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Analysis of lactic acid bacteria communities and their seasonal variations in a spontaneously fermented dairy product (Malian fènè) by applying a cultivation/genotype-based binary model

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ABSTRACT

Seasonal fluctuations of lactic acid bacteria (LAB) communities were analysed in spontaneous fermentation processes of the Malian sour milk fènè through the design of a novel flow-chart protocol for rapid typing of putative LAB. The protocol is based on cultivation on semi-selective complex agar media, followed by a binary scheme for genus- and species-specific genotypic identification. Presumptive LAB ($n = 1583$) were isolated and typed from 98 samples. Fènè samples displayed 10^8 cfu mL^{-1} in all seasons. Seasonal fluctuations indicated a higher prevalence of *Streptococcus* spp. and *Enterococcus* spp. in the cold and hot season, respectively. Analysis of enterococci ($n = 596$) and streptococci ($n = 454$) revealed the predominance of *Streptococcus infantarius* subsp. *infantarius* over *Streptococcus thermophilus*. The binary culture-based protocol is a rapid tool to follow LAB species diversity and seasonal variations in any complex dairy microbial ecosystem including the cultivation of strains for subsequent characterisation.

1. Introduction

Understanding the role of a complex microbiota in terms of composition-based quality and food safety, microbial loading and diversity throughout spontaneous fermentation processes of artisanal dairy products is a challenging task. Selective or semi-selective media have been used in the past for phenotypic isolation of microbes in spontaneous fermentation processes. These culture-dependent methods were occasionally combined with genotypic molecular identification techniques for selected species determination (De Vuyst, Vrancken, Ravyts, Rimaux, & Weckx, 2009; Mathara, Schillinger, Kutima, Mbugua, & Holzapfel, 2004) resulting in a higher fidelity.

In the last ten years, culture-independent methods targeting directly nucleic acid extracts have become popular for diversity analysis of a complex microbiota of food (Rantsiou & Cocolin, 2006). However, such approaches are not applicable in laboratories with only limited molecular biology equipment. Furthermore, viable and pure bacterial single-strain cultures are often desired and required for subsequent strain characterisations including adapted starter culture aimed to develop for traditional fermentation processes. Therefore, the aim was to develop a cultivation/genotype-based binary identification model for detection, quantification, isolation, identification and monitoring of lactic acid bacteria (LAB) representing the key microorganisms in most milk fermentations. This model approach was applied to determine seasonal variations in fènè, a traditional fermented milk of Mali and the principle protein source for people living in rural areas where spontaneous fermentation is the sole option for preserving milk. LAB were assumed to play a key role among variable communities of microbes transmitted from the immediate surroundings and contributing to the spontaneous souring process of traditionally fermented milks in Asia (Yu et al., 2009) and Africa (Kebede, Viljoen, Gadaga, Narvhus, & Lourens-Hattingh, 2007). Lactobacilli, lactococci and enterococci have been established as the traditional predominant genera among LAB in indigenous fermented dairy products (Beukes, Bester, & Mostert, 2001; Gonfa, Foster, & Holzapfel, 2001; Mathara et al., 2004; Obodai & Dodd, 2006).

Our on-site approach focused on seasonal diversification and unequivocal typing down to species or strain level of predominating LAB in fènè by isolating them on semi-selective culture media and by a combination of molecular biology typing protocols for

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accurate identification. The predominating microorganisms which were detected belong to safe fermentative LAB species as well as to problematic enterococci and streptococci whose presence in fènè may pose a threat to human health.

2. Methods

2.1. Sample collection and milk fermentation

Fènè production was investigated in Mali during the hot (February and March 2005), cold (November and December 2005) and rainy (June and July 2006) seasons at the Bagan Yiri Wa Ton communal dairy cooperative (small-scale facility) and a home-scale/domestic production site located in the village of Kasséla, 40 km from the capital Bamako. Small-scale and home-scale fènè fermentation processes were sampled and processed twice per season at seven-day intervals. The communal pool of raw milk delivered by approximately 50 farmers was heated for 50 min from ambient temperature (30.6 ± 4.2 °C) to 87 ± 5 °C. Twenty-litre aliquots were transferred into 30-L plastic vats, covered with a mosquito net and permitted to cool down to ambient temperatures.

Spontaneous fermentation occurring afterwards for about 22 h on site at ambient temperatures was monitored. For this, two 50-mL samples were collected each time from raw (named Lm) and cooled (F0) milk and from fermenting milk after 2 (F2), 4 (F4), 8 (F8), 18 (F18) and 22 h (Ff). Ff represents the ready-to-eat fènè at its final fermentation stage. Samples were transported on ice, stored between 4 and 8 °C and finally analysed within 24 h at the Laboratoire Central Vétérinaire, Bamako. Milk and environmental temperatures as well as pH (mobile pH-meter 604, Metrohm AG, Herisau, Switzerland) were measured immediately after sample collection.

2.2. Microbiological analysis

Samples originated from milk and fermented dairy products described in Section 2.1, the production environment and equipment of the small-scale dairy cooperative. Samples were analysed for their microbiota using the agar media de Man–Rogosa-–Sharpe (MRS; pH 6.4, M17) and Azide Maltose (KFS), all from Labo-Life Sàrl, Pully, Switzerland.

Resident airborne microbiota were analysed with agar media plates (9.4 cm in diameter) placed for 1 h on the mosquito net covering the fermentation vat and at a distance of 1 m apart from the vat to isolate potential contaminating bacteria. Further samples were taken with a sterile cotton swab from fermentation vat surfaces and streaked on MRS, M17 and KFS agar media.

Bacterial counts of milk samples were determined through serial dilution (10^{-1}–10^{-8}) in sterilised peptone solution (0.85%, w/v, NaCl and 0.1%, w/v, peptone; VWR-International, Dietikon, Switzerland). Aliquots of 0.1 mL were spread in duplicate on selective media; plate count agar (PCA; Becton Dickinson, Allschwil, Switzerland) for the counting of aerobic mesophiles, MRS agar for presumptive lactobacilli, M17 agar for presumptive lactococci, KFS agar for presumptive enterococci and violet red bile dextrose agar (VRBD; VWR-International) for presumptive *Enterobacteriaceae*. MRS plates were incubated at 37 °C for 48 h using anaerobic jars with an atmosphere generation system (AnaeroGen, Oxoid AG, Basel, Switzerland). PCA and M17 plates were incubated at 30 °C for 24 h, KFS plates at 43 °C for 48 h and VRBD plates at 37 °C for 24 h, all under aerobic conditions in a standard incubator.

Five colonies per dilution and medium were picked from MRS, M17 and KFS agar media based on colour, shape and colony size differences and purified by streak plating at least three times on the same medium. Isolates were phenotypically pre-typed by Gram-staining (Gregersen, 1978), catalase activity testing (3% H_2O_2) and microscopic examination. Isolates from MRS, M17 and KFS agar media were sub-cultured for 24 h in 2-mL Cryo-Vials (Huber & Co, Reinach, Switzerland) containing 1 mL of MRS, M17 and brain heart infusion (BHI) medium (Becton Dickinson), respectively. Cultures were then transported within 24 h at ambient temperature to the laboratory at ETH Zurich, mixed with 0.7 mL of sterile glycerol (98%) and stored at −80 °C.

2.3. Identification of presumptive lactic acid bacteria by molecular typing

Gram-positive, catalase-negative bacterial colonies from milk sources growing on MRS, KFS or M17 agar media were handled as putative coccoid or rod-shaped LAB. After DNA isolation (Goldenberger, Perschil, Ritzler, & Altwegg, 1995) they were further typed using PCR assays according to the flow-chart presented in Fig. 1. The volume of 1.5 μL (approx. 5–15 ng) of such DNA preparations served as template in PCR amplifications performed in a Biometra®TGradient Cycler (BioLabo, Châtel-St. Denis, Switzerland). Reaction mixtures, adjusted amplification PCR protocols to enhance specificity and references for oligonucleotide primers are displayed in Supplementary Table S1 and reference strains in Table 1.

Coccoid-shaped presumptive LAB were first typed with an *Enterococcus* genus-specific PCR (Ke et al., 1999) whereas rod-shaped isolates were analysed with the *Lactobacillus* genus-specific PCR (Dubernet, Desmasures, & Gueguen, 2002) (Fig.1). Non-*Enterococcus* coccoid isolates were subsequently typed with *Lactococcus* genus- and species-specific PCR–Restriction Fragment Length Polymorphism (RFLP) assays (Deasy, Rea, Fitzgerald, Cogan, & Beresford, 2000; Pu, Dobos, Limsowtin, & Powell, 2002). Non-*Lactococcus* isolates were further investigated with a *Streptococcus thermophilus*-specific- (Lick, Keller, Bockelmann, & Heller, 1996) and a *Streptococcus*-genus-specific PCR assay (Picard et al., 2004). Lactococci had to be typed prior to streptococci and excluded from the *Streptococcus* genus-specific PCR assay due to false-positive DNA amplification of *Lactococcus lactis* subspecies.

Isolates assigned to the genus *Streptococcus* were further analysed for the presence of members of the *Streptococcus bovis/Streptococcus equinus* complex (SBSEC) (Chen et al., 2008). Presumptive coccoid LAB, which were not assigned to one of the genera *Enterococcus*, *Lactococcus* or *Streptococcus* through the PCR assays mentioned above, were subjected to *Pediococcus*-specific typing (Mora, Fortina, Parini, & Manachini, 1997). Isolates not assigned to *Pediococcus* and other cocci were subsequently analysed with a *Weissella* genus- and species-specific PCR–RFLP assay (Jang et al., 2002). Rod-shaped isolates assigned to the genus *Lactobacillus* were further typed at species level with a multiplex PCR approach (Song et al., 2000). Bacteria belonging to the *Lactobacillus plantarum*-group were indicated as *Lb. plantarum*. Rod-shaped isolates not assigned to the genus *Lactobacillus* were subjected to the *Weissella* genus-specific PCR assay as described above. Some streptococci were typed by partial 16S rRNA gene sequencing (Microsynth, Balgach, Switzerland) to species level using PCR products generated by universal primers (Greisen, Loeffelholz, Purohit, & Leong, 1994). NCBI database matching (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to analyse 16S rRNA gene sequences, which were subsequently compared for sequence identity through the fasta comparison tool (http://fasta.bioch.virginia.edu/fasta_www2/fasta_www.cgi).

Typing at strain level to cluster all isolates assigned to *Lc. lactis* subsp. *lactis* and species within the genera *Lactobacillus*, *Weissella* and *Pediococcus* was performed by a rep-PCR assay according to an adapted protocol of Kostinek et al. (2005) using the $(GTG)_5$ primer (Gevers, Huys, & Swings, 2001) and a reduced replication time of 3 min.

Pulsed-field gel electrophoresis (PFGE; Olive & Bean, 1999) was used as an additional typing tool at strain level. For PFGE analysis,

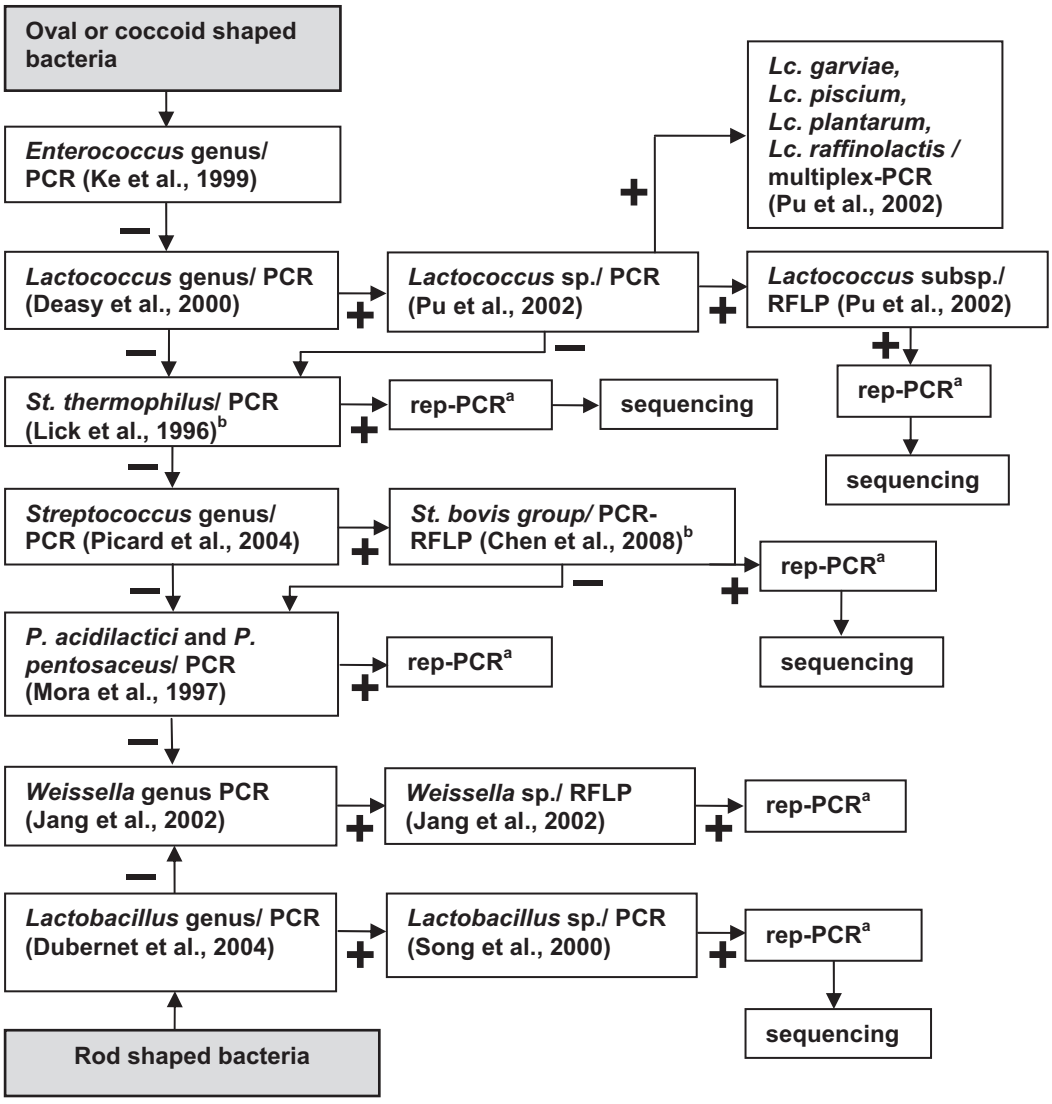

Fig. 1. Flow chart of molecular protocol used for typing presumptive lactic acid bacteria: +, positive result in preceding step; −, negative result in preceding step. Superscript letter a indicates adapted and modified from Gevers et al. (2001) and Kostinek et al. (2005); superscript letter b indicates recommended improvement of methodological flow chart: perform *St. bovis* group PCR–RFLP (Chen et al., 2008) in position of *St. thermophilus* PCR (Lick et al., 1996).

cells were grown to an optical density (OD_{600}) of 6.0 ± 0.5 for lactobacilli in MRS, 2.4 ± 0.3 for *Streptococcus infantarius* subsp. *infantarius* in M17 and 0.6 ± 0.1 for *Lc. lactis* subsp. *lactis* in M17. DNA plugs were processed according to Simpson, Stanton, Fitzgerald, and Ross (2002). DNA isolated from *St. infantarius* subsp. *infantarius* (Bouton, Guyot, & Grappin, 1998), lactobacilli (Coeuret, Dubernet, Bernardeau, Gueguen, & Vernoux, 2003) and *Lc. lactis* subsp. *lactis* (Tanskanen, Tulloch, Hillier, & Davidson, 1990) was digested with *Sma*I, *Not*I and *Sma*I, respectively. PFGE and rep-PCR fingerprints were grouped with the unweighted pair-group method using the arithmetic averages clustering algorithm (UPGMA) (Sneath & Sokal, 1973). Dendrograms were constructed with the program http://www.tinet.org/~debb/UPGMA (Garcia-Vallve, Romeu, & Palau, 2000).

DNA from agarose gel electrophoresis was stained with ethidium bromide and visualised with a UV transilluminator. Gel images were captured using the AlphaImager™ system (Alpha Innotech Corporation, San Leandro, California, USA). The digitised images were normalised to a TriDye 100-bp and 1-kb DNA ladder (BioConcept, Allschwil, Switzerland).

Strain diversity (D) from different sources (Table 2) was calculated per selected species or genus according to Simpson's index of diversity (ID) with $D = 1 - ID$ (Hunter & Gaston, 1988; Simpson, 1949) and 95% confidence interval (CI) recommendations (Grundmann, Hori, & Tanner, 2001). Values close to 1 indicate high diversity, and values close to 0 indicate low diversity.

2.4. Statistical analysis

Bacterial counts were normalised by $\log_{10}$ transformation. Differences between means of bacterial counts analysed between seasons, pH and temperature were tested for statistical significance ($p < 0.05$) using the Tukey's-*b* test of SPSS (ver. 16.0 SPSS, Chicago, IL, USA).

3. Results and discussion

3.1. Enumeration and isolation of presumptive lactic acid bacteria from fènè samples

3.1.1. Microbial growth dynamics of spontaneous fènè fermentation processes

The aim of this study was to develop a cultivation/genotype-based binary identification model for detection, quantification,

Table 1
Reference strains used in this study.

Bacterial strains[a]
E. faecalis DSM 20478^T
Lb. acidophilus DSM 20079^T
Lb. casei subsp. *casei* ATCC 334
Lb. delbrueckii subsp. *lactis* FBC FAM-10991
Lb. delbrueckii subsp. *bulgaricus* DSM 20081^T
Lb. fermentum DSM 20052^T
Lb. plantarum DSM 20174
Lb. salivarius FBC DC 6
Lb. reuteri FBC SD 2112
Lc. garvieae DSM 20684^T
Lc. lactis subsp. *lactis* DSM 20481^T
Lc. lactis subsp. *cremoris* DSM 20069^T
Lc. raffinolactis DSM 20443^T
P. acidilactici DSM 20284^T
P. pentosaceus DSM 20336^T
St. bovis DSM 20480^T
St. infantarius subsp. *infantarius* CCUG 43820^T
St. infantarius subsp. *coli* CCUG 43822
St. alactolyticus DSM 20782^T
St. gallolyticus subsp. *gallolyticus* DSM 16831^T
St. gallolyticus subsp. *macedonicus* DSM 15879^T
St. thermophilus DSM 20617^T
W. confusa DSM 20196^T
W. paramesenteroides DSM 20288^T

[a] Abbreviations of the genera are: *E.*, *Enterococcus*; *Lb.*, *Lactobacillus*; *Lc.*, *Lactococcus*; *P.*, *Pediococcus*; *St.*, *Streptococcus*; *W.*, *Weissella*. The superscript letter T indicates type strain of a species or subspecies. Culture collection abbreviations are: DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany; ATCC, American Type Culture Collection, Rockville, USA; FBC, Collection of the Laboratory of Food Biotechnology, ETH Zurich, Switzerland; CCUG, Culture Collection, University of Gothenburg, Sweden.

isolation, identification and monitoring of LAB and test it on the example of fènè, a traditional Malian fermented milk. To elucidate the microbial growth dynamics of the entire fènè fermentation process, a broad microbial diversity analysis was performed by classical plating techniques of ready-to-eat fènè at small- and home-scale level. Bacterial counts on each agar medium (KFS, M17, MRS and PCA) increased gradually throughout the fermentation from 2 $\log_{10}$ cfu mL^{-1} (F0) to over 8 $\log_{10}$ cfu mL^{-1} (Ff). The initial high microbial load of raw milk of nearly 6 $\log_{10}$ cfu mL^{-1} was partially but not completely reduced by the milk-heating step (F0 sample, Fig. 2A) before fermentation under non-aseptic conditions. Presumptive enterococci were not detected on KFS agar media until the fourth hour (F4) of fènè fermentation, but very high final counts at over 8 $\log_{10}$ cfu mL^{-1} were found (Fig. 2A). A pH decrease was detected when the cell density was higher than 7 $\log_{10}$ cfu mL^{-1}, which occurred approximately after 8 h of fermentation (Fig. 2A).

3.1.2. Seasonal fluctuations of presumptive lactic acid bacteria in fènè

Significant ($p < 0.05$) seasonal fluctuations of bacterial counts on the five different agar media were observed at the end of spontaneous 22-h fermentation processes at both small-scale and home-scale level, respectively (Table 3). Counts were significantly lower during the rainy season than in the hot season on PCA and the LAB-selecting media MRS, KFS and M17. A reduction of counts on MRS from 9.2 ± 0.5 to 7.9 ± 0.5 $\log_{10}$ cfu mL^{-1} was measured for the hot and rainy seasons, respectively (Table 3).

Total *Enterobacteriaceae* counts (on VRBD agar medium) were higher than 7.5 $\log_{10}$ cfu mL^{-1} throughout the three seasons. The average ambient temperatures and pH remained constant throughout the seasons (Table 3). The titres of presumptive LAB in fènè were similar to Western-type yoghurt whereas pH values of 4.4–4.8 of fènè were slightly higher compared with pH values of 4.1–4.4 of traditional yoghurt and possibly insufficient to suppress *Enterobacteriaceae*. This could explain the relatively high prevalence of presumptive *Enterobacteriaceae* in fènè (Table 3). Furthermore, fermentation initiation relied on the autochthonous bacterial communities present in small quantities in the fermentation vessel, which may explain the slow acidification in comparison with starter culture initiated fermentations.

