delbrueckii* strain TJA 31, *L. paracasei* strain TJB 4 and *L. helveticus* strains TJA 10 were selected for further studies. When inoculated singly at approx. 1×10^7 CFU /ml in pasteurized goat milk, each of the strains grew well in the milk, but showed quite different acidification behavior. The *L. plantarum* TJA 26B showed only a ca. 1 log increase in growth to reach a final level of ca. 1×10^8 CFU /ml (Fig. 2). This growth led to only a moderate pH decrease

Table 3. Genome data[a] of selected *Lactobacillus* strains from Tajikistani fermented milk.

Strain	TJA 10	TJA 26B	TJA 31	TJB 4
GenBank Accession No.	QNXC00000000	QXEU00000000	QNXB00000000	QXET00000000
No. of contigs	254	122	54	244
Largest contig	61,465	341,241	424,489	180,575
N50	21,570	131,900	107,123	51,965
GC-content (mol%)	36.73	44.5	49.79	46.4
Total length (bp[1])	1,889,241 bp	3,243,521 bp	1,742,687 bp	2,945,278 bp
Plasmid sequence	n.d.[2]	n.d.	n.d.	n.d.
CDS (coding sequence)	2124	3233	1861	3186
tRNA	52	54	66	56
rRNA	6	4	10	11
ncRNA	3	4	3	3
Bacteriocin	Helveticin J	Plantaricin EF, N, and JK	n.d.	n.d.
Citrate lyase	+[3]	+	n.d.	+
Acetolactate synthase	+	+	n.d.	+
Acquired antibiotic resistance genes	n.d.	n.d.	n.d.	n.d.

[1]; Base pair, [2]; not detected, [3]; gene detected.

from pH 6.6 to ca. 5.4 (Fig. 3). The *L. delbrueckii* TJA 31 also grew from ca. 1×10^6 CFU /ml to ca. 1×10^8 CFU /ml and was able to reduce the pH down to pH 5.0. The *L. paracasei* strain TJB 4 and *L. helveticus* strains TJA 10 grew from 10^6 CFU /ml to almost 1×10^9 CFU /ml and ca. 5×10^8 CFU /ml, respectively (Fig. 2). These strains showed the highest pH lowering activity and reduced the pH from 6.5 to 4.5 or 3.8, respectively (Fig. 3). When the goat's milk was inoculated with the four starter strains in combination with the yeast *K. marxianus* MBT-5698, the LAB counts on MRS agar increased from ca. 1×10^7 CFU /ml to ca. 1×10^9 CFU /ml. The yeast counts on YGC medium increased from 5×10^5 CFU /ml to approx. 5×10^6 (data not shown). This co-inoculation of potential starter strains with yeast

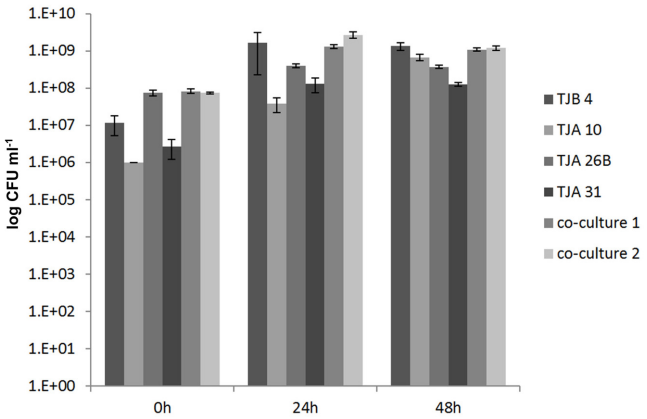

Fig. 2. Determination of total lactic acid bacteria counts in pasteurized goat's milk after inoculation starter cultures using MRS agar plates.

Co-culture 1: four starter lactic acid bacteria with the yeast *Kluyveromyces marxianus* MBT-5698, co-culture 2: four starter lactic acid bacteria with *S. thermophilus* MBT-2 and *Kluyveromyces marxianus* MBT-5698. Counts shown are from triplicate determinations with indicating a standard error.

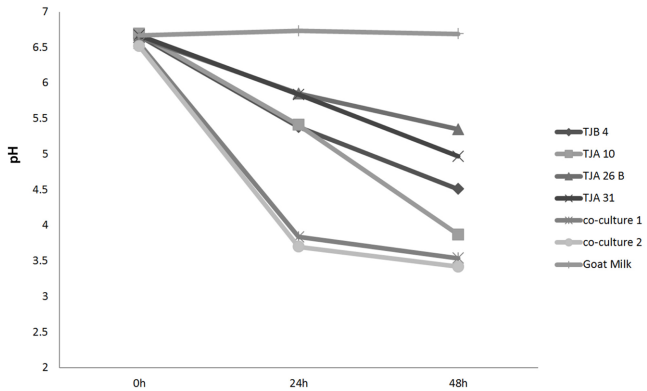

Fig. 3. pH development of goat's milk inoculated with starter lactic acid bacteria and co-culture with a *S. thermophilus* and yeast.

Co-culture 1: four starter LAB with the yeast *Kluyveromyces marxianus* MBT-5698, co-culture 2: four starter LAB with *S. thermophilus* MBT-2 and *Kluyveromyces marxianus* MBT-5698. Goat milk without inoculation of LAB as negative control.

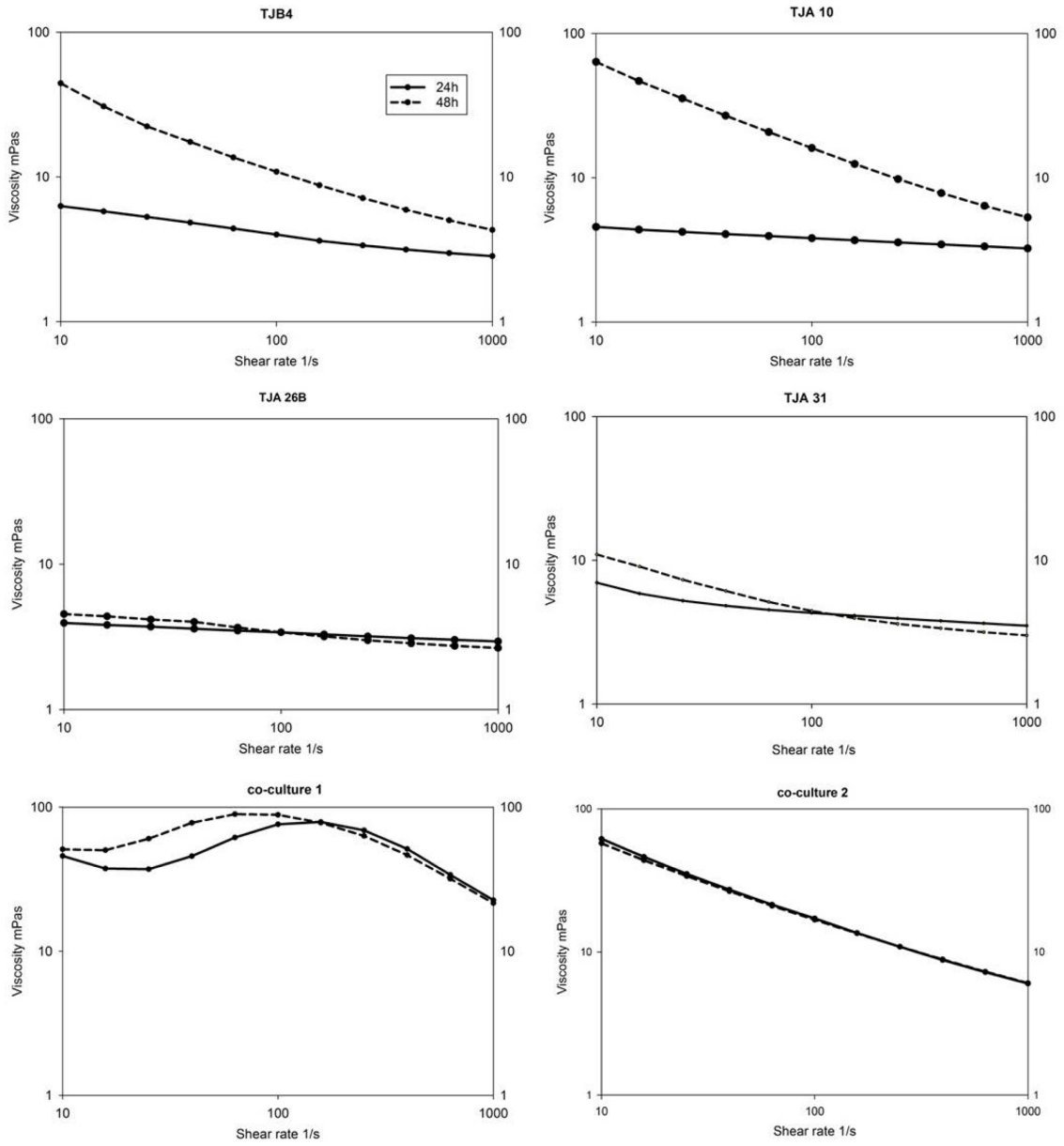

Fig. 4. Flow curves of the fermented milk products after 24 h (solid line) and after 48 h (dotted line) of fermentation.

led to the lowest determined pH of ca. pH 3.4 (Fig. 3). Similarly, the co-inoculation of the four potential starters together with *S. thermophilus* MBT-2 and the yeast *K. marxianus* MBT-5698 also led to a LAB count on MRS of ca. 1×10^9 CFU /ml after 24 h, while the yeast count increased from 5×10^4 CFU /ml to almost 1×10^6 CFU /ml (data not shown). In this case the pH of the fermentation decreased to pH 3.5 (Fig. 3).

The flow curves of the different cultures are shown in Fig. 4. All samples, except co-cultures 1 and 2, showed similar flow behavior. After 24 h no significant increase in the viscosity was detected. This corresponded well with the results of the pH measurements (Fig. 3). The pH values of samples TJB 4, TJA 10, TJA 26B, and TJA 31 were higher than 5.5 (Fig. 3) and thus no gelation of the milk could occur, as indicated in Fig. 4. The co-cultures on the other hand, show pH values lower than 4.0, and that gelation was nearly completed. Co-culture 2 showed a typical flow-thinning behavior. Viscosity decreased with higher shear rates, which were caused by aggregate destruction. Co-culture 1 shows shear-thickening behavior in the range of 10 to 100/s and shear-thinning behavior in the range above

