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The contribution of fast growing, psychrotrophic microorganisms on biodiversity of refrigerated raw cow's milk with high bacterial counts and their food spoilage potential

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ABSTRACT

Storage of raw milk in the bulk tank creates an environment which selects for psychrotrophic bacteria. Results from earlier studies suggested that the microbiota of bulk tank milk with high bacterial counts is dominated by single, cold-adapted species with high growth rates at low temperatures. We checked this assumption in more detail and analyzed the microbial diversity of 48 samples from bulk tank raw cow's milk with bacterial counts > 100,000 cfu/mL from different geographic regions by culture-dependent and –independent methods.

Contrary to presumptions from earlier studies, only the minority (24%) of samples was dominated by a single bacterial species and diversity was not correlated with bacterial counts. The dominating species in this group of samples were identified as psychrotrophic *Acinetobacter* and *Pseudomonas* species, related to poor hygiene and spoilage, or mesophilic, mastitis-related *Streptococcus* species and *Escherichia coli*. This shows that storage of raw milk under refrigeration does not always lead to a selection of cold-adapted bacteria.

Approximately half of the raw milk isolates showed either lipolytic or proteolytic activity at 10 °C or 4 °C. Consistent or increased enzymatic activity at cold temperatures was detected for *Acinetobacter* spp. and *Pseudomonas* spp., but also for genera with minor abundance, e.g. *Carnobacterium* and *Arthrobacter*.

1. Introduction

Raw milk provides a highly nutritious habitat which is susceptible for microbial contamination because it contains various macronutrients (carbohydrates, fats and proteins), micronutrients (vitamins, amino acids and minerals), an almost neutral pH (6.4–6.7) and high water activity (Quigley et al., 2013). It facilitates high growth rates for various microbial contaminants, especially if storage temperatures are within the growth range of these microorganisms. The sources of contaminations are versatile, including the cow's udder surface, feed, feces or the milking equipment (Quigley et al., 2013; Vacheyrou et al., 2011).

The bacterial load of raw milk affects quality of the processed milk and dairy products and high bacterial counts may cause substantial economic losses for the dairy producer (Zwilling et al., 2016). Despite storage under refrigeration, bacteria are able to grow in raw milk, leading to high bacterial counts. This condition creates a selective environment for psychrotrophic bacteria which are able to outgrow the accompanying microorganisms even at optimal storage temperatures of 4 °C due to high growth rates (Lafarge et al., 2004). De Jonghe et al. (2011) simulated the outgrowth of *Pseudomonas* species in raw milk on a laboratory scale and emphasized that a prolonged storage time leads to elevated bacterial counts. Especially the genus *Pseudomonas* was identified as main dominating contaminant of refrigerated raw milk and was in focus of various investigations (Cousin, 1982; De Jonghe et al., 2011; Ercolini et al., 2009; Raats et al., 2011; Sørhaug and Stepaniak, 1997).

Psychrotrophic bacteria like *Pseudomonas* spp. are able to produce proteolytic or lipolytic enzymes, which degrade components of raw milk, leading to failures in flavor and texture (De Jonghe et al., 2011; Fricker et al., 2011; Ribeiro Júnior et al., 2018; Von Neubeck et al., 2015). Extracellular proteases and lipases may also affect shelf life of processed milk because some are able to withstand even ultra-high-temperature treatment (Alves et al., 2016; Sørhaug and Stepaniak, 1997). Especially poor hygiene and biofilm formation in storage tanks or milking equipment are a major source of elevated bacterial counts in raw milk (Cousin, 1982; Marchand et al., 2012).

Until now, only few studies analyzed the microbial composition of raw milk from the bulk tank with high total bacterial counts (**TBC**). These studies suggested that single bacterial species dominated high TBC raw milk (Holm et al., 2004; Rodrigues et al., 2017). Because some

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of our preliminary analyses indicated the presence of a more diverse microbiota in raw milk with high bacterial counts, we checked the diversity of high TBC raw milk for a larger sample size and with samples from two distant geographic regions in Germany. Additionally, we analyzed proteolytic and lipolytic activity at different temperatures, to assess the food spoilage potential of the dominant microbial contaminants from raw milk. In order to explore the source of bacterial contamination, we also tested the biofilm-forming capacity of the dominant microbial contaminants isolated from raw milk. Raw milk samples taken by the local authorities from the bulk tank are routinely conserved with a chemical conserving agent. Therefore, we preliminary tested the use of conserved raw milk for cultivation-dependent analyses.

2. Material and methods

2.1. Raw milk samples

Raw milk samples were obtained from the state control association LKV North Rhine-Westphalia and the institute for milk analyses IfM Verden. The samples originated from different dairy farms in North Rhine-Westphalia and Lower Saxony (Germany) and were taken at three points in time (March, April and July) over a period of two years. Samples were taken by these two labs from the bulk tank by the time the milk was collected from the farmer to the dairy. Samples were preserved with azidiol (0.4% v/v) by the LKV or boric acid (0.6% w/v) by the IfM immediately after sampling (Barcina et al., 1987; Gill, 1932). TBC was determined routinely at the LKV and IfM using flow cytometry (BVL, 2002). About 1.5% of these samples showed a TBC ≥100,000 cfu/mL and were classified as class 2 raw milk according to the German Milk Quality Regulation (2010). From this subset, 48 samples were selected and included in this study. Aliquots (20 mL) of these raw milk samples were transported on ice to our laboratory within 24 h of sample collection and culture-dependent and -independent analyses were started on the same day.

2.2. Preservation of raw milk

Bactericidal effects of azidiol or boric acid against raw milk associated microorganisms were tested before analyses of the raw milk samples. Unpreserved and preserved raw milk was stored at 4 °C for 4d and the Standard Plate Count (**SPC**) was determined on tryptic soy agar (**TSA**, Merck) after 3 h, 24 h and 4d. The bactericidal effect of preservatives was measured as decrease of SPC during storage at 4 °C.

2.3. Cultivation procedure

Raw milk samples were serially diluted in Ringer's solution and the SPC was determined as colony forming units per mL (cfu/mL) on TSA at 30 °C after 48 h. Additionally, the number of psychrotrophs was determined on TSA at 10 °C after 7d (Scherer and Neuhaus, 2006).

2.4. Isolation, differentiation and identification of isolates

Numbers and percentages of colonies with different morphotypes were determined on plates with 30–300 colonies. Morphotypes were determined by means of macroscopic criteria, as well as cell-morphology, gram-staining behavior and oxidase activity. A representative number of isolates (up to 5) from each morphotype and from each cultivation temperature and raw milk sample were subcultivated on TSA for further analyses of their fatty acid profiles and 16S rRNA gene sequences. Oxidase activity was tested with Bactident oxidase test strips (Merck), gram-staining behavior was determined according to Gerhardt et al. (1981) and cell morphology was observed with a Zeiss Axio Observer phase-contrast microscope. Analyses of the fatty acid (**FA**) patterns was performed as described previously by Weber et al. (2014). Extraction of genomic DNA, amplification and sequencing of partial and complete 16S rRNA gene sequences were performed as described previously (Buchholz-Cleven et al., 1997; Wiertz et al., 2013). Sequences were manually edited with Chromas Lite 2.1.1. (Technelysium Pty Ltd, South Brisbane, AU) and assembled with BioEdit 7.2.5 (Hall, 1999). Isolates were identified based on their 16S rRNA gene sequences using the Basic local alignment search tool (BLAST; Altschul et al., 1990). Presumptive *E. coli* isolates were additionally identified using the API 20E test system (BioMèrieux, France) according to the manufacturer's specifications.

2.5. Extraction of DNA from raw milk and construction of 16S rRNA gene clone libraries

Lysis of bacterial cells and extraction of DNA from raw milk samples (10 mL) was performed as described previously (Weber et al., 2014), using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germany) in an enzymatic approach with lysozyme (20 mg/mL; Sigma-Aldrich, USA) and mutanolysin (stock solution: 4000 U in 0.1 M potassium phosphate buffer, pH 6.2; Sigma-Aldrich, USA). 16S rRNA genes were amplified using the universal bacterial primer 8F and 1492R (Buchholz-Cleven et al., 1997) and PCR products were purified using the QIAquick PCR purification kit (Qiagen, Germany). Clone libraries were established as described by Weber et al. (2014). PCR products were cloned using the pGEM®-T vector system (Promega, USA) and transformed into competent *E. coli* JM109 ($> 1 \times 10^8$ cfu/µg DNA; Promega, USA). Clones from each clone library were randomly selected and inserts were amplified using M13F and M13R primer (Messing, 1983). PCR products were purified, sequenced as described above, and clone sequences (partial 16S rRNA sequences with 600 bp or full 16S rRNA sequences with 1,500 bp) were identified by comparison to the sequences of type strains using BLAST. Clone sequences were checked for chimeric sequences using DECIPHER v9.21 (Wright et al., 2012). For each raw milk sample, 5 clone sequences were analyzed. If these sequences were not identical, up to 13 additional clones were sequenced to calculate the diversity of the clone libraries per sample.

2.6. Determination of diversity of raw milk samples

16S rRNA gene sequences of clones were classified into operational taxonomic units (**OTU**) at > 99% gene sequence similarity using USEARCH v9 (Edgar, 2010). Coverage was calculated in order to assess the completeness of total sequence diversity according to Good (1953). Diversity of raw milk samples was determined by calculating the Shannon diversity index (H) and the species evenness (J) (Shannon and Weaver, 1969). For the culture-dependent approach, numbers of different morphotypes on the TSA plate with the lowest dilution and less than 300 colonies were used to calculate indices H and J.

2.7. Lipolytic and proteolytic activity

Lipolytic activity of representative isolates was analyzed on tributyrin agar (TSA supplemented with 1.0% v/v tributyrin) and proteolytic activity was analyzed on skim milk agar (TSA with 5.0% w/v skim milk powder) (Harrigan, 1998). Both tests were performed at 30 °C for 48 h and at 10 °C and 4 °C for 7d as described before by Hahne et al. (2018). Strains were considered positive for proteolysis or lipolysis by formation of a transparent halo around the colonies.

2.8. Microtiter plate assay for biofilm formation

The ability of representative isolates to form biofilms was analyzed in 96-well polystyrene microtiter plates (Greiner Bio-One, USA) according to Kolari et al. (2001). Each well with 200 µL tryptic soy broth (**TSB**) was inoculated with 2 µL of overnight cultures, adjusted to McFarland Standard No. 0.5 (approx. 1.5×10^8 cfu/mL). Uninoculated

Table 1
Bacterial isolates and clones recovered from raw cow's milk with high TBC. GenBank accession numbers are given for full or partial 16S rRNA gene sequences.

Isolates	Acc.-No.	Clones	Acc.-No.	Taxon
JZ RK-19 (n = 10)	GenBank: MH119668	K111 (n = 37)	GenBank: MH128602	*Acinetobacter albensis*
JZ R-39	GenBank: MH119673	K78 (n = 4)	GenBank: MH128608	*Acinetobacter baumannii*
		C_K103	GenBank: MH128607	*Acinetobacter bereziniae*
		B_K311	GenBank: MH128611	*Acinetobacter bohemicus*
JZ R-85	GenBank: MH119672			*Acinetobacter gerneri*
JZ RK-34 (n = 4)	GenBank: MH119669	B_K325 (n = 10)	GenBank: MH128604	*Acinetobacter guillouiae*
JZ RK-116 (n = 14)	GenBank: MH119667	B_K128 (n = 53)	GenBank: MH128597	*Acinetobacter johnsonii*
		B_K155	GenBank: MH128609	*Acinetobacter lwoffii*
JZ R-104 (n = 2)	GenBank: MH119670			*Acinetobacter parvus*
JZ R-155	GenBank: MH119671			*Acinetobacter* sp. 1
		B_K66 (n = 14)	GenBank: MH128613	*Acinetobacter* sp. 2
		B_K465 (n = 3)	GenBank: MH128598	*Acinetobacter* sp. 3
		C_K8	GenBank: MH128599	*Acinetobacter* sp. 4
		K641	GenBank: MH128600	*Acinetobacter* sp. 5
		B_K193	GenBank: MH128601	*Acinetobacter* sp. 6
		C_K6	GenBank: MH128612	*Acinetobacter* sp. 7
		B_K216	GenBank: MH128606	*Acinetobacter* sp. 8
		K662	GenBank: MH128610	*Acinetobacter* sp. 9
		B_K555	GenBank: MH128605	*Acinetobacter* sp. 10
		B_K563	GenBank: MH128675	*Aeromicrobium* sp.
JZ RK-62	GenBank: MH119684			*Aeromonas rivipollensis*
		K717 (n = 2)	GenBank: MH128660	*Aerococcus suis*
		B_K24 (n = 6)	GenBank: MH128661	*Aerococcus viridans*
		K673	GenBank: MH128665	*Alloiococcus* sp. 1
		K675	GenBank: MH128666	*Alloiococcus* sp. 2
JZ RK-114	GenBank: MH119716			*Arthrobacter oryzae*
JZ RK-74, JZ RK-36	GenBank: MH119717, GenBank: MH119718			*Arthrobacter psychrolactophilus*
JZ R-35	GenBank: MH119719			*Arthrobacter* sp. 1
JZ R-183 (n = 3)	GenBank: MH119720			*Arthrobacter* sp. 2
JZ R-7 (n = 5)	GenBank: MH119706			*Bacillus licheniformis*
JZ R-10	GenBank: MH119707			*Bacillus idriensis*
DI-4	GenBank: MH119708			*Bacillus persicus*
		K678	GenBank: MH128629	*Bdellovibrio* sp.
DI-9	GenBank: MH119743			*Bergeyella* sp.
JZ R-8	GenBank: MH119725			*Brachybacterium conglomeratum*
		C_K18	GenBank: MH128672	*Brachybacterium paraconglomeratum*
JZ R-27 (n = 3)	GenBank: MH119726			*Brevibacterium frigoritolerans*
		K759	GenBank: MH128685	*Brumimicrobium* sp.
JZ RK-71 (n = 6)	GenBank: MH119709	B_K477 (n = 7), K775 (n = 2)	GenBank: MH128647, GenBank: MH128648	*Carnobacterium maltaromaticum*
JZ RK-43	GenBank: MH119710	K226	GenBank: MH128649	*Carnobacterium divergens*
JZ R-43	GenBank: MH119731			*Calidifontibacter* sp.
JZ RK-33 (n = 4)	GenBank: MH119734	B_K4 (n = 4)	GenBank: MH128677	*Chryseobacterium carnipullorum*
JZ RK-54	GenBank: MH119735	K664	GenBank: MH128678	*Chryseobacterium vrystaatense*
JZ RK-16 (n = 3)	GenBank: MH119736			*Chryseobacterium joostei*
JZ R-108	GenBank: MH119737	K666 (n = 5)	GenBank: MH128679	*Chryseobacterium bovis*
JZ R-187	GenBank: MH119738			*Chryseobacterium balustinum*
JZ R-180	GenBank: MH119739			*Chryseobacterium montanum*
		K3 (n = 9)	GenBank: MH128681	*Chryseobacterium haifense*
JZ RK-118	GenBank: MH119740			*Chryseobacterium* sp. 1
		B_K191 (n = 4)	GenBank: MH128682	*Chryseobacterium* sp. 2
		B_K230	GenBank: MH128683	*Chryseobacterium* sp. 3
JZ R-64 (n = 2)	GenBank: MH119675			*Citrobacter freundii*
		C_K20 (n = 2)	GenBank: MH128616	*Citrobacter gillenii*
JZ R-132 (n = 2)	GenBank: MH119721			*Corynebacterium casei*
JZ R-70	GenBank: MH119722			*Corynebacterium frankenforstense*
JZ R-26	GenBank: MH119723			*Corynebacterium xerosis*
JZ R-177	GenBank: MH119724			*"Corynebacterium phoceense"*
DI-6	GenBank: MH119733			*Dietzia* sp.
		K608 (n = 2)	GenBank: MH128628	*Dyella* sp.
DI-10	GenBank: MH119744			*Empedobacter stercoris*
DI-13	GenBank: MH119711	B_K19 (n = 3)	GenBank: MH128650	*Enterococcus faecalis*
		K50	GenBank: MH128651	*Enterococcus camelliae*
		B_K206	GenBank: MH128652	*Enterococcus devriesei*
		K2.3 (n = 5)	GenBank: MH128654	*Enterococcus durans*
DI-39 (n = 3)	GenBank: MH119674	K5.15 (n = 29), K5.17	GenBank: MH128614, GenBank: MH128615	*Escherichia coli*
		K704 (n = 2)	GenBank: MH128663	*Facklamia* sp.
		K631	GenBank: MH128664	*Facklamia tabacinasalis*
		K679	GenBank: MH128686	*Fluviicola taffensis*
		K665 (n = 2)	GenBank: MH128662	*Globicatella* sp.
JZ R-210 (n = 3)	GenBank: MH119676	C_K92 (n = 4)	GenBank: MH128617	*Hafnia paralvei*
		B_K339 (n = 4)	GenBank: MH128618	*Hafnia alvei*

(*continued on next page*)

Table 1 (*continued*)

Isolates	Acc.-No.	Clones	Acc.-No.	Taxon
		B_K553	GenBank: MH128673	*Janibacter* sp.
JZ R-12	GenBank: MH119713			*Jeotgalicoccus psychrophilus*
		K706	GenBank: MH128656	*Jeotgalicoccus schoeneichii*
		K2.20	GenBank: MH128676	*Kocuria kristinae*
JZ RK-121	GenBank: MH119714	C_K63	GenBank: MH128670	*Kurthia gibsonii*
JZ RK-40 (n = 5)	GenBank: MH119694	B_K251 (n = 11), B_K275 (n = 5)	GenBank: MH128640, GenBank: MH128641	*Lactococcus lactis*
JZ RK-47 (n = 3)	GenBank: MH119695	C_K105 (n = 2)	GenBank: MH128642	*Lactococcus raffinolactis*
JZ RK-78 (n = 4)	GenBank: MH119696	K766 (n = 10)	GenBank: MH128643	*Lactococcus piscium*
JZ R-138	GenBank: MH119697	B_K280 (n = 3)	GenBank: MH128644	*Lactococcus garvieae*
		K633	GenBank: MH128657	*Lactobacillus plantarum*
		B_K296	GenBank: MH128658	*Lactobacillus casei*
JZ R-194 (n = 2)	GenBank: MH119677	C_K70 (n = 6)	GenBank: MH128619	*Lelliottia jeotgali*
JZ R-79	GenBank: MH119730			*"Leucobacter margaritiformis"*
		B_K160	GenBank: MH128667	*Leuconostoc lactis*
		B_K485 (n = 3)	GenBank: MH128668	*Leuconostoc mesenteroides*
		B_K277	GenBank: MH128659	*Listeria monocytogenes*
DI-44	GenBank: MH119712	K626	GenBank: MH128655	*"Macrococcus bohemicus"*
JZ R-90	GenBank: MH119727			*Microbacterium neimengense*
JZ R-184	GenBank: MH119728			*Microbacterium maritypicum*
JZ R-124	GenBank: MH119729			*Micrococcus luteus*
		K263	GenBank: MH128671	*Micrococcoides* sp.
JZ R-139 (n = 2)	GenBank: MH119685	K168 (n = 5)	GenBank: MH128623	*Moraxella osloensis*
JH14	GenBank: MH119686	B_K195	GenBank: MH128624	*Ochrobactrum anthropi*
		B_K554	GenBank: MH128674	*Ornithinimicrobium* sp.
JZ R-93	GenBank: MH119689			*Paracoccus aestuariivivens*
		K609	GenBank: MH128625	*Paracoccus carotinifaciens*
JZ R-134	GenBank: MH119680			*Pelistega* sp.
JZ R-83	GenBank: MH119679			*Providencia alcalifaciens*
JZ R-38	GenBank: MH119660			*Pseudomonas aeruginosa*
JZ RK-57 (n = 10)	GenBank: MH119657	B_K163 (n = 16)	GenBank: MH128587	*Pseudomonas azotoformans*
		B_K265	GenBank: MH128590	*Pseudomonas brenneri*
JZ R-133 (n = 6)	GenBank: MH119658	B_K198 (n = 4)	GenBank: MH128588	*Pseudomonas fluorescens*
JZ RK-32 (n = 2)	GenBank: MH119666	B_K171 (n = 19)	GenBank: MH128593	*Pseudomonas fragi*
JZ RK-60 (n = 3)	GenBank: MH119664	B_K54 (n = 3)	GenBank: MH128591	*Pseudomonas helleri*
		B_K126	GenBank: MH128596	*"Pseudomonas kribbensis"*
JZ R-127	GenBank: MH119662			*Pseudomonas mucidolens*
JZ RK-27	GenBank: MH119661			*Pseudomonas arsenicoxydans*
		B_K168	GenBank: MH128595	*Pseudomonas* sp.
JZ R-192	GenBank: MH119663			*Pseudomonas protegens*
		B_K304	GenBank: MH128594	*Pseudomonas rhizosphaerae*
JZ R-125	GenBank: MH119656	B_K161 (n = 4)	GenBank: MH128586	*Pseudomonas synxantha*
JH13 (n = 3)	GenBank: MH119665	B_K462 (n = 3)	GenBank: MH128592	*Pseudomonas weihenstephanensis*
JZ R-170 (n = 2)	GenBank: MH119659	B_K52	GenBank: MH128589	*Pseudomonas yamanorum*
		K692 (n = 2)	GenBank: MH128632	*Pseudoxanthomonas mexicana*
JZ R-135	GenBank: MH119681			*Psychrobacