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RESEARCH LETTER

A novel multiplex PCR/RFLP assay for the identification of *Streptococcus bovis*/*Streptococcus equinus* complex members from dairy microbial communities based on the 16S rRNA gene

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SBSEC; dairy streptococci; *Streptococcus infantarius*; *Streptococcus gallolyticus*; dairy fermentation; PCR/RFLP identification assay.

Abstract

The *Streptococcus bovis*/*Streptococcus equinus* complex (SBSEC) comprises pathogenic species associated with different degrees with human infections but also spontaneously fermented dairy products. We aimed therefore at developing a specific identification assay for the SBSEC targeting the 16S rRNA gene comprising a multiplex PCR followed by a differentiating restriction fragment length polymorphisms (RFLP). The multiplex PCR assay was positively applied on 200 SBSEC isolates including reference strains. The assay did not yield false-positive amplifications with strains of closely related bacteria and isolates of non-SBSEC streptococci, lactococci, enterococci, and other genera of dairy origin. The downstream RFLP using MseI and XbaI enabled further discrimination of *Streptococcus infantarius*/*S. bovis* (biotype II.1) from *Streptococcus gallolyticus* (biotype I and II.2)/*Streptococcus alactolyticus* and *S. equinus*. Furthermore, the newly developed primers can be used directly for Sanger sequencing. Conclusively, this novel PCR/RFLP assay is applicable in the complex dairy microbial communities and provides an important tool to assess the prevalence of members of the SBSEC in dairy products.

Introduction

The *Streptococcus bovis/Streptococcus equinus* complex (SBSEC) comprises a large variety of species and subspecies of which especially *Streptococcus infantarius* subsp. *infantarius* and potentially other members of the SBSEC were reported as the predominant lactic acid bacteria (LAB) in spontaneously fermented African milk products (Abdelgadir *et al.*, 2008; Wullschleger, 2009; Jans, 2011). Members of the SBSEC were also detected in Mexican, Greek, and Italian cheese, fermented Mexican maize drink, or fermented Bangladeshi milk (Tsakalidou *et al.*, 1998; Díaz-Ruiz *et al.*, 2003; Pacini *et al.*, 2006; Rashid *et al.*, 2009; Renye *et al.*, 2011). First discrimination of SBSEC has been based on phenotypic classification schemes that were greatly revised with the ability of 16S rRNA gene phylogenetic analysis (Poyart *et al.*, 2002; Schlegel *et al.*, 2003).

The genes *sodA* (Poyart *et al.*, 1998, 2002) and *groESL* (Chen *et al.*, 2008) were targeted for PCR assay in combination with sequencing and restriction fragment length polymorphism (RFLP) for the identification of members of the SBSEC. A further assay was developed specifically for *Streptococcus gallolyticus* subsp. *macedonicus* based on the 16S rRNA gene (Papadelli *et al.*, 2003). Alternative approaches proposed the utilization of MALDI-TOF for species identification based on the *sodA* gene (Hinse *et al.*, 2011). Most of these assays were developed using blood-derived clinical cultures of the SBSEC or restricted to a single species. In contrast to blood samples, raw dairy products were shown to contain a large diversity of different lactococci, enterococci, *Streptococcus thermophilus*, or *Streptococcus agalactiae* (Delbès *et al.*, 2007; Younan & Bornstein, 2007; Franciosi *et al.*, 2009; Giannino *et al.*, 2009; Jans, 2011), which increases the requirements regarding specificity of the primers. Even though the genes *groESL* or *sodA* provide improved capability to differentiate between species and subspecies, the 16S rRNA gene is still regarded as the recommended target for the initial identification of novel bacteria for which the higher degree of conservation of the 16S rRNA gene can be of

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advantage (Glazunova *et al.*, 2009). This gene is one of the most important genotypic markers for bacterial taxonomy (Yarza *et al.*, 2008), and a large number of 16S rRNA gene sequences are available for downstream comparison and further analysis (Benson *et al.*, 2009). It therefore represents an ideal target for the analysis of complex and less-studied ecological niches such as the human microbiota (Grice *et al.*, 2008; Liu *et al.*, 2008) or spontaneous food fermentations, e.g., the African dairy environment. The high-density and complex microbial communities in these niches could result in unexpected genetic modifications through horizontal gene transfer (HGT), which was observed for African *S. infantarius* subsp. *infantarius* as well as for *S. thermophilus* and other LAB (Hols *et al.*, 2005; Makarova *et al.*, 2006; Jans, 2011). HGT is affecting nearly all genes within prokaryotic genomes; some genes including the 16S rRNA gene are, however, hypothesized to be less affected by HGT (Jain *et al.*, 1999).