Bacterial counts on different media of pooled raw milk (Fig. 2) corresponded to previous findings from Mali, where the main source of contamination was related to milk containers in combination with high temperatures and poor hygiene (Bonfoh et al., 2003). High counts of *Enterobacteriaceae* are therefore possibly due to recontaminations after the initial heating and during the subsequent processing steps and equipment used (Hetzel et al.,

Table 2
Lactic acid bacteria typed to strain level isolated from spontaneous fènè fermentation processes during hot, cold and rainy seasons and environmental isolates[a] from fermentation vats and surrounding air.

Bacterial species[b]	Total isolates	Hot season	Cold season	Rainy season	Number of strains per species	Diversity index	CI lower	CI higher
Lactobacillus spp.[c]	10 (0.6%)	0 (0.0%)	8 (0.7%) [3]	2 (0.8%)	8	0.96	0.89	1.02
Lb. fermentum	77 (4.9%)	10 (5.5%)	64 (5.5%) [22]	3 (1.2%)	18	0.78	0.69	0.87
Lb. plantarum	24 (1.5%)	0 (0.0%)	21 (1.8%) [19]	3 (1.2%)	9	0.90	0.85	0.96
Lb. delbrueckii spp.	2 (0.1%)	2 (1.1%)	0 (0.0%)	0 (0.0%)	2	1.0	1.00	1.00
Lc. lactis subsp. *lactis*	62 (3.9%)	3 (1.6%)	55 (4.8%)	4 (1.6%)	21	0.85	0.77	0.92
Lc. garvieae	11 (0.7%)	0 (0.0%)	11 (1.0%)	0 (0.0%)	–	–		
Streptococcus spp.[d]	454 (28.7%)	33 (18.0%)	377 (32.6%) [38]	44 (18.1%) [1]	10[e]	0.48	0.34	0.61
W. paramesenteroides	11 (0.7%)	0 (0.0%)	6 (0.5%) [1]	5 (2.1%)	3	0.64	0.51	0.76
W. confusa	75 (4.7%)	4 (2.2%)	68 (5.9%) [6]	3 (1.2%)	21	0.92	0.90	0.95
P. acidilactici	8 (0.5%)	0 (0.0%)	3 (0.3%)	5 (2.1%) [2]	4	0.82	0.70	0.94
P. pentosaceus	36 (2.3%)	0 (0.0%)	35 (3.0%) [7]	1 (0.4%)	30	0.99	0.97	1.00
Enterococcus spp.	596 (37.7%)	124 67.8%)	356 (30.8%) [63]	116 (47.7%) [18]	–	–	–	–
Not typed	217 (13.7%)	7 (3.8%)	153 (13.2%) [28]	57 (23.5%) [5]	–	–	–	–
Total isolates (strains)	1583 (100%)[f]	183 (100%)[f]	1157 (100%)[f]	243 (100%)[f]				

[a] Number of environmental isolates give in square parentheses.
[b] Abbreviations of genera are indicated in Table 1; – indicates value not determined.
[c] Lactobacilli that could not be typed to species level (results not shown).
[d] 87 streptococci isolates were typed as *St. infantarius* subsp. *infantarius* with PCR (Chen et al., 2008; Lick et al., 1996), rep-PCR and 16S rRNA gene sequencing.
[e] 79 streptococci isolates were typed with PCR (Chen et al., 2008; Lick et al., 1996) and 16S rRNA gene sequencing as *St. infantarius* subsp. *infantarius* and clustered with rep-PCR into 10 clusters.
[f] Due to the display of only 3 significant numbers, rounding issues can lead to total values different from 100.0%. Percentages were calculated relative to the total number of isolates per season.

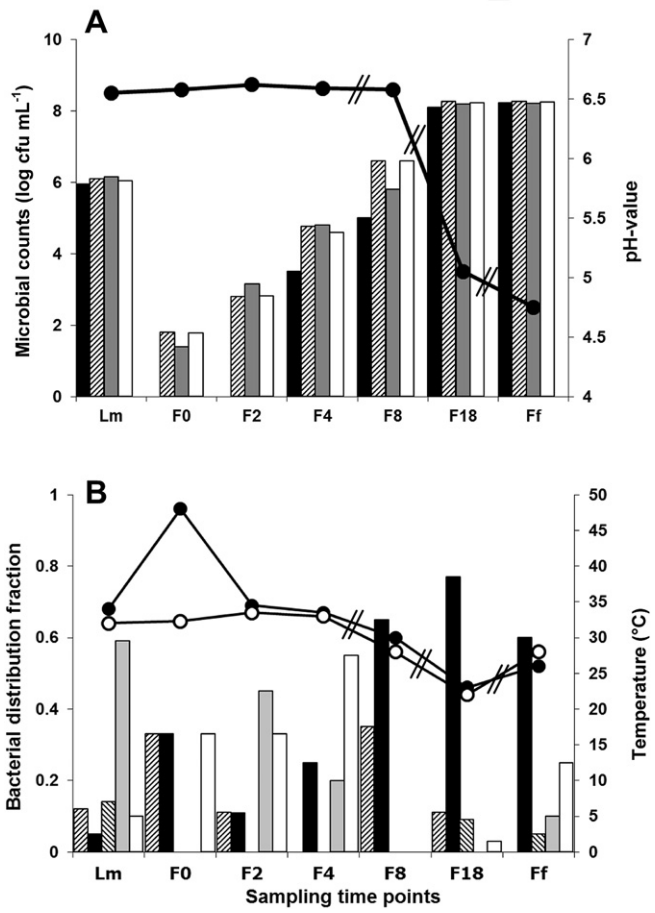

Fig. 2. Analysis of microbiota during a spontaneous fènè fermentation processes at small-scale heated milk fènè production during the cold season. Panel A displays bacterial counts on KFS (■), M17 (▨), MRS (▩) and PCA (□) agar media at a detection limit above 10^2 cfu mL^{-1} and indicates pH values (●). Panel B summarises the relative bacterial distribution obtained after molecular typing of genera and species isolated from MRS-agar media (■, *Streptococcus* spp. and *St. infantarius* subsp. *infantarius*; ▨, *Enterococcus* spp.; ▩, *Lb. fermentum*, *W. confusa* and *P. pentosaceus*; ▧, *Lc. lactis* subsp. *lactis*; □, not typed isolates and small numbers of *Lc. garvieae*, *W. paramesenteroides*, *P. acidilactici*, *Lb. plantarum* and *Lactobacillus* spp.) and temperatures (○, ambient and ●, milk): line breaks indicate nonlinear time-scale exceeding the 2 h measuring interval. Abbreviations of the sampling points: Lm, pooled milk; F0, start point of fermentation; F2, F4, F8, and F18, 2 h, 4 h, 8 h, and 8 h after fermentation started, respectively; Ff, final product after 22 h.

2004) or insufficient acidification by the spontaneous fermentation. The presence of *Enterobacteriaceae* indicates putative health risks for consumers. The high presence of presumptive LAB suggests fènè as an ideal dairy fermentation to apply the binary typing model developed in this study to determine species diversity and seasonal variations.

3.2. Application of the binary identification model to type bacterial isolates at genus, species and strain level

3.2.1. Genus and species diversity during fènè fermentations reveals high prevalence of St. infantarius *subsp.* infantarius *and seasonal variations*

The binary LAB typing system (Fig. 1) was evaluated and applied to identify presumptive LAB isolates sampled during spontaneous fènè fermentation in the hot, cold and rainy season as illustrated for the cold season in Fig. 2. A total of 1583 catalase-negative, Gram-positive cocci and rods were isolated during three seasons from 98 fènè samples and typed to species or subspecies level with PCR and RFLP assays (Fig. 1, Supplementary Table S1). All isolates, except for 217, could be grouped either into three different genera or nine species of LAB (Table 2). Two hundred and thirteen of these 1583 isolates originated from fermentation plastic vats (before filling) or surrounding air (indicated in brackets in Table 2). LAB from these environmental sources presumably contaminated previously heated milk. Indeed, enterococci, streptococci and most LAB species as identified in fènè were detected most frequently in the environment coming into contact with milk (Table 2) and reflect the environmental habitat of enterococci (Ogier & Serror, 2008).

Growth dynamics of microbial communities during dairy fermentations are important to indicate the role of different species during the process. MRS medium was selected to illustrate and follow Gram-positive and catalase-negative presumptive *Lactobacillus* isolates during fènè fermentation since they are expected to be favoured on this medium. The isolates from MRS medium were identified and grouped according to the typing approach presented in this paper (Fig. 1). Surprisingly, a broad range of non-*Lactobacillus* LAB (Fig. 2B) was detected on this medium. Streptococci prevailed during the fermentation process reaching between 33% (F0) and over 60% in the samples F8, F18 and Ff (Fig. 2B). *Pediococcus pentosaceus* was detected in the samples Lm, F2 and F4 with 50%, 31% and 14%, respectively, and *Weissella confusa* was found in small numbers in unheated milk (Lm), F2 and F4 with 6%–13% and together with *Lactobacillus fermentum* both with 5% in the final product (Ff) (results of single species not displayed). *Lc. lactis* subsp. *lactis* were only detected in unheated milk (Lm), after 18 h (F18) and in the final product (Ff) with 13%, 9% and 5%, respectively (Fig. 2B).

Similarly, isolates from M17 and KFS agar media (Fig. 2A) were typed. Only approximately 36% of the M17 coccoid isolates from the Ff sample were lactococci besides a majority of streptococci and enterococci (data not shown). Enterococci and streptococci were among the first colonising bacteria of milk. They were persistently present at high titres in all successive samples analysed (Fig. 2B) indicating a potential contribution to the fermentation process. However, enterococci are also implicated in the spread of antibiotic resistances and nosocomial infections and therefore not recommended for human consumption (Haug, Tanner, Lacroix, Stevens, & Meile, 2011; Leuschner et al., 2010; Teuber, Meile, & Schwarz, 1999).

Table 3
Bacterial counts on non-selective and selective agar media and pH of four data points as well as ambient and milk temperatures (*T*) of ready to eat fènè after 22 h of spontaneous fermentation per season.[a]

Analysis season	pH	Temperatures (°C)		MRS	Bacterial counts (log_{10} cfu mL^{-1})			
		Ambient	Milk		M17	KFS	PCA	VRBD
Hot	4.4 ± 0.2^{a}	31.5 ± 5.3^{a}	33.9 ± 3.4^{a}	$9.2 \pm 0.5^{a,Y,x}$	$9.2 \pm 0.3^{a,Y,x}$	$8.8 \pm 0.3^{a,x}$	$9.4 \pm 0.2^{a,Y}$	$7.5 \pm 0.5^{a,Z}$
Cold	4.6 ± 0.3^{a}	30.1 ± 4.0^{a}	33.6 ± 6.0^{a}	$8.9 \pm 0.5^{a,Y}$	$9.0 \pm 0.4^{a,Y}$	$8.6 \pm 0.4^{a,b,Y}$	$9.0 \pm 0.5^{a,b,Y}$	$7.7 \pm 0.7^{a,Z}$
Rainy	4.8 ± 0.7^{a}	30.2 ± 6.5^{a}	33.5 ± 5.3^{a}	$7.9 \pm 0.5^{b,Z}$	$8.2 \pm 0.8^{b,Z}$	$7.8 \pm 1.0^{b,Z}$	$8.4 \pm 0.6^{b,Z}$	$7.9 \pm 0.7^{a,Z}$

[a] Temperatures recorded at each sampling point throughout the fermentation process in the correspondent season. Column values with different superscript lowercase letters (seasons) and row values with different superscript upper case letters are significantly different by Tukey's test ($p < 0.05$). Data are means and standard deviations of four sampling points comprising two independent fermentations at small-scale and two at home-scale production taken during the same analysis period.

Interestingly, *Streptococcus* spp. and *Enterococcus* spp. displayed seasonal variations with a peak in the cold season yielding 32.6% of all isolates and the hot season yielding 67.8% of all isolates, respectively (Table 2), indicating possible temperature dependency of bacterial communities during fènè fermentation.

All 454 *Streptococcus* isolates were first analysed with the *St. thermophilus*-specific PCR assay (Lick et al., 1996; Fig. 1, Supplementary Table S1). Out of these, 79 positive isolates were grouped into 10 clusters with the rep-PCR assay (Gevers et al., 2001; Fig. 3, Table 2). Partial 16S rRNA gene sequences with a length between 500 and 1450 bp of presumptive *St. thermophilus* strains 11FA-1, 6C, 6BY-11b, 3AG, 11EQ-2, 11GJ-2; 8DF-1; 13AF; 13DW and 13AY-1 were sequenced. Each isolate represents one cluster of presumptive *St. thermophilus*. Surprisingly, their 16S rRNA gene sequences displayed over 99.0% sequence identity with the 16S rRNA gene of *St. infantarius* subsp. *infantarius*, whereas the highest sequence identity detected with that of *St. thermophilus* DSM 20617^T was only 94.0%. Eight additional isolates selected randomly from the 375 streptococci, testing negative for the *St. thermophilus*-specific PCR assay (Lick et al., 1996), were subjected to 16S rRNA gene sequencing. These eight *Streptococcus* isolates exhibited over 98.0% 16S rRNA gene sequence identity with that of *St. infantarius* subsp. *infantarius*.

In summary, out of a total of 454 streptococci, 79 plus eight random isolates were identified as *St. infantarius* subsp. *infantarius*, indicating that this is the predominant species of streptococci isolated from spontaneous fènè fermentation processes similar to data reported for the fermented camel milk products gariss (Sudan) and suusac (Kenya) (Abdelgadir, Nielsen, Hamad, & Jakobsen, 2008; Jans, Bugnard, Njage, Lacroix, & Meile, 2012a).

St. infantarius subsp. *infantarius* is a member of the *St. bovis/St. equinus* complex (SBSEC) (Schlegel, Grimont, Ageron, Grimont, & Bouvet, 2003). Certain species of the SBSEC are associated with several human and animal infections (Herrera, Min Kwon, & Ricke, 2009), which could indicate putative health risks for consumers. The conspicuous absence of *St. thermophilus* may be explained by the ambient temperature (Fig. 2B) which was too low compared with the optimal growth temperature range between 40 and 45 °C for this species (Zirnstein & Hutkins, 2004). In addition, this species might be outcompeted by *St. infantarius* subsp. *infantarius* strains, a potential pathogenic subspecies (Corredoira, Alonso, Coira, & Varela, 2008), of which we recently showed evidence that an African variant adapted to the dairy niche during evolution (Jans et al., 2012b).

Our approach demonstrated, therefore, that the PCR assay based on the *lacZ* gene for identifying *St. thermophilus* (Lick et al., 1996) was not able to differentiate *St. thermophilus* from bacteria of the SBSEC isolated in our study. Further discriminating tools for presumptive *St. thermophilus* such as 16S rRNA gene sequencing had therefore to be implemented. Through integration of other PCR assays, this binary model typing scheme can be leveraged to any foodstuffs containing LAB as fermentative microbiota, e.g., for other LAB such as *Leuconostoc* spp., meat-related LAB or SBSEC (Chen et al., 2008; Jans, Lacroix, & Meile, 2012c; Marty, Buchs, Eugster-Meier, Lacroix, & Meile, 2012). It is further recommended to screen for members of the SBSEC prior to screening for *St. thermophilus* (Fig. 1).

The remaining 217 unidentified isolates (13.7%) were not characterised. They might represent false negative or so far not identified Gram-positive/catalase-negative bacteria including also LAB species other than those identified during the present study. This fact demands further development of the typing scheme (Fig. 1). Some isolates positive for the *Lactococcus* genus-specific PCR (Deasy et al., 2000) could not be sub-typed into *Lactococcus* species by multiplex PCR (Pu et al., 2002). However, all of them were positive for the *Streptococcus* genus-specific PCR (Picard et al., 2004). Other LAB, such as the mastitis agent *Lc. garvieae*, were less regularly isolated from fermentation processes (Teixeira et al., 1996).

Conclusively, the presence of large groups of *Lactobacillus* spp., *Lactococcus* spp. and *Streptococcus* spp. indicated the need to further investigate the strain diversity within the predominant genera and species.

3.2.2. Strain diversity of spontaneous fènè fermentation processes

Reliable species identification is a prerequisite for strain fluctuation studies during fermentation. Strain clustering was performed by rep-PCR since this less time-consuming method was evaluated to result in the same clusters as PFGE for 37 tested isolates from the four species *Lb. plantarum*, *Lb. fermentum*, *St. infantarius* subsp. *infantarius* and *Lc. lactis* (data not shown). A total of 126 different strains from 384 isolates belonging to ten different species were found in fènè samples (Table 2). The highest strain diversity indices were observed for *P. pentosaceus* ($D = 0.99$) whereas *St. infantarius* subsp. *infantarius* exhibited the lowest strain diversity ($D = 0.48$) since ten strains out of 79 isolates were classified. Among environmental LAB (as described above) one *Pediococcus acidilactici* strain and four *Lb. fermentum* strains were

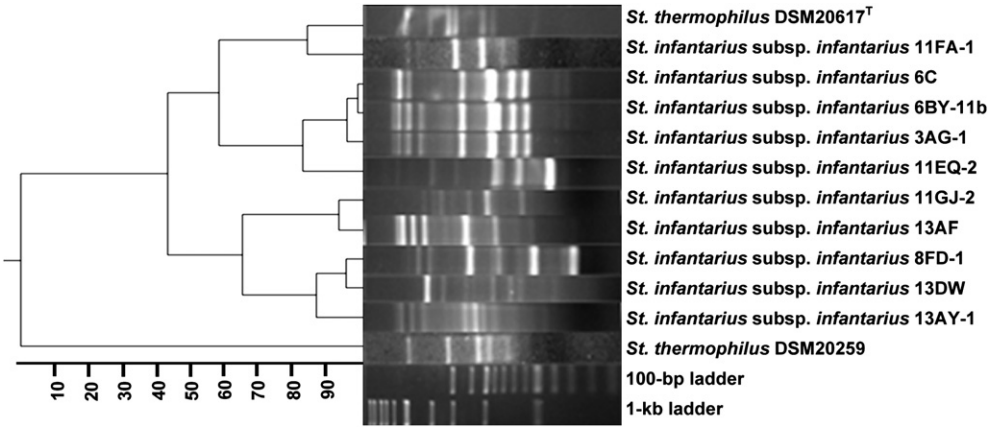

Fig. 3. Dendrogram calculated on rep-PCR fingerprints of *Streptococcus infantarius* subsp. *infantarius* isolates from fènè and from reference strains, generated via the unweighted pair-group method using arithmetic averages clustering algorithm (UPGMA) with correlation indicated as % values of the corresponding Pearson correlation coefficient.

found in empty fermentation vats, air and fermented milk by comparing their PFGE profiles (data not shown).

Strain detection within a single fènè fermentation process varied with respect to time as follows: only a total of seven strains comprising *Lc. lactis* subsp. *lactis* (1 strain), *St. infantarius* subsp. *infantarius* (1 strain), *W. confusa* (3 strains) and *Lb. fermentum* (2 strains) were detected both in the first 8 h of a spontaneous fermentation process and in the corresponding final products from 18 spontaneous fermentations from three seasons. Six strains comprising *Lc. lactis* subsp. *lactis* (1), *St. infantarius* subsp. *infantarius* (1), *W. confusa* (2) and *Weissella paramesenteroides* (2) were detected only in the first 8 h of the fermentation whereas 27 strains comprising *Lc. lactis* subsp. *lactis* (7), *St. infantarius* subsp. *infantarius* (3), *P. pentosaceus* (8), *W. confusa* (7) and *Lb. fermentum* (2) were only found in the final products. A total of 86 strains among 126 strains were only isolated once during spontaneous fermentation processes or from environmental samples.

Strain diversity fluctuated greatly, with only three strains of *Lc. lactis* subsp. *lactis* (2) and *St. infantarius* subsp. *infantarius* (1) found in both the first and the second fermentation process sampled during a single season and, seasonally with only four out of 126 strains isolated at least in two seasons. These four strains included *Lb. plantarum* (1) and *St. infantarius* subsp. *infantarius* (3) present in two seasons, with one strain of *St. infantarius* subsp. *infantarius* found in all three seasons.

Fluctuations in strain diversity among different species (Table 2) were found from fermentation to fermentation during the same season. The fact that only a few strains were detected both at early stages of fermentation and in the final product underscores the importance of multiple sampling throughout complex fermentation processes.

4. Conclusions

We regard our culture-based typing strategy organised in a logical flow-chart setup as a universal methodological approach to identify catalase-negative Gram-positive bacteria isolated from a dairy environment for subsequent further strain characterisation and starter culture development.

The typing protocol applied in this study with a Malian sour milk enabled us to type 87% out of over 1500 Gram-positive, catalase-negative presumptive LAB isolates cultured on three classical agar media. It was a valuable tool in determining the diversity and growth dynamics of LAB communities dominating in a complex fermented food matrix and its production environment.

This approach facilitates immediate availability of viable, typed and predominant LAB strains as potential competitive starter cultures. The authors therefore also stress the flexibility and adaptability of this methodology through integration of novel PCR assays. The detection of predominant enterococci and *St. infantarius* subsp. *infantarius* in the fermented fènè suggests potential health risks to consumers, the importance to elucidate their role in the fermentation process and introduce a competitive adapted starter culture.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.idairyj.2012.08.001.

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