Table 4. Antibiotic resistance tests of isolates.

Strain	Minimum inhibitory concentration (μg/ml)						
	Amp	Ery	Tetra	Strep	Chlor	Gent	Van
L. delbruecki							
TJA 1	0.25	0.125	1	2	1	8	0.25
TJA 2	Sensitive[b]	0.25	16	16	2	2	0.5
TJA 9	0.5	0.125	16	16	4	8	0.5
TJA 12	16	0.5	8	8	4	4	0.5
TJA 17	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TJA 19	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TJA 21	Sensitive	0.5	16	8	2	2	0.5
TJA 28	Sensitive	0.25	16	32	1	2	1
TJA 29	Sensitive	Sensitive	16	32	1	16	0.5
TJA 31	0.25	0.5	8	8	2	4	0.5
TJA 32	1	0.25	1	4	4	2	0.25
L. helveticus							
TJA 6A	Sensitive	0.125	>256	1	2	0.25	>256
TJA 10	0.25	0.125	32	4	2	4	0.25
TJA 16	Sensitive	2	16	8	1	4	0.5
TJA 20	Sensitive	Sensitive	8	4	1	4	0.5
TJA 24A	0.25	Sensitive	8	2	1	4	0.5
TJA 27	0.25	Sensitive	16	8	2	8	0.5
L. plantarum							
TJA 7A	Sensitive	0.25	>256	2	2	0.5	>256
TJA 11S	0.125	0.25	16	2	2	2	0.5
TJA 11B	Sensitive	0.25	>256	4	4	0.25	>256
TJA 26B	0.125	0.25	128	2	4	0.25	>256
L. pentosus							
TJA 26S	Sensitive	0.125	8	4	2	0.5	>256
L. paracasei							
TJB 4	0.25	0.125	8	4	2	0.5	>256
Breakpoints[a] for *L. plantarum*	2	1	32	n.r.	8	16	n.r.
Breakpoints[a] for *Lactobacillus* obligate homofermentative	1	1	4	16	4	16	2

[a] Breakpoints according to EFSA [22], [b] Growth inhibition occurs at 0.06 μg/ml As the least concentration, n.r: not required. n.d. : no bacterial growth on ISO medium
Amp: ampicillin, Ery: erythromycin, Tetra: tetracycline, Strep: streptomycin, Chlor: chloramphenicol, Gent: gentamicin, Van: Vancomycin.
The value '>256' means no growth inhibition occurred and this was the maximum concentration tested.

100/s after 24 as well as after 48 h.

Antibiotic Resistance Profile

In order to characterize the antibiotic resistances of the different lactobacilli, the LSM medium of Klare *et al*. [21], consisting of a mixture of 90% Iso-Sensitest and 10% MRS medium, was used to test the sensitivity towards 7 different antibiotics including ampicillin, erythromycin, tetracycline, streptomycin, chloramphenicol, gentamicin and vancomycin. In this study, most of lactobacilli strains were able to grow in the LSM medium, except for the TJA 17 and TJA 19 *L. delbrueckii* strains (Table 4). All *L. plantarum* strains isolated from Tajikistani fermented milk were sensitive to ampicillin, erythromycin, chloramphenicol and gentamicin, whereas all strains except *L. plantarum* TJA 11S were resistant to vancomycin. In this study, none of the strains, except the *L. helveticus* TJA 16 strain, were resistant to erythromycin and none of these strains were resistant to chloramphenicol. In the present study, however, almost all strains except *L. delbrueckii* strains TJA 28 and TJA 29 were

susceptible to streptomycin and all strains were susceptible to gentamicin. The strains in this study therefore were generally susceptible to most antibiotics tested and this allowed a selection of strains as safe starter strains. Thus, the strains *L. delbrueckii* TJA 31, *L. paracasei* TJB 4 and *L. helveticus* TJA 10 tested for their application as starter culture in goat's milk did not display any potentially transferable resistances towards antibiotics such as tetracycline, erythromycin, ampicillin, chloramphenicol and streptomycin. Strain *L. plantarum* TJA 26B on the other hand, showed a relatively high MIC value of 128 μg /ml for tetracycline. However, the annotated sequence data showed that none of the four selected strains possessed any acquired antibiotic resistance genes (Table 3). Accordingly, no determinant for acquired tetracycline resistance could be determined to occur in the genome sequence of *L. plantarum* TJA 26B. In addition, no plasmid sequences could be determined in all four strains. Therefore, there appears to be no apparent danger of these strains with regard to transferable antibiotic resistances.

Discussion

L. delbrueckii subsp. *bulgaricus* is a microorganism that is known to be well adapted to the milk environment and together with *S. thermophilus* is a recognized starter bacterium for the production of yoghurt in Western countries. It was therefore not surprising to find the microorganism associated with the Tajikistani fermented milk products. According to Dellaglio *et al*. [24] and Adimpong *et al*. [25], the subspecies *L. bulgaricus* (*L. delbrueckii* subsp. *bulgaricus*) is the only subsp. which is unable to ferment sucrose. As all *L. delbrueckii* strains in this study could also not ferment sucrose (Table 2), it is likely that these strains belong to the *L. delbrueckii* subsp. *bulgaricus*. The only strain identified as *L. paracasei* by 16S rRNA gene sequencing clustered apart from the *L. delbrueckii* strains in group I in the RAPD-dendrogram. This strain produced only the L- lactic acid enantiomer and grew at 15 but not at 45°C, which is typical for *L. paracasei* [26]. *L. plantarum* strains were commonly isolated and thus appeared to play role as predominant isolates in the fermentation of Tajikistani milk products. A previous study of Torriani *et al*. [27] showed that *L. plantarum* and *L. pentosus* are genotypically and phenotypically closely related. Thus *L. plantarum* and *L. pentosus* are difficult to distinguish on the basis of 16S rRNA gene sequences. Based on the similar RAPD profiles of the four isolates, it is possible that isolate TJA 26S which was identified by 16S rRNA sequencing as *L. pentosus*, may indeed also be a *L. plantarum* strain. These four strains which clustered together in subgroup II in the M13 RAPD fingerprint analysis (Fig. 1) were able to grow at 15°C but not 45°C, produced DL-lactic acid and did not produce CO_2 from glucose metabolism (Table 1). These characteristics are typical for *L. plantarum* and *L. pentosus* [28] strains and thus fitted well with the 16S rRNA gene sequence results. In our study, these four strains were also able to use melizitose as a carbohydrate source (Table 2), which indicates that these strains are indeed *L. plantarum* and *L. pentosus*, rather than *L. paraplantarum*, the latter which cannot ferment melizitose [28, 29]. These strains also produced both D- and L-lactic acid enantiomers, which were also characteristic for these bacteria [30].

Most of the *L. helveticus* strains isolated from Tajikistani fermented milk were able to utilize the carbohydrates galactose, fructose, trehalose and mannose, which have been described in a previous study [30]. *L. helveticus* is also a well-known strain for its role in the manufacture of some Swiss-type hard cheeses [31] and its presence in milk fermentations.

The RAPD strain typing method is a rapid, accurate and sensitive method for monitoring particular strains in a fermentation to which specific starter cultures were added [32]. However, the RAPD fingerprint technique only has limited potential identifying different *Lactobacillus* strains at the species level [27]. Thus, 16S rRNA gene sequence analysis was carried out to identify bacteria in this study. Nevertheless, our results showed that RAPD analysis in most cases grouped well the strains according to their 16S rRNA gene sequence-determined identities. Accordingly, the *L. delbrueckii* and *L. helveticus* strains all clustered in separate groups, only group II contained both *L. helveticus* and the difficult to distinguish *L. pentosus* and *L. plantarum* strains.

Traditionally, *L. helveticus* and *L. delbrueckii* are considered as thermophilic lactobacilli used as starter cultures in the hard cheeses production, such as Swiss type cheeses or long-ripened Italian cheeses produced at elevated temperatures [31]. Accordingly, all these strains grew well at 45°C (Table 2). *L. helveticus* strains have also been reported to be proteolytic, producing certain peptides with health-promoting properties during milk or dairy food fermentation [33]. Furthermore, probiotic features of *L. helveticus*, such as prevention of gastrointestinal infections, protective effects against pathogens, modulation of host immune response and adherence to epithelial cells were well described by Slattery *et al*. [34]. These lactobacilli, thus, were interesting for further study regarding their technological characteristics

during the goat milk fermentation.

When single strains of each species, *i.e. L. plantarum* TJA 26B, *L. delbrueckii* TJA 31, *L. paracasei* TJB 4 and *L. helveticus* TJA 10 were inoculated singly in pasteurized goat milk, each of the strains grew well but showed quite different acidification behavior, from pH 3.8 for *L. helveticus* TJA 10, to pH 5.4 for *L. plantarum* TJA 26B. When the goat's milk was inoculated with all four strains including the yeast *K. marxianus* MBT-5698, this co-inoculation led to the lowest determined pH of ca. pH 3.4. Similarly, the co-inoculation of the four potential starters together with *S. thermophilus* MBT-2 and the *K. marxianus* MBT-5698 also led to a low pH of the fermentation of 3.5. Clearly, therefore, as the aim for the use of starter cultures was to increase food safety, our results suggested that combinations of starters with yeasts and streptococci should be used to guarantee a deep enough acidification to below pH 4.0, as this would prevent the growth of most foodborne pathogenic bacteria.

The lower pH brought about by the co-inoculation of the starter strains with the yeast may be explained for the symbiosis of *S. thermophilus* and *L. delbrueckii* in yoghurt fermentation (also known as proto-cooperation) [35]. Alternatively, the growth of the yeast may have improved conditions for growth of the cultures which did not grow well in the goat's milk, *i.e. L. plantarum* TJA 26B and *L. helveticus* TJA 10, which only grew to ca. 1×10^8 CFU /ml (Fig. 2). The yeast may have stimulated their growth by providing more anaerobic conditions when using the oxygen in the medium for its respiratory growth, or by providing growth-stimulating factors such as possibly vitamins, trace elements or amino acids. Indeed, a previous study of Plessas et al. [36] showed that synergistic effects could be determined during growth of *L. helveticus*, *L. delbrueckii* subsp. *bulgaricus* and *K. marxianus*. The authors suggested that this was also due especially to the presence of *K. marxianus*, which provides the LAB with growth factors such as vitamins, which in turn promote growth and lead to increased lactic acid production [36].

The flow curves of the different cultures in milk showed no significant increase in the viscosity, while they showed that gelation of the milk after fermentation with the co-cultures was nearly completed. Co-culture 2 showed a typical flow-thinning behavior. Viscosity decreased with higher shear rates, which were caused by aggregate destruction. Co-culture 1 showed shear-thickening behavior in the range of 10 to 100/s and shear-thinning behavior in the range above 100/s after 24 as well as after 48 h. This was probably caused by aggregates which blocked each other in the shear gap at lower shear rates and become destructed at higher shear rates. After 48 hours, cultures TJB 4, TJA 10, TJA 26B, and TJA 31 also showed shear-thinning behavior. The maximum viscosity directly corresponded to the lowest pH value.

There have been some previous reports on aminoglycoside resistances among *Lactobacillus* spp. such as *L. casei* and *L. delbrueckii* subsp. *bulgaricus* [37]. Whether antibiotic resistances are problematic depends on whether they can be transferable to other bacteria, i.e. whether they reside on mobile elements. In previous studies, several genes encoding antibiotic resistance determinants were identified from lactobacilli strains such as aph(3)-IIIa and ant(6) aminoglycoside resistance genes, as well as chloramphenicol (cat) [38], erythromycin-resistance (erm) [39] and tetracycline (tet) resistance genes [37, 40]. The tetracycline resistance gene tet(S), for example, was shown to be located on a plasmid in the probiotic *L. plantarum* strain CCUG 43738 [41]. Most antibiotic resistances of LAB strains seem to be intrinsic; however, in some cases, transferable resistances may occur, and according to the EFSA's Qualified Presumption of Safety (QPS) decision tree, these bacteria should be tested for transferable resistance genes before being considered as starter cultures for use in foods. A similar, intrinsic antibiotic resistance of *L. plantarum* towards vancomycin was previously reported [21, 37, 42]. Ammor *et al*. [43] reported lactobacilli to be commonly resistant towards aminoglycosides such as gentamicin, kanamycin and streptomycin, and susceptible to other protein synthesis inhibitors. Generally, the isolates in this study showed little antibiotic resistances and this allowed the selection of susceptible strains for use as potential starter cultures.

In this study, therefore, the lactobacilli from Tajikistani fermented milk could be identified to consist of *L. delbrueckii* subsp. *bulgaricus*, *L. helveticus*, *L. pentosus* and *L. paracasei* strains. When these strains were co-inoculated together with the yeast *K. marxianus*, a synergistic growth stimulation and increased acid production associated with a lowered pH could be observed. The co-inoculation led to the lowering of pH levels below 5.5 which allowed gelling of milk. Most *Lactobacillus* isolates from Tajikistani fermented milk were generally susceptible to antibiotics. The whole genome sequence data showed that the four representative strains did not have any acquired antibiotic resistance genes; this would therefore not hinder the consideration of these strains as potential starter cultures. In addition, the whole genome sequence data showed that two strains possessed bacteriocin genes, which may be important for contributing to the safety of the products and to

fermentation success. Furthermore, three strains possessed a gene which is important for diacetyl production, *i.e*. the citrate lyase gene. The metabolism of citrate to α-acetolactate is part of the metabolic pathway for the production of diacetyl. Diacetyl formation occurs spontaneously from α-acetolactate by decarboxylation, without specific enzymatic reaction [44]. The presence of this gene indicated that these three strains are potentially capable of producing the aroma compound diacetyl.

The low incidence of antibiotic resistance in the strains isolated from Yaghnob fermented milk could be related to the absence of use of antibiotics in this population: in fact, antibiotic therapy has not been used and traditional medicine with herbs has been mostly utilized to treat diseases. The microorganisms associated with the fermentation of Tajikistani milk have so far not been described. The results of this study clearly show that the microorganisms associated with fermentation are *K. marxianus* (as previously reported by [36]) and the *Lactobacillus* spp. identified in this study as consisting of *L. delbrueckii* subsp. *bulgaricus*, *L. plantarum*, *L. helveticus* and *L. paracasei*. The definition of these microorganisms as important for fermentation is a critical first step for the development of starter cultures and for paving the road to future industrial applications.

Nucleotide Sequence Accession Number

The whole-genome shotgun project of Tajikistani starter cultures can be accessed through BioProject number PRJNA479758 and has been deposited at DDB/ENA/GenBank under the accession no. listed in Table 3.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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