Comparative analysis of bacterial community composition in bulk tank raw milk by culture-dependent and culture-independent methods using the viability dye propidium monoazide

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ABSTRACT

Microbial diversity of 3 raw milk samples after 72 h of storage at 4°C in a bulk tank was analyzed by culture-dependent and -independent methods. The culture-dependent approach was based on the isolation of bacteria on complex and selective media, chemotaxonomic differentiation of isolates, and subsequent identification by 16S rRNA gene sequencing. The culture-independent approach included the treatment of raw milk with the dye propidium monoazide before direct DNA extraction by mechanic and enzymatic cell lysis approaches, and cloning and sequencing of the 16S rRNA genes. The selective detection of viable bacteria improved the comparability between bacterial compositions of raw milk based on culture-dependent and -independent methods, which was the major objective of this study. Several bacterial species of the phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* were detected by the culture-dependent method, whereas mainly bacteria of the phylum *Proteobacteria* as well as low proportions of the phyla *Bacteroidetes* and *Actinobacteria* were detected by the culture-independent method. This led to the conclusion that the phylum *Firmicutes* was strongly discriminated by the culture-independent approach. Generally, species richness detected by the culture-dependent method was higher than that detected by the culture-independent method for all samples. However, few taxa could be detected solely by the direct DNA-based method. In conclusion, the combination of culture-dependent and -independent methods led to the detection of the highest bacterial diversity for the raw milk samples analyzed. It was shown that DNA extraction from raw milk as the essential step in culture-independent methods causes the discrimination of taxa by incomplete cell lysis. Treatment of raw milk with the viability dye propidium monoazide

was optimized for the application in raw milk without former removal of milk ingredients and proved to be a suitable tool to ensure comparability of bacterial diversity depicted by both methods.

Key words: propidium monoazide, bacterial community, raw milk

INTRODUCTION

Bacterial community structure in bulk tank raw milk is an important factor influencing quality and safety of the product milk itself as well as of processed dairy products, as it reflects hygienic conditions during milking and storage of raw milk in the bulk tank. Contamination of raw milk during the milking process depends on udder and teat microbiota, which is influenced by the stable environment. Primarily gram-positive bacteria, such as staphylococci, corynebacteria, bacilli, and the genus *Kocuria*, but also some gram-negative species of the family *Enterobacteriaceae* can contaminate raw milk from this source (Vacheyrou et al., 2011; Verdier-Metz et al., 2012). Other sources of contamination are the milking machine and the bulk tank itself, where biofilms can establish if cleaning and disinfection procedures are insufficient (Hayes et al., 2001; Teh et al., 2011). Especially psychrotolerant species, such as the gram-negative genera *Acinetobacter* and mainly *Pseudomonas*, may contribute to product spoilage, as they are able to proliferate at low storage temperatures and become the dominating genera in cold-stored raw milk (Raats et al., 2011). Moreover, some species were shown to produce heat-resistant lipases and proteases (Hantsis-Zacharov and Halpern, 2007), which may cause product spoilage even after pasteurization of the milk. The level of contamination is influenced by hygienic conditions and milking practice as well as cleaning and disinfection success of the milking equipment and bulk tank (Verdier-Metz et al., 2009; Pantoja et al., 2011).

Analyses of raw milk microbiota are based on culture-dependent methods (Hantsis-Zacharov and Halpern, 2007; Vacheyrou et al., 2011), direct molecular methods

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(Lafarge et al., 2004; Verdier-Metz et al., 2009), or a combination of both (Rasolofoa et al., 2010; Fricker et al., 2011; Verdier-Metz et al., 2012). Culture-dependent methods are more time consuming than culture-independent methods and incubation conditions and media influence the detected community composition. Moreover, bacteria that entered the “viable but nonculturable” state described by Oliver (2005) cannot be detected by culturing methods. In contrast, culture-independent methods using PCR amplification of 16S rRNA genes are less time consuming and well established in non-food research areas. They are also of increasing interest in dairy research (Giraffa and Neviani, 2001; Justé et al., 2008). However, direct comparison between species diversity determined by culture-dependent and -independent methods could lead to misinterpretations, as direct DNA-based methods detect also dead cells as well as extracellular DNA, which has been shown to persist in different matrices from a few days up to weeks (Josephson et al., 1993; McKillip et al., 1999). This is especially important for food products, as spoilage and the risk potential of pathogens depend on their viability. This challenge can be overcome by using viable PCR (**vPCR**), which makes use of one of the dyes ethidium monoazide or propidium monoazide (**PMA**). These dyes can selectively penetrate cells with impaired membrane integrity, where they intercalate into DNA molecules. Photoactivation by intense light produces covalent bonds between dye and DNA molecules, hindering PCR amplification of DNA molecules carrying this modification (Nogva et al., 2003; Nocker et al., 2006).

Viable PCR using PMA has been applied to few dairy products so far. The use of PMA was described for application in fermented milk (García-Cayuela et al., 2009), cheese (Desfossés-Foucault et al., 2012), and pasteurized (Soejima et al., 2012) and UHT milk (Cattani et al., 2013) for selective detection of certain lactic acid bacteria, spoiling organisms, or pathogens. Moreover, in most studies, the milk matrix was removed by proteolysis and washing steps before PMA treatment, and no information exists about interactions of PMA with milk components potentially hindering successful live-dead discrimination. Intense washing steps preceding PMA treatment and DNA extraction could result in loss of bacteria, though, and would reduce the detectable bacterial diversity by DNA-based methods.

To our knowledge, vPCR using PMA has not yet been applied to analyze bacterial community structure in raw milk. Therefore, the aim of this study was the adaptation of PMA-treatment and DNA-extraction methods for use in the raw milk matrix. We focused on the development of raw milk PMA treatment without preceding purification from certain milk compounds

and the application of as gently as possible washing steps before DNA extraction. Moreover, the depicted bacterial community structure gained by application of vPCR combined with cloning was compared with the community structure presented by traditional culture-dependent techniques to assess the coverage of bacterial sequences obtained by direct DNA extraction from raw milk.

MATERIALS AND METHODS

Raw Milk Sampling

Three raw milk samples were taken from the bulk tank (Kryos 6BII; WestfaliaSurge Japy SAS, Saint Apollinaire, France) of a research farm of the University of Bonn (Königswinter, Germany) after 72 h of cold storage at 4°C. Here, raw milk of about 60 cows of the breed German Holstein is collected twice daily using modern milking equipment and cold stored in the bulk tank for 72 h before pick up by the purchaser. Until further processing in the laboratory on the same day, milk samples were transported under refrigerated conditions. The samples, further referred to as milk sample 1 (**M1**) and milk sample 3 (**M3**), were taken in April of 2 consecutive years (2012 and 2013), whereas milk sample 2 (**M2**) was taken in August 2012. Milk sample 1 was analyzed by culturing methods, M2 was analyzed using the culture-independent method, and M3 was analyzed using both methods in a parallel approach.

Enumeration and Isolation of Bacteria

Raw milk was serially diluted in Ringer's solution and 100- μ L aliquots of each dilution as well as the undiluted milk sample were spread in duplicate on the following media (all by Merck KGaA, Darmstadt, Germany): Trypticase soy agar (**TSA**) for total bacterial count (**TBC**), violet red bile agar (**VRBA**) for coliform count (**CC**), skim milk plate count agar, and de Man, Rogosa, and Sharpe (**MRS**) agar for the detection of lactic acid bacteria. Plates were incubated for 48 h at 30°C or at 37°C (VRBA). The MRS agar was incubated anaerobically at 30°C. From each medium, a variety of morphologically different colonies was randomly selected for isolation and purified on TSA for subsequent identification.

Differentiation and Identification of Isolates

Isolates were characterized according to their colony and cell morphology, visualized by light microscopy (BH2; Olympus Europa SE & Co. KG, Hamburg,

Germany). Further differentiation was achieved via Gram staining (Gerhardt et al., 1981), KOH lysis test and detection of L-alanine aminopeptidase by use of Bactident L-alanine aminopeptidase test strips (Merck KGaA). Fatty acid profiles of the isolates were determined after growth on TSA for 48 h at 30°C. Fatty acid methyl ester preparation followed the methods described by Sasser (1990). Fatty acid methyl ester extracts were analyzed by GC-MS with a gas chromatograph (model 7890A; Agilent Technologies Germany GmbH, Waldbronn, Germany) equipped with a 5% phenyl methyl silicone capillary column and a mass spectrometer (model 5975C; Agilent Technologies Germany GmbH) as described previously (Lipski and Altendorf, 1997). Fatty acid methyl esters were identified by their retention time and by their mass spectra (ChemStation; Agilent Technologies Germany GmbH). Double-bond positions of monounsaturated fatty acids were located by dimethyl disulfide derivatization (Sigma-Aldrich Chemie GmbH, Hamburg, Germany) as described by Nichols et al. (1986). Isolates were differentiated into groups according to their FA profiles.

The DNA extraction, PCR amplification, and sequencing of 16S rRNA genes were performed as described previously (Wiertz et al., 2013). The isolates were identified by searching their 16S rRNA gene sequence against the databases EzTaxon-e server (Kim et al., 2012) and GenBank using the Basic local alignment search tool (BLAST; Altschul et al., 1990) at the National Center for Biotechnology Information (NCBI) website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

PMA Treatment of Raw Milk

A preliminary test was conducted with raw milk inoculated with the isolate *Pantoea agglomerans* M1_I4, followed by either heat inactivation (100°C for 10 min) or not, and subsequent PMA treatment [stock solution: 20 mM PMA (Biotium Inc., Hayward, CA) in 20% dimethyl sulfoxide (AppliChem GmbH, Darmstadt, Germany)] at a final concentration of 100 μ M. A heat-inactivated and a viable sample without PMA treatment were used as control. Success of heat inactivation was tested by cultivation on TSA. In the main trial, 2 aliquots of 10 mL of raw milk were treated with 50 μ L of PMA stock solution, resulting in a final concentration of 100 μ M. Two aliquots of 10 mL of untreated raw milk and 2 aliquots of 10 mL of inoculated raw milk (M2 inoculated with *Pantoea agglomerans* M1_I4 and *Microbacterium ginsengiterrae* M1_I6, and M3 inoculated with *Escherichia coli* DSM 498 and *Staphylococcus aureus* DSM 1104) served as controls for successful live-dead discrimination and DNA extraction, respectively.

Propidium monoazide-treated samples were incubated in the dark for 5 min with periodic mixing. For PMA induction, 1-mL aliquots were filled into 2-mL reaction tubes and placed horizontally onto aluminum foil covered with ice from both sides, laying in a glass vessel, which was shaken on a platform shaker at 150 rpm. Light exposure was conducted with a 400-W halogen lamp (R7s, 9,000 lumen; Osram GmbH, Augsburg, Germany) at a 20-cm distance to the tubes for 5 min.

DNA Extraction from Raw Milk and PCR Amplification

Raw milk was centrifuged at $13,000 \times g$ for 20 min at 4°C. The pellet was washed with 0.9% NaCl and after repeated centrifugation under identical conditions, cell lysis and DNA extraction were carried out using 2 different approaches of the Qiagen DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany) as modifications of the manufacturer's protocol. In an enzymatic approach, the cell pellet was suspended in 600 μ L of lysis buffer [20 mM Tris HCl (pH 8.2), 2 mM EDTA, and 1.2% Triton X-100] containing lysozyme (5 mg/mL), and 30 μ L of mutanolysin (stock solution: $\geq 4,000$ U in 0.1 M potassium phosphate buffer, pH 6.2; all chemicals by Sigma-Aldrich Biochemie GmbH). After 1.5 h of incubation at 37 °C, 400 μ L of buffer AL and 50 μ L of Proteinase K (both from Qiagen GmbH) were added and an overnight incubation step at 55°C with gentle shaking followed. In a mechanical lysis approach, the cell pellet was suspended in 1 mL of 0.9% NaCl; transferred to a 2-mL reaction tube, which was filled with sterile 2-mm glass beads (Merck KGaA); and mixed for 10 min. After transfer of this mix to a 15-mL reaction tube, 360 μ L of buffer ATL (Qiagen GmbH) and 50 μ L of proteinase K were added and an overnight incubation step at 55°C with gentle shaking followed. The next day, 400 μ L of buffer AL was added, which was followed by an incubation step for 30 min at 70°C. Four hundred microliters of ethanol (Merck KGaA, Darmstadt, Germany) was added to all mixes, solutions were transferred into DNeasy mini columns, and further DNA extraction was carried out according to the manufacturer's instructions. The 16S rRNA genes were amplified using primer pair GM3F and GM4R (Muyzer et al., 1995) and PCR products were purified using the QIAquick PCR purification kit (Qiagen GmbH).