Therefore, the objective was to utilize the high conservation of the 16S rRNA gene to develop an identification assay applicable to all species within the SBSEC allowing clear differentiation from other streptococci, enterococci, and lactococci regularly found in the dairy environment. The availability of large sets of nucleotide sequences from all members of the SBSEC including dairy isolates (Jans, 2011) enabled the design of a subsequent RFLP for the discrimination of SBSEC species groups. Furthermore, the primers were designed to work with Sanger sequencing for downstream sequence analysis. The assay was then evaluated against reference strains and isolated species of dairy microbial communities.

Materials and methods

Bacterial reference strains listed in Table 1 were obtained from the Culture Collection University of Gothenburg (CCUG, Gothenburg, Sweden), the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), and the National Collection of Type Cultures (NCTC, Porton Down, UK). Stock cultures of all strains were stored at −80 °C in 30% (v/v) glycerol. Generally, streptococci were grown anaerobically at 37 °C for 16–24 h on BHI agar (Biolife, Milan, Italy) and on M17 agar (Biolife) for SBSEC, *Streptococcus salivarius*, *S. thermophilus*, and *Streptococcus vestibularis*. *Lactococcus* and *Leuconostoc* strains were propagated aerobically at 30 °C for 16–24 h on M17 (Biolife) and MRS (Biolife), respectively. Lactobacilli and pediococci were incubated anaerobically at 37 °C on MRS agar (Biolife) for 1–2 days. Anaerobic agar media incubation was performed with AnaeroGen packs (Oxoid, Pratteln, Switzerland) in jars. All chemicals and enzymes used in this study were obtained from Sigma-Aldrich (Buchs, Switzerland) unless otherwise noted.

Additional tests to confirm the specificity of the PCR primers were performed with isolates obtained from camel milk products, which were previously identified using species-specific PCR-based methods, 16S rRNA gene sequencing and a modified rep-PCR assay (Gevers *et al.*, 2001; Wullschleger, 2009; Jans, 2011). They included the following number of isolates and species: six *Enterococcus faecalis*, 24 *Enterococcus faecium*, 35 *Lactococcus lactis* subsp. *lactis*, five *S. agalactiae*, 192 *S. infantarius* subsp. *infantarius*, five *Streptococcus gallolyticus*, and 42 *S. thermophilus* (Jans, 2011).

Primer design and restriction site analysis

Sequences of the 16S rRNA gene of multiple strains per species of the SBSEC were obtained from GenBank (Table 1). The DNA sequences were aligned in BioEdit (Hall, 1999) using ClustalW and analyzed for conserved regions specific for SBSEC (Fig. 1). The primers were designed to amplify fragments of 1119 and 1120 bp of the 16S rRNA gene of *S. bovis*/*Streptococcus infantarius* (biotypes II.1) and *S. gallolyticus* (biotypes I and II.2), respectively. Four separate forward primers and one reverse primer were designed to target all members within the SBSEC (Fig. 1). The forward primers were designed in a region of the 16S rRNA gene adjacent to the primer position previously used to discriminate *S. gallolyticus* subsp. *macedonicus* (Papadelli *et al.*, 2003).

The amplified section of the 16S rRNA gene was *in silico* analyzed for species-specific mutations leading to different restriction enzyme profiles in CLC Sequence Viewer version 6.0.2 (CLC bio, Aarhus, Denmark). MseI and XbaI restriction sites discriminating the *S. gallolyticus* (biotypes I and II.2) cluster from the *S. bovis/S. infantarius* (biotypes II.1) cluster were identified *in silico* (Fig. 2). The expected fragments were 278 and 842 bp for XbaI-digested PCR products of *S. gallolyticus*. The expected MseI profile for *S. gallolyticus* contains three fragments between 17–28 bp and single fragments of 88, 136, 196, 227, and 411 bp. The expected MseI profile for *S. bovis/S. infantarius* contains single fragments of 16, 17, 46, 88, 136, 152, 253, and 411 bp. *Streptococcus equinus* was expected to display the MseI profile of *S. bovis/S. infantarius* and the XbaI profile of *S. gallolyticus*.