Construction of 16S rRNA Gene Clone Libraries

Purified PCR-products of both lysis approaches were cloned using the pGEM-T vector system and

transformed into competent *E. coli* JM 109 cells as specified by the manufacturer (Promega Corp., Madison, WI). From each clone library, inserts of randomly selected clones were amplified using primer pair M13F and M13R (Messing, 1983). The PCR products were confirmed by electrophoresis, purified, and sequenced as described above. All sequences were checked using the Pintail program (Ashelford et al., 2005) and chimeric sequences were excluded from further analyses. Approved sequences were compared with the public databases described above.

Statistical Analysis and Construction of Phylogenetic Trees

To assess the completeness of total sequence diversity determined by the number of sequenced clone inserts, Rarefaction analysis was conducted using the program Analytic Rarefaction (<http://www.uga.edu/~strata/software>) and coverage was calculated according to Good (1953), with assignment of sequences to the same operational taxonomic unit (OTU) at $\geq 99\%$ sequence similarity (Singleton et al., 2001). Shannon-Index and equitability were calculated for each sample (Shannon and Weaver, 1969).

For each OTU and method of investigation, one representative sequence was used for phylogenetic tree construction using the MEGA 5.1 program (Tamura et al., 2011). Operational taxonomic unit sequences and sequences of their closest relative type strains taken from the databases used for species identification were aligned using the ClustalW program (Thompson et al., 1994). A neighbor-joining phylogenetic tree (Saitou and Nei, 1987) was constructed and assessed by bootstrap analysis with 1,000 resamplings. Representative sequences obtained in this study were deposited in the European Nucleotide Archive under the accession numbers HG934344 to HG934384.

RESULTS

Bacterial Counts and Grouping of Isolates

Total bacterial counts were 3.5×10^3 cfu/mL for M1 and 2.9×10^3 cfu/mL for M3, whereas TBC for M2 was about 2 log levels higher (1.9×10^5 cfu/mL). Samples M1 and M3 also contained similar concentrations of coliform bacteria with 3.0×10^2 cfu/mL for M1 and 8.4×10^2 cfu/mL for M3. The CC for M2 was higher than for M1 and M3 (1.7×10^5 cfu/mL). Isolates were divided into 22 groups/subgroups according to their morphological features, Gram-staining, and FA profiles. Profile data of these groups and subgroups are given in the Appendix (Tables A1 to A4).

Bacterial Community Structure as Determined by the Culture-Dependent Method

At least one representative isolate from each group or subgroup was identified by 16S rRNA gene sequencing. Species identification as well as number of identified isolates per species are presented in Table 1. Isolates were assigned to a species if sequence similarity between isolate and the type strain of a species was $\geq 98\%$. Due to the differentiation according to FA profiles, percentages of each phylum in raw milk samples M1 and M3 were calculated on the basis of total number of isolates, as shown in Table 2. Sample M1 contained 39% of species belonging to each of the phyla *Actinobacteria* and *Firmicutes*, with most in the genera *Corynebacterium* and *Staphylococcus*. Sixteen percent of species belonged to the phylum *Proteobacteria*, with *Acinetobacter* as the predominant genus and 1 isolate was identified as *Flavobacterium* spp. affiliated with the phylum *Bacteroidetes*. Predominant in sample M3 was the phylum *Proteobacteria*, to which 57% of the isolates were assigned. The most abundant genera were *Escherichia*, *Acinetobacter*, and *Pseudomonas*. The phylum *Firmicutes* was represented by 24% of the isolates, most of them belonging to different species of the genus *Staphylococcus*. The phyla *Actinobacteria*, with a high species richness of the genus *Corynebacterium*, and *Bacteroidetes* were represented by 13 and 5% of the isolates, respectively.

Optimization of PMA Treatment for Raw Milk

As shown in Figure 1, after PMA treatment (100 μ M final concentration) of inoculated and heat-inactivated raw milk, a significant reduction in PCR product band intensity occurred compared with the untreated sample containing heat-inactivated bacteria. The PMA treatment at a final concentration of 100 μ M did not restrain DNA extraction of living bacteria in raw milk and subsequent PCR amplification, as band intensities of the untreated sample inoculated with living cells and the PMA treated sample were similar (Figure 1). A lower PMA concentration of 50 μ M was not sufficient to prevent PCR amplification of DNA from heat-inactivated cells (data not shown).

Bacterial Community Structure as Determined by the Culture-Independent Method

Polymerase chain reaction product bands could be detected for the PMA-treated enzymatic and mechanic cell lysis approaches of sample M2 (Figure 2). As the band intensity of the mechanical approach was weak, a second PCR with the same primers was conducted

Table 1. Species and genera detected in bulk tank raw milk samples as determined by culture-dependent methods and clone libraries¹

Phylum	Genus and species	No. of sequences by sample ²				Representative clones/isolates	ENA accession no.
		M1 I	M2 C	M3 I	M3 C		
Actinobacteria	<i>Corynebacterium xerosis</i>	1		2		M3_I17	HG934361
	<i>Corynebacterium confusum</i>			1		M3_I13	HG934372
	<i>Corynebacterium casei</i>			1		M3_I10	HG934371
	<i>Corynebacterium</i> spp.				2	M3_C20	HG934346
	<i>Corynebacterium frankenforstense</i>	2		1		M1_I13	HG934357
	<i>Rhodococcus qingshengii</i>	1				M1_I12	HG934365
	<i>Kocuria atrinae</i>	1				M1_I5	HG934363
	<i>Kocuria salsicia</i>			1		M3_I9	HG934379
	<i>Curtobacterium flaccumfaciens</i>	1				M1_I17	HG934367
	<i>Microbacterium ginsengiterrae</i>	1				M1_I6	HG934356
	<i>Microbacterium oxydans</i>			1		M3_I7	HG934375
	Total for group	7	0	7	2		
Firmicutes	<i>Lactococcus lactis</i> ssp. <i>lactis</i>	2				M1_I15	HG934359
	<i>Pediococcus pentosaceus</i>	2				M1_I16	HG934366
	<i>Aerococcus viridans</i>	1		1		M3_I5	HG934358
	<i>Bacillus subtilis</i> ssp. <i>inaquosorum</i>			1		M3_I11	HG934380
	<i>Staphylococcus chromogenes</i>	1		1		M3_I12	HG934378
	<i>Staphylococcus arlettae</i>			1		M3_I6	HG934377
	<i>Staphylococcus equorum</i> ssp. <i>equorum</i>			1		M3_I1	HG934376
	<i>Staphylococcus cohnii</i> ssp. <i>cohnii</i>	1				M1_I1	HG934360
	Total for group	7	0	5	0		
	<i>Ochrobactrum lupini</i>	1				M1_I11	HG934364
Proteobacteria	<i>Sphingomonas aerolata</i>	1				M1_I19	HG934368
	<i>Ralstonia pickettii</i>		2			M2_C37	HG934355
	<i>Escherichia coli</i> taxonomic group		13	2	2	M2_C14	HG934384
	<i>Lelliottia amnigena</i>			1		M3_I19	HG934373
	<i>Pantoea agglomerans</i>	1	9			M2_C38	HG934354
	<i>Aeromonas media</i>		3			M2_C13	HG934350
	<i>Aeromonas punctata</i> ssp. <i>caviae</i>		8			M2_C40	HG934351
	<i>Pseudomonas azotoformans</i>			2	2	M3_C7	HG934348
	<i>Pseudomonas extremaustralis</i>				4	M3_C19	HG934349
	<i>Acinetobacter</i> spp. 1	1				M1_I20	HG934383
	<i>Acinetobacter</i> spp. 2	1				M1_I7	HG934382
	<i>Acinetobacter lwoffii</i>		1			M2_C29	HG934353
	<i>Acinetobacter johnsonii</i>		4			M2_C27	HG934352
	<i>Acinetobacter</i> spp.				2	M3_C5	HG934381
	<i>Acinetobacter guillouiae</i>		1	2	12	M3_C6	HG934344
	Total for group	5	41	7	22		
	<i>Pedobacter panaciterrae</i>				2	M3_C28	HG934347
Bacteroidetes	<i>Sphingobacterium caeni</i>			1		M3_I2	HG934374
	<i>Flavobacterium</i> spp.	1				M1_I3	HG934362
	<i>Chryseobacterium bovis</i>				3	M3_C12	HG934345
	<i>Chryseobacterium soli</i>			1		M3_I3	HG934369
	<i>Chryseobacterium vrystaatense</i>			1		M3_I8	HG934370
	Total for group	1	0	3	5		
Total for all		20	41	22	29		

¹Genus/species affiliation was determined as sequence similarity to type strains obtained by the BLAST database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>; Altschul et al., 1990) and EzTaxon-e server (Kim et al., 2012). One representative sequence for each species was deposited in the European Nucleotide Archive (ENA; <http://www.ebi.ac.uk/ena/>) with the given accession numbers.

²I = isolate sequence; C = clone library sequence.

using the first product as template. This resulted in brighter product band intensity. In the case of this sample, PCR products from PMA-treated milk of both lysis approaches were used to construct clone libraries. In contrast, the enzymatic lysis approach for the PMA-treated sample M3 resulted in a weak PCR product band intensity, which gave only a low yield of clones. To increase the amount of PCR product, the DNA extract was further concentrated and a second PCR was performed, resulting in brighter product band intensity

that gave a higher number of clones. Neither a second PCR of the first product nor further concentration of the DNA extract led to a detectable product from the mechanical cell lysis approach of sample M3. Therefore, no clone library was constructed for this approach. As can be seen in Tables 1 and 2, all 41 sequenced inserts of sample M2 belonged to species of the phylum *Proteobacteria*, with *Escherichia*, *Aeromonas*, and *Pantoea* as predominant genera. On the basis of all 29 sequenced inserts of sample M3, the majority (76%) was also as-

Table 2. Abundance of detected genera related to the 3 tank milk samples and 2 approaches¹

Phylum	Genus	FA group	No. of isolates by sample		No. of clone sequences by sample	
			M1	M3	M2	M3
<i>Actinobacteria</i>	<i>Corynebacterium</i>	1A, 2, 20	10	5		2
	<i>Rhodococcus</i>	1B	1			
	<i>Kocuria</i>	6C, 16	2	2		
	<i>Curtobacterium</i>	7B	7			
	<i>Microbacterium</i>	6A, 14B	2	1		
	Total for group (%)		22 (39%)	8 (13%)	0 (0%)	2 (7%)
<i>Firmicutes</i>	<i>Lactococcus</i>	3A, 3B	5 ²			
	<i>Pediococcus</i>	5B	6 ²			
	<i>Aerococcus</i>	4	3	1		
	<i>Bacillus</i>	14C		1		
	<i>Staphylococcus</i>	6B, 8, 15	8	13		
	Total for group (%)		22 (39%)	15 (24%)	0 (0%)	0 (0%)
<i>Proteobacteria</i>	<i>Ochrobactrum</i>	13	1			
	<i>Sphingomonas</i>	12	1			
	<i>Ralstonia</i>				2	
	<i>Escherichia</i>	19A		14 ³	13	2
	<i>Lelliottia</i>	19B		1		
	<i>Pantoea</i>	11	1		9	
	<i>Aeromonas</i>				11	
	<i>Pseudomonas</i>	18		7		6
	<i>Acinetobacter</i>	10A, 10B, 17	6	13	6	14
	Total for group (%)		9 (16%)	35 (57%)	41 (100%)	22 (76%)
<i>Bacteroidetes</i>	<i>Pedobacter</i>					2
	<i>Sphingobacterium</i>	22		1		
	<i>Flavobacterium</i>	9A	1			
	<i>Chryseobacterium</i>	21A, 21B		2		3
	Total for group (%)		1 (2%)	3 (5%)	0 (0%)	5 (17%)
Not sequenced (%)			3 (5%)	1 (2%)		
Total for all			57	62	41	29

¹Genus affiliation of nonsequenced isolates was determined according to the FA profile groups (see Appendix Tables A1 to A4 for detailed information). Phylum percentages were calculated for each sample and method and are given in parentheses.

²Two of the 5 isolates identified as the genus *Lactococcus* and all 6 isolates identified as the genus *Pediococcus* were obtained from de Man, Rogosa, and Sharpe (MRS) agar, a selective medium for lactic acid bacteria. Thus, the overall abundance of these genera in relation to total number of isolates might have been overestimated.

³Five of the 14 isolates identified as the genus *Escherichia* were obtained from violet red bile agar, a selective medium for coliform bacteria. Thus, overall abundance of this genus in relation to total number of isolates might have been overestimated.

signed to the *Proteobacteria*, predominated by sequences of the genera *Acinetobacter* and *Pseudomonas*, followed by 17% of the sequences belonging to the phylum *Bacteroidetes* and 2 sequences (7%) affiliated to the genus *Corynebacterium* of the phylum *Actinobacteria*.

Coverage for cloned sequences was calculated as 95.1% for sample M2 and 100% for sample M3. Rarefaction analysis showed achievement of the plateau phase of the curve after sequencing of 29 clone inserts for sample M3, but a slight increase of the trend line for sample M2. This indicates that bacterial community diversity was almost completely covered (M2) or completely covered (M3; Figure 3).

Overall Bacterial Community Structure as Determined by Culture-Dependent and Culture-Independent Methods

The phylogenetic tree (Figure 4) represents evolutionary distance between the isolate and clone sequences

obtained from all 3 milk samples and their most closely related type strains. Sequence labels illustrate the origin of the sequence (milk samples M1 to M3), the method applied [isolation (I) or cloning (C)], and the total number of similar sequences from the respective approach in parentheses. One representative sequence for each OTU and its European Nucleotide Archive accession number can be found in Table 1. Most sequences showed at least 98% similarity to known type strains, except for the isolates M1_I3 with only 94% sequence similarity to type strain *Flavobacterium ummariense* DS-12^T, M1_I7 with 97.1% sequence similarity to type strain *Acinetobacter johnsonii* CIP 64.6^T, M1_I20 with 96.6% sequence similarity to type strain *Acinetobacter junii* CIP 64.5^T, and clone sequence M3_C20 with similarity of 95.9% to type strain *Corynebacterium doosanense* CAU 212^T. These sequences indicate the presence of yet undescribed species.

Whereas the culture-dependent approach resulted in the isolation of bacteria of the phyla *Actinobacteria*,

Figure 1. Polymerase chain reaction products on a 1% agarose gel of the intact or heat-inactivated isolate *Pantoea agglomerans* M1_I4 in raw milk treated or not treated with propidium monoazide (PMA) at a final concentration of 100 μ M before DNA extraction. Lanes 1 and 2 = raw milk inoculated with intact isolate with PMA; lanes 3 and 4 = inoculated and heat-inactivated raw milk with PMA; lanes 5 and 6 = raw milk inoculated with intact isolate without PMA; lanes 7 and 8 = inoculated and heat-inactivated raw milk without PMA; N = negative control (PCR reaction without bacterial DNA); M = marker (Hyper Ladder I; Bioline GmbH, Luckenwalde, Germany).

Firmicutes, *Proteobacteria*, and *Bacteroidetes*, with 17 different species from 14 genera in sample M1 and 18 different species from 12 genera in sample M3, the clone libraries for samples M2 and M3 did not depict similar species richness. For M2, 8 species from 5 genera of the phylum *Proteobacteria* were detected in the clone libraries for both lysis approaches, whereas the

clone libraries from the enzymatic lysis approach of M3 resulted in the most sequences being from the phylum *Proteobacteria*, followed by *Bacteroidetes* and 2 *Actinobacteria* sequences with 8 species of 6 genera overall.

Shannon diversity, calculated for the set of isolates obtained from samples M1 and M3, were 2.79 and 2.84, respectively, with equitability values of 0.98. Shannon indices and equitabilities for samples M2 and M3, analyzed by the culture-independent method, were lower, with values of 1.76 and 1.79, and 0.85 and 0.86, respectively. Thus, detected species diversity of the same habitat was higher for the samples M1 and M3 analyzed by the culture-dependent method than for the samples M2 and M3 analyzed by the direct, culture-independent approach. An equitability value of almost 1 also indicates an even distribution of species within the isolates, whereas lower values for samples M2 and M3 investigated by the culture-independent method indicate higher tendency toward dominance of individual species.

DISCUSSION

To our knowledge, this is the first study comparing culture-dependent and culture-independent methods for bacterial community analysis of bulk tank raw milk comprising the application of viable PCR with PMA for live-dead discrimination of bacterial cells before DNA extraction. This ensures comparability of the depicted bacterial community structure of both methods.

Figure 2. Polymerase chain reaction products on a 1% agarose gel from raw milk sample 2 (M2; left) and raw milk sample 3 (M3; right) treated with 100 μ M propidium monoazide (PMA) before DNA extraction using enzymatic and mechanic cell lysis approaches. Lanes 1 and 4a = M2 + PMA; lanes 2 and 5 = M2 without PMA; lane 3 = M2 inoculated with isolate *Microbacterium ginsengiterrae* M1_I6 without PMA; lane 4b = reamplification of the PCR product from 4a; lane 6 = M2 inoculated with isolate *Pantoea agglomerans* M1_I4 without PMA; lanes 7a and 10 = M3 + PMA; lane 7b = M3 + PMA after further concentration of the DNA extract; lanes 8a and 11 = M3 without PMA; lane 8b = M3 without PMA after further concentration of the DNA extract; lanes 9 and 12 = M3 inoculated with a mixture of *Escherichia coli* DSM 498 and *Staphylococcus aureus* DSM 1104 without PMA; N = negative control (PCR reaction without DNA); M = marker (Hyper Ladder I; Bioline GmbH, Luckenwalde, Germany).

Figure 3. Rarefaction analysis of clone sequences obtained from raw milk sample M2 and raw milk sample M3 conducted with the program Analytic Rarefaction (<http://www.uga.edu/~strata/software>). OTU = operational taxonomic units.

Total bacterial counts and CC for milk samples M1 and M3 reflected very good quality of the raw milk that had been stored in the bulk tank at 4°C for 72 h before sampling. In milk sample M2, after similar storage conditions, TBC and CC were of a higher magnitude, each in the range of 10^5 cfu/mL, thus reflecting the poor quality of this sample. Short-term increases of bacterial counts caused by rapid proliferation of streptococci or *Enterobacteriaceae* are a well-known phenomenon for bulk tank milk (Hayes et al., 2001). Similar cell counts from TBC and CC suggested the dominance of *Enterobacteriaceae* for sample M2. The detection of clone sequences dominated by *Enterobacteriaceae* sequences support this assumption. In contrast, for samples M1 and M3 the CC were substantially lower than the TBC. This supports the assumption that sample M2 was not representative of bacterial community composition and TBC of the analyzed bulk tank can be considered as a spike (Hayes et al., 2001) for this sample.

Most bacterial genera detected in the milk samples analyzed here have already been reported for raw milk as well as teat skin and farm environment by other authors (Fricker et al., 2011; Vacheyrou et al., 2011; Verdier-Metz et al., 2012). Based on the culturing approach, samples M1 and M3 were dominated by gram-positive isolates of the phyla *Actinobacteria* and *Firmicutes*, probably originating from teat skin, whereas gram-negative isolates of the phylum *Proteobacteria* and *Bacteroidetes* formed the minority in both samples. However, TBC and CC for both samples were similar. In accordance with Rasolofo et al. (2010), on the third day of refrigerated storage of raw milk, species of the genera *Staphylococcus*, *Corynebacterium*, and *Acinetobacter* accounted for high percentages of bacteria isolated from samples M1 and M3. Moreover, the genus *Pseudomonas* was isolated quite often from sample M3.