DNA isolation and PCR/RFLP conditions

DNA was isolated from bacterial single colonies with a short cell lysis in 0.1 M Tris-HCl-EDTA and Triton-X100 buffer at pH 8.0 (Goldenberger *et al.*, 1995). All PCR reactions were performed with 1 μL (approximately 5–20 ng) of extracted DNA, 1 μM of each primer, 12.5 μL 2 × PCR Master Mix (Fermentas, Le Mont-sur-Lausanne,

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Table 1. Reference and type strains including GenBank accession numbers used for primer design and controls during the SBSEC-PCR assay targeting specifically the 16S rRNA gene of members of the SBSEC

Species	Strain* and GenBank accession/reference number	SBSEC-PCR/RFLP result
Enterococcus durans	DSM 20633^T	No amplification
Enterococcus faecalis	DSM 20478^T	No amplification
Enterococcus faecium	DSM 20477^T	No amplification
Enterococcus malodoratus	DSM 20681^T	No amplification
Lactococcus garviae	DSM 20684^T	No amplification
Lactococcus lactis subsp. *cremoris*	DSM 20069^T	No amplification
Lactococcus lactis subsp. *lactis*	DSM 20481^T, DSM 20729	No amplification
Lactococcus plantarum	DSM 20686^T	No amplification
Lactococcus raffinolactis	DSM 20443^T	No amplification
Leuconostoc mesenteroides	9, LOC M37.6, M7-1	No amplification
Pediococcus acidilactici	DSM 20284^T	No amplification
Pediococcus damnosus	DSM 20331^T	No amplification
Pediococcus pentosaceus	DSM 20336	No amplification
Streptococcus agalactiae	CCUG 4208^T	No amplification
Streptococcus alactolyticus	DSM 20728^T, DJF_VP45 (EU728776)	1120bp; R_{MseI}: *S. gallolyticus*; R_{XbaI}: *S. gallolyticus*
Streptococcus anginosus	DSM 20563^T	No amplification
Streptococcus bovis	DSM 20480^T, ATCC 27960 (AB002481), ATCC 33317^T (AB002482), HDP90084 (AF429764)	1119bp; R_{MseI}: *S. infantarius*; R_{XbaI}: *S. infantarius*
Streptococcus cristatus	DSM 8249^T	No amplification
Streptococcus equinus	DSM 20558^T, ATCC 9812^T (AF429765)	1119bp; R_{MseI}: *S. infantarius*; R_{XbaI}: *S. gallolyticus*
Streptococcus gallolyticus subsp. *gallolyticus*	DSM 16831^T, ACM 3611 (X94337), ATCC 43143 (AP012053), ATCC BAA-2069 (FR824043), UCN34 (NC_013798)	1120bp; R_{MseI}: *S. gallolyticus*; R_{XbaI}: *S. gallolyticus*
Streptococcus gallolyticus subsp. *macedonicus*	DSM 15879^T, NZRCC 20100^T (AF088900), ACA-DC 206 (NR_037002)	1120bp; R_{MseI}: *S. gallolyticus*; R_{XbaI}: *S. gallolyticus*
Streptococcus gallolyticus subsp. *pasteurianus*	DSM 15351^T, ATCC 43144 (AP012054)	1120bp; R_{MseI}: *S. gallolyticus*; R_{XbaI}: *S. gallolyticus*
Streptococcus gordonii	DSM 6777^T	No amplification
Streptococcus infantarius subsp. *coli* (=*S. lutetiensis*)	CCUG 43822, HDP90246^T (AF429763)	1119bp; R_{MseI}: *S. infantarius*; R_{XbaI}: *S. infantarius*
Streptococcus infantarius subsp. *infantarius*	CCUG 43820^T, ATCC BAA-102^T (NZ_DS572679), HDP90056^T (AF429762)	1119bp; R_{MseI}: *S. infantarius*; R_{XbaI}: *S. infantarius*
Streptococcus infantarius subsp. *infantarius*	CJ18† (HQ662525)	1119bp; R_{MseI}: *S. infantarius*; R_{XbaI}: *S. infantarius*
Streptococcus intermedius	DSM 20573^T	No amplification
Streptococcus mitis	DSM 12643^T	No amplification
Streptococcus mutans	DSM 20523^T	No amplification
Streptococcus oralis	DSM20627^T	No amplification
Streptococcus parasanguinis	DSM 6778^T	No amplification
Streptococcus pneumoniae	ATCC 49619, D39, R6	No amplification
Streptococcus pyogenes	ATCC 19615, SF370 ATCC 47803	No amplification
Streptococcus salivarius	ATCC 13419, ATCC 9759, NCTC 8606, NCTC 8618^T, SK101, SK128	No amplification
Streptococcus sanguinis	DSM 20567^T	No amplification
Streptococcus thermophilus	DSM 20259, DSM 20479, DSM 20617^T, S205, S206, S207, S209, S210, S211, S212, S213	No amplification
Streptococcus vestibularis	CCUG 24686, CCUG 24688, CCUG 29269, CCUG 47110, DSM 5636^T	No amplification
Vagococcus carniphilus	DSM 17031^T	No amplification
Vagococcus elongates	CCUG 51423^T	No amplification
Vagococcus fessus	DSM 15697^T	No amplification

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Table 1. Continued

Species	Strain* and GenBank accession/reference number	SBSEC-PCR/RFLP result
Vagococcus fluvialis	DSM 5731^T	No amplification
Vagococcus lutrae	DSM 15741	No amplification
Vagococcus salmoninarum	DSM 6633^T	No amplification
Weissella confusa	DSM 20196^T	No amplification
Weissella paramesenteroides	DSM 20288^T	No amplification

R_{MseI}, MseI restriction profile; R_{XbaI}, XbaI restriction profile.
*Source of strains: CCUG, Culture Collection University of Gothenburg; DSM/DSMZ, German Collection of Microorganisms and Cell Cultures; and NCTC, National Collection of Type Cultures.
†Strain isolated from African fermented camel milk (Jans, 2011).

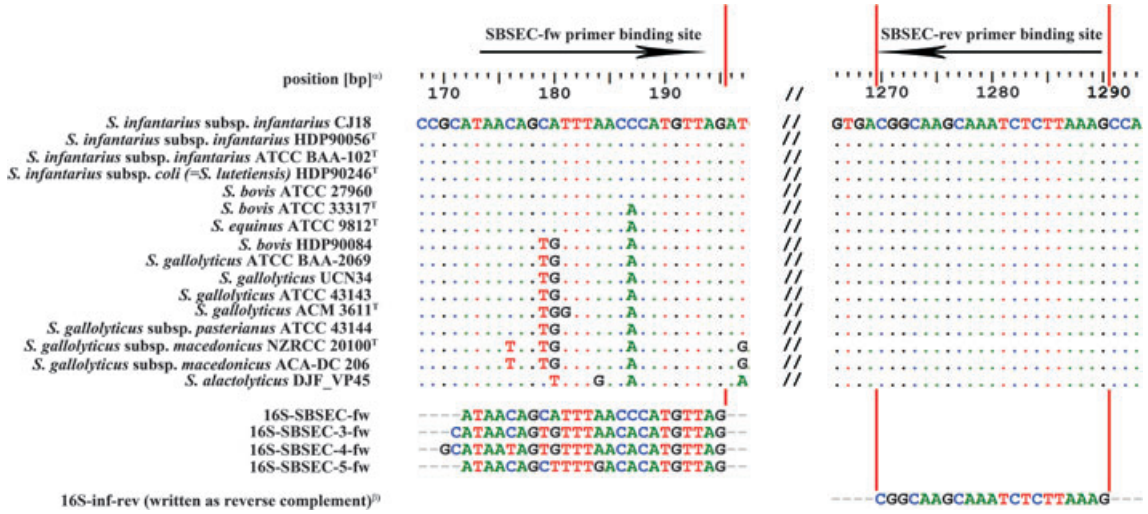

Fig. 1. Forward (fw) and reverse (rev) primer binding sites for the SBSEC multiplex PCR assay on the 16S rRNA gene. α)Position relative to the 16S rRNA gene of *Streptococcus infantarius* subsp. *infantarius* CJ18 (HQ662525). β)Primer 16S-inf-rev written as reverse complement for alignment purpose, original sequence: 5′-CTT TAA GAG ATT TGC TTG CCG-3′.