Propidium monoazide treatment as well as DNA-extraction methods were optimized for raw milk in preliminary experiments. The experiments were conducted with *S. aureus* DSM 1104 and *E. coli* DSM 498 in nutrient broth and milk (data not shown) and confirmed that PMA photoinduction did not reduce the number of colony-forming units per milliliter of these test strains and, therefore, had no effect on the viability of bacteria. Propidium monoazide treatment at a final concentration of 100 μ M had no effect on DNA extraction from living bacteria. This was in accord with other reports of other authors about PMA-treated samples of living bacteria at a final concentration of 100 μ M (Bae and Wuertz, 2009; Nocker et al., 2009). Nocker et al. (2006) observed occurrence of DNA loss from living *E. coli* O157:H7 only after increasing the final PMA concentration to 240 μ M, whereas ethidium monoazide was shown to exert cytotoxic effects as well as the ability to penetrate into living cells at lower concentrations (Nocker et al., 2006; Cawthorn and Witthuhn, 2008). Thus, PMA treatment is better suited for reliable and almost complete depiction of mixed bacterial communities of unknown composition (Fittipaldi et al., 2012). Somatic cell eukaryotic DNA as well as milk compounds may be able to reduce effective PMA concentration by chemical adsorption or by shielding of bacterial cells during photoactivation. This effect has been discussed for compounds of environmental samples by other authors (Bae and Wuertz, 2009; Fittipaldi et al., 2012). However, our preliminary experiments with inoculated and heat-inactivated raw milk showed no negative effect of the milk matrix on live-dead discrimination (Figure 1).

Cell lysis and DNA extraction using silica gel columns of the DNeasy Blood and Tissue Kit (Qiagen GmbH) followed modified protocols of the manufacturer and literature. As in the present study, Di Pinto et al. (2007) increased amounts of the kit's lysis buffers and of proteinase K for bacterial cell disruption and DNA extraction from complex food matrices. Milk compounds, such as lipids, whey proteins, and caseins, may function as possible PCR inhibitors (Wilson, 1997) and were successfully removed by centrifugation, washing steps, and proteinase K treatment, as PCR amplification of 16S rRNA genes for clone library construction succeeded.

Species diversity in samples M1 and M3 detected by the culture-dependent approach was higher and more evenly distributed than diversity and distribution values for the samples M2 and M3 investigated culture independently. This was demonstrated by calculation of the Shannon index and equitability. Especially the gram-positive phyla *Firmicutes* and *Actinobacteria* were underrepresented in the clone libraries (Tables 1

Figure 4. Neighbor-joining phylogenetic tree of isolates (I) and clone sequences (C), constructed the “partial deletion” mode and a cut-off value of 70%. Numbers indicate the strain number of the respective isolate or the number of the clone sequence and numbers in parentheses give the total numbers of sequences with $\geq 99\%$ similarity gained by the respective approach. Correctness of topology was validated by bootstrap with 1,000 repeats; bootstrap values $\geq 70\%$ are presented.

and 2). Detection of species belonging to the phylum *Firmicutes* in samples M1 and M3 by the culture-dependent approach, but not by the direct approach, demonstrated that representatives of this phylum were discriminated by the culture-independent approach used in this study. Because a high number of sequences from gram-negative *Proteobacteria* were detected within the clone libraries for samples M2 and M3, we concluded that DNA extraction by silica gel columns and PCR amplification were not inhibited by the raw milk matrix. A subsequent experiment proved that the raw milk matrix had an inhibitory effect on enzymatic cell lysis of *Staphylococcus chromogenes* M3_I12 using species-specific primers but not on cell lysis of the isolate *Corynebacterium xerosis* M3_I17. In contrast to the *Staphylococcus* strain, this representative of the *Actinobacteria* could be clearly detected within the milk matrix (data not shown). Both strains were isolated from sample M3. Other authors also concluded that direct DNA extraction from milk samples may be hampered by sample compounds (Cremonesi et al., 2006; Yeung, 2012), which has crucial influence on the depicted bacterial diversity.

In most other studies investigating raw milk by culture-independent methods, preferential DNA extraction of gram-negative bacteria has not been observed (Rasolofo et al., 2010; Raats et al., 2011; Quigley et al., 2013). Nevertheless, Fricker et al. (2011) detected a higher proportion of gram-negative bacteria within clone libraries compared with culture-dependent methods and none of the cloned sequences was assigned to the genus *Staphylococcus*. This is in accordance with our study. Although 2 *Staphylococcus* sequences could also be detected by cloning of 16S rRNA genes after DNA extraction directly from raw milk, Delbès et al. (2007) detected higher diversity of the genus *Staphylococcus* by culturing approaches. For cultivation-independent detection of staphylococci, the enzymatic lysis approach described in our study could possibly be optimized by additional use of lysostaphin, as reported by Verdier-Metz et al. (2012), or by a combination of mechanical and enzymatic cell lysis.

In contrast to the cultivation approach, the phylum *Actinobacteria* was poorly represented in the clone libraries of samples M2 and M3. This may be attributed to the high GC (guanine-cytosine) content of their DNA (Suzuki and Giovannoni, 1996; von Wintzingerode et al., 1997). Preferred amplification of sequences with low GC content probably occurs more frequently in mixtures of different 16S rRNA genes.

The mechanical cell lysis approach resulted in a detectable PCR product after a second amplification only for sample M2 with increased TBC, whereas for M3, no detectable product could be achieved at all. This meth-

od of cell lysis is probably better suited for raw milk containing large amounts of bacteria because the probability of repeated collisions with glass beads as well as frictional forces between the cells themselves rises with increased cell numbers. All chimeric sequences detected in sample M2 originated from the PCR product of the mechanical lysis approach. The occurrence of these artifacts may be related to the application of a second PCR to the first product using the same primers.

Polymerase chain reaction product yield was quite low for the enzymatic approach as well, but could be increased by concentration of the DNA extract of sample M3. Despite the low DNA concentrations obtained after direct extraction from 10 mL of raw milk, we refrained from the use of higher raw milk volumes, as we considered saturation of the silica columns by bacterial and bovine DNA. Other studies described successful DNA extraction from raw milk sample volumes of 250 μ L (Verdier-Metz et al., 2009) or 1 mL (Delbès et al., 2007; Quigley et al., 2013), so evidence exists that representative DNA extracts may also be obtained from lower amounts of raw milk. Yuan et al. (2012) showed that the extracted amount of DNA is not related to the detected microbial diversity. Even PCR products derived from small amounts of DNA, such as in the present study, are sufficient for an almost complete representation of bacterial diversity in the sample. The rarefaction analysis and calculation of coverage for samples M2 and M3 demonstrated that the diversity within the PCR products was almost completely covered by the sequencing approach. However, the bias introduced by cell lysis, DNA extraction, and PCR amplification could not be assessed by our diversity analyses.