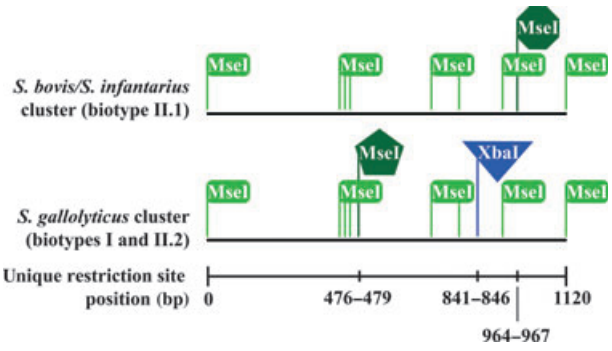

Fig. 2. Restriction site analysis of the 1119 and 1120-bp SBSEC-PCR 16S rRNA gene product of *Streptococcus bovis/Streptococcus infantarius* cluster (biotype II.1) and *Streptococcus gallolyticus* cluster (biotype I and II.2), respectively. Both species harbor 8 conserved MseI restriction sites (light green). Unique restriction sites were detected for MseI at 476–479 bp for *S. gallolyticus* (pentagon shape) and at 946–967 bp for *S. infantarius* (octagon shape). Further unique restriction by XbaI (triangle) was determined for *S. gallolyticus* at 841–846 bp.

Switzerland), and distilled DNase-free H_2O (Fermentas) to a final volume of 25 μL. Oligonucleotides were obtained from Microsynth (Balgach, Switzerland). The PCR assay was performed in a Biometra® TGradient Cycler (Biolabo, Châtel-St-Denis, Switzerland) according to the following protocol: initial denaturation at 95 °C for 3 min followed by 35 cycles of 30 s denaturation at 95 °C, 30 s annealing at 62 °C, and 60 s replication at 72 °C. A final replication was performed at 72 °C for 7 min. The reaction was subsequently cooled to 4 °C until analysis.

Successful PCR products were purified using the GFX DNA purification kit (GE Healthcare Europe, Glattbrugg, Switzerland). Restriction enzymes for the RFLP assay were obtained from New England Biolabs (NEB, Ipswich, MA) and used according to specifications. Reaction volumes and purified PCR products were adjusted to a final volume of 11.5 μL per reaction and digested at 37 °C for 2 h. Enzymes were used at a final concentration of 2 and 3 U μL^{-1} for XbaI and MseI, respectively. Restriction

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digestions were performed separately for XbaI and MseI on aliquots of the original purified PCR product.

Amplified DNA and RFLP products were analyzed by 1% and 2% agarose gel electrophoresis (Euroclone, Milan, Italy), respectively. DNA fragments were visualized with ethidium bromide staining (2.5 mg L^{-1}). A 100-bp TriDye DNA standard (BioConcept, Allschwil, Switzerland) was used as DNA size marker.

Results and discussion

The identification of all SBSEC reference strains (Table 1) as well as 192 *S. infantarius* and five *S. gallolyticus* isolates was successfully performed using the multiplex PCR/RFLP assay developed in this study. The specificity of the multiplex PCR assay was confirmed with various streptococcal species closely related to the SBSEC as well as other LAB often present in raw milk products (Table 1). The PCR assay yielded the desired 1.1-kb fragment only with DNA of SBSEC strains corresponding to the expected product of 1119–1120 bp (Fig. 3a). It did not yield false-positive amplification of non-SBSEC reference strains or dairy isolates of closely related species commonly detected in raw milk products, such as enterococci, lactococci, and other streptococci. Especially, *S. agalactiae* (group B streptococci) and group C streptococci regularly detected from milk of mastitic animals (Younan & Bornstein, 2007; Whiley & Hardie, 2009; Jans, 2011) were *in silico* evaluated to yield a potentially false-positive result when using other assays such as the 324-fold degenerate *groESL* primers (Chen *et al.*, 2008). The clear discrimination of SBSEC from other streptococcal groups is a clear advantage of the assay developed in our study considering the potential commensal microbial communities of raw dairy products. In contrast to other assays such as the general *Streptococcus* genus-specific assay targeting the *sodA* gene, the assay developed in this study does not require downstream sequencing for species identification (Poyart *et al.*, 1998). Nevertheless, the primers developed in our study were designed to be compatible with the emerging wide availability of sequencing technologies. Primers 16S-SBSEC-fw and 16S-inf-rev were successfully used in Sanger sequencing performed on two independently obtained amplicons of strain CJ18. RFLP yields the required differentiation power and can be easily performed in-house by most laboratories. However, sequencing can provide an even higher level of detail of the entire amplicon for subsequent phylogenetic analysis, database comparisons, and potential clustering of isolates. RFLP only differentiates isolates and their amplicons based on the position of individual restriction enzyme recognition sites but does not deliver information on sequence differences possibly existing between these sites.

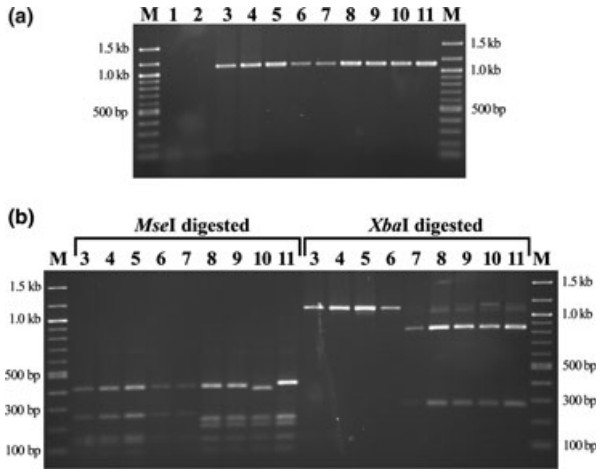

Fig. 3. Electrophoretic separation of DNA fragments after SBSEC-specific multiplex PCR amplification (a) and RFLP assay (b). Initial PCR fragment analysis and subsequent RFLP analysis were performed on a 1% and 2% agarose gel, respectively. Amplified DNA fragments were digested using MseI and XbaI restriction enzymes in two parallel and separate reactions. M: 100-bp DNA size marker; (1) no DNA control, (2) *Streptococcus thermophilus* DSM 20259, (3) *Streptococcus infantarius* subsp. *infantarius* CCUG 43820^T, (4) *S. infantarius* subsp. *coli* (=*Streptococcus lutetiensis*) CCUG 43822, (5) *S. infantarius* subsp. *infantarius* CJ18, (6) *Streptococcus bovis* DSM 20480^T, (7) *Streptococcus equinus* DSM 20558^T, (8) *Streptococcus gallolyticus* subsp. *gallolyticus* DSM 16831^T, (9) *Streptococcus gallolyticus* subsp. *pasteurianus* DSM 15351^T, (10) *Streptococcus gallolyticus* subsp. *macedonicus* DSM 15879^T, and (11) *Streptococcus alactolyticus* DSM 20728^T.

The RFLP assay performed in separate reactions for MseI and XbaI was consistent among the reference strains of the SBSEC used in this study. Three RFLP profile groups were distinguished (Fig. 3b): (1) the *S. gallolyticus* species including *Streptococcus alactolyticus* featured the expected specific MseI and XbaI profiles; (2) the *S. bovis* and *S. infantarius/S. lutetiensis* species were not digested by XbaI and featured the expected group-specific MseI profile; and (3) the *S. equinus* PCR fragment was digested by XbaI but featured the *S. bovis/S. infantarius* MseI profile (Table 1 and Fig. 3b).

The involvement of members of the SBSEC in food fermentations seems to be larger than previously expected (Tsakalidou *et al.*, 1998; Díaz-Ruiz *et al.*, 2003; Abdelgadir *et al.*, 2008; Wullschleger, 2009; Jans, 2011). Therefore, the PCR assay developed in this study allows the rapid screening of isolates to identify members of SBSEC within the complex microbial communities of spontaneous food fermentations. Despite a high sequence identity of 98.5% within the amplified DNA fragment, the restriction digestion of PCR products yielded the important discrimination of species into three major SBSEC groups

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and the differentiation of the *S. gallolyticus* cluster (former biotype I and biotype II.2) from the *S. bovis*/*S. infantarius* cluster (biotype II.1). This separation is also of clinical relevance because of the association of different infections (Schlegel *et al.*, 2003; Beck *et al.*, 2008). A benefit of the 16S rRNA gene over the *groESL* is the high conservation and low variability within the 16S rRNA gene that reduces the risk of misidentifying a species, especially when investigating novel and complex microbial niches of previously unstudied sources such as raw dairy products, where diverse microbial communities can be found (Clarridge, 2004; Delbès *et al.*, 2007; Chen *et al.*, 2008; Giannino *et al.*, 2009; Jans, 2011). To conclude, a reliable assay to identify members of SBSEC from complex microbial communities such as fermented dairy products was developed and validated. The importance of this novel assay is in the investigation of the increasing reports of members of the SBSEC being involved in food fermentations to assess their prevalence and role during the fermentation with respect to food safety. Furthermore, the simplicity of the assay allows the application of this method in laboratories without direct access to current sequencing technologies, such as in Africa, where members of the SBSEC seem to play a large role in dairy fermentations while still offering the optional direct Sanger sequencing.

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