The results of the present study showed that assessment of bacteriological quality of milk samples was possible using the well-established culturing method for determination of TBC and CC. Via grouping of the isolates and subsequent identification of representative individuals for each group, percentages of each phylum were calculated with reference to total number of isolates (Table 2). Thus, estimations could be made for the original culturable bacterial community composition at the time of sampling. Nevertheless, complete coverage of the microbiota by culturing methods is unrealistic, as a preselection of bacteria is performed by choice of media, incubation conditions, and temperatures as well as selection of morphologically significantly different colonies for isolation. Lebaron et al. (1998) described large species variability within similar colony morphotypes from marine environmental samples. Consequently, different species within the same colony morphotype could have been underestimated in the present study. The detection of different species within the same genus by the culture-dependent method and by the culture-

independent method could be a hint for the correctness of this assumption.

Selective media, such as VRBA for coliform bacteria and MRS agar for lactic acid bacteria, provide particularly good growth conditions for the respective bacteria. By isolation from these media, percentages of corresponding genera in the original milk sample might be overestimated by relation to the total number of isolates. This could be the case for the genera *Lactococcus* and *Pediococcus* from sample M1 as well as for the *E. coli* isolates from sample M3. Two of the 5 *Lactococcus* isolates and all 6 *Pediococcus* isolates were obtained from MRS agar, whereas 5 of the 14 *Escherichia* isolates were obtained from VRBA. Consequently, their percentages in Table 2 might have been overestimated. Another disadvantage of culture-dependent methods is that they are more time consuming than culture-independent methods. Moreover, they are not able to detect bacteria in the “viable but nonculturable” state (Oliver, 2005). However, isolation techniques are and will be fundamental for the in-depth characterization of exceptional strains, which may represent new species from this source. The microbial diversity of raw milk is still a growing area of research (Wiertz et al., 2013).

In contrast, culture-independent methods, based on detection of DNA should be able to provide a more complete picture of the community of living bacteria in raw milk. Successful introduction of PMA treatment for the exclusive detection of living bacteria is an important tool to overcome the detection of DNA from dead cells, a striking disadvantage of molecular methods. The results of the present study stress the importance of effective bacterial cell lysis as a crucial step toward the almost complete detection of the living bacterial community present in raw milk. Although it was shown that the phylum *Firmicutes* is strongly discriminated by the lysis approaches used, the importance of culture-independent methods was emphasized. As can be seen in Tables 1 and 2, different species of the same genus were detected by the respective methods. The genera *Aeromonas* and *Ralstonia* as well as the genus *Sphingobacterium* were only detected in the clone libraries of samples M2 and M3, respectively. With the endpoint PCR method used in the present study, no quantitative statements about bacterial community composition of the bulk tank raw milk samples could be made. After achievement of more effective cell lysis and direct DNA extraction, fast quantitative molecular methods could be developed to capture the bacterial community structure in raw milk almost completely.

The results of the present study lead to the conclusion that the combination of culture-dependent and -independent methods is the best choice to cover the bacterial community composition of bulk tank raw

milk. This result is in accordance with the conclusions of Delbès et al. (2007) and Fricker et al. (2011). In our study, PMA treatment was successfully introduced for the milk matrix and was found to be applicable in this matrix for direct detection of viable cells. This could help to focus direct methods for those populations that may represent a risk for product quality and safety.

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APPENDIX

Table A1. Fatty acid profiles of the isolate groups 1 to 5 (with SD in parentheses) and the number of isolates for each group, cell morphology, and Gram-staining results

Item	Group and subgroup ¹							
	1		2	3		4	5	
	1A	1B		3A	3B		5A	5B
No. of isolates	9	1	5	3	2	4	1	6
Morphology	R	R	R	C/R	C	C	C	C
Gram stain	+	+	+	+	+	+	+	+
FA (%)								
10:0			0.4 (1.0)					
12:0			0.3 (0.8)			0.7 (0.6)		0.3 (0.7)
14:0		3.7		9.4 (0.6)	21.6 (7.0)	4.6 (2.2)	0.7	0.5 (0.7)
<i>iso</i> 15:0								
<i>anteiso</i> 15:0								
<i>cis</i> -11 15:1	0.2 (0.3)				1.1 (1.6)			
<i>iso</i> 16:0								
<i>cis</i> -7 16:1						18.8 (3.8)		
<i>cis</i> -9 16:1			0.7 (1.5)		0.4 (0.4)			0.1 (0.2)
<i>cis</i> -10 16:1		16.3						
<i>trans</i> -9 16:1								
16:0	42.8 (5.0)	51.4	1.8 (1.2)	82.0 (2.0)	59.1 (10.1)	47.8 (17.8)	0.6	0.6 (0.8)
<i>iso</i> 17:0			0.1 (0.3)				59.5	76.2 (6.8)
<i>anteiso</i> 17:0								
ECL 16.7 ²	4.0 (1.3)		0.0 (0.1)					
<i>cis</i> -6 17:1			0.4 (0.9)					
17:0			0.2 (0.2)					
<i>cis</i> -9 18:1	51.6 (4.9)	15.3	80.4 (3.5)			21.5 (12.6)		
<i>cis</i> -11 18:1				2.7 (0.4)	5.2 (1.2)	2.1 (2.5)	36.9	20.5 (7.4)
18:0	1.3 (0.3)		15.6 (6.9)		0.1 (0.1)	3.9 (0.8)	2.3	2.0 (1.0)
10-Methyl 18:0	0.1 (0.2)	13.2						
<i>cis</i> -7 19:1								
19:0								
<i>cyclo</i> 11–12 19:0				5.9 (1.1)	12.5 (3.4)			
11-Methoxy 17:0						0.9 (1.5)		

¹R = rod shaped; C = coccoid; + = gram positive.²ECL = equivalent chain length.

Table A2. Fatty acid profiles of the isolate groups 6 to 10 (with SD in parentheses) and the number of isolates for each group, cell morphology, and Gram-staining results

Item	Group and subgroup ¹									
	6			7		8	9		10	
	6A	6B	6C	7A	7B		9A	9B	10A	10B
No. of isolates	2	7	2	1	7	2	1	1	4	2
Morphology	C/R	C	C	R	R	C	R	R	C/R	C/R
Gram stain	+	+	+	+	+	+	–	v	–	v
FA (%)										
10:0									2.1 (2.8)	
12:0									5.0 (2.3)	9.8 (0.9)
<i>iso</i> 13:0		0.3 (0.4)								
12:0 3OH									0.2 (0.4)	
ECL 13.5 ²								11.3		
<i>iso</i> 14:0	0.1 (0.1)		0.2 (0.3)							
14:0			0.5 (0.0)							0.1 (0.1)
<i>iso</i> 15:0	4.2 (1.3)	9.3 (2.3)	1.0 (0.9)	3.5	0.2 (0.2)	24.4 (13.1)	80.8	80.5		
<i>anteiso</i> 15:0	62.2 (4.8)	82.4 (6.1)	87.4 (5.6)	95.1	61.4 (8.7)	61.8 (19.9)	8.7			
<i>iso</i> 16:0	1.8 (2.1)		1.8 (1.5)	0.7	3.2 (2.7)	0.3 (0.4)	0.5			
<i>cis</i> -9 16:1								6.6	21.6 (5.4)	52.9 (7.1)
16:0	0.3 (0.5)		1.6 (0.3)		0.6 (1.0)				27.6 (9.3)	22.3 (8.6)
<i>iso cis</i> -9 17:1							9.9	1.2		
<i>iso</i> 17:0	4.1 (5.5)	0.9 (0.6)				3.8 (2.4)				0.8 (1.2)
<i>anteiso</i> 17:0	24.8 (4.0)	7.2 (5.4)	7.4 (5.7)	0.8	34.4 (10.6)	8.3 (3.8)				
<i>cis</i> -9 18:1									39.0 (3.9)	10.3 (2.1)
<i>cis</i> -11 18:1									3.0 (3.5)	3.8 (0.1)
18:0	0.9 (1.3)	0.1 (0.1)				0.8 (0.3)			1.3 (2.3)	
<i>cis</i> -7 19:1	1.0 (1.4)									
19:0	0.3 (0.5)									
20:0	0.3 (0.4)					0.4 (0.6)				

¹R = rod shaped; C = coccoid; + = gram positive; – = gram negative; v = gram variable.²ECL = equivalent chain length.

Table A3. Fatty acid profiles of the isolate groups 11 to 17 (with SD in parentheses) and the number of isolates for each group, cell morphology, and Gram-staining results

Item	Group and subgroup ¹								
	11	12	13	14			15	16	17
				14A	14B	14C			
No. of isolates	1	1	1	1	1	1	12	2	13
Morphology	C	R	R	C	R	R	C	C	R
Gram stain	+	v	+	+	v	+	+ / v	+	- / v
FA (%)									
10:0									0.1 (0.1)
12:0	2.3								5.8 (0.9)
<i>iso</i> 13:0							0.1 (0.2)		
12:0 3OH									0.4 (0.2)
<i>iso</i> 14:0								0.4 (0.1)	
14:0	3.8	0.8							
<i>iso</i> 15:0				1.0	3.2	6.8	3.3 (0.8)	0.1 (0.1)	
<i>anteiso</i> 15:0				75.6	72.6	77.0	92.5 (2.1)	91.6 (1.6)	
15:0		0.6							
14:0 2OH		3.0							
<i>iso</i> 16:0					10.2			6.0 (1.4)	
<i>cis</i> -9 16:1	26.5	28.3	0.4						8.6 (3.0)
<i>cis</i> -11 16:1		1.2							
16:0	57.8	20.0	5.9				0.1 (0.4)		44.3 (3.0)
<i>iso</i> 17:0				1.3	0.5	4.1	0.5 (0.3)		
<i>anteiso</i> 17:0				19.3	13.5	12.0	3.4 (1.6)	1.9 (0.0)	
<i>cis</i> -11 17:1		2.5							
<i>cyclo</i> 17:0	6.2								
17:0			0.7						
<i>cis</i> -9 18:1									40.3 (3.5)
<i>cis</i> -11 18:1	3.4	41.8	57.2						
18:0		0.3	6.8	0.9			0.2 (0.3)		0.5 (0.6)
<i>cis</i> -11 11-methyl 18:1		1.5	0.5						
<i>iso</i> 19:0				0.3					
<i>anteiso</i> 19:0				1.4					
<i>cyclo</i> 11–12 19:0			28.4						
20:0				0.2					

¹R = rod shaped; C = coccoid; + = gram positive; - = gram negative; v = gram variable.

Table A4. Fatty acid profiles of the isolate groups 18 to 22 (with SD in parentheses) and the number of isolates for each group, cell morphology, and Gram-staining results

Item	Group and subgroup ¹						
	19			20	21		22
	18	19A	19B		21A	21B	
No. of isolates	7	14	1	1	1	1	1
Morphology	R	R	R	C	R	R	R
Gram stain	—	—/v	—	+	—	—	—
FA (%)							
ECL 10.85 ²		3.6 (1.3)	2.9				
10:0 3OH	0.1 (0.1)						
12:0	1.6 (0.2)	2.4 (0.2)	0.8				
<i>iso</i> 13:0						0.5	
12:0 2OH	0.2 (0.1)						
12:0 3OH	0.2 (0.2)						
ECL 13.5					9.0	9.5	
14:0		2.2 (0.2)	1.9				0.7
<i>iso</i> 15:0					53.1	83.6	49.4
<i>anteiso</i> 15:0					8.8		0.7
15:0		0.2 (0.1)	0.7				
14:0 3OH		0.3 (0.2)	0.2				
<i>iso</i> 16:0					0.1		
<i>cis</i> -9 16:1	24.0 (3.2)	9.1 (2.0)	42.6				39.6
<i>iso</i> 15:0 2OH					1.9	0.7	
16:0	66.9 (4.3)	56.9 (4.1)	42.2	8.8	0.3	0.1	8.5
<i>iso</i> 15:0 3OH					0.1	0.1	0.4
<i>iso cis</i> -9 17:1					24.5	4.9	
<i>iso</i> 17:0					0.7		
ECL 16.7				64.4			
<i>cyclo</i> 17:0	2.3 (1.4)	4.1 (1.6)	0.1				
17:0		0.1 (0.1)	0.5				
16:0 3OH							0.3
<i>cis</i> -9 18:1				26.2			
<i>cis</i> -11 18:1	4.5 (1.0)	21.0 (2.8)	8.1				
18:0	0.0 (0.1)			0.6			
<i>iso</i> 17:0 3OH					1.4	0.6	0.5

¹R = rod shaped; C = coccoid; + = gram-positive; — = gram negative; v = gram variable.²ECL = equivalent chain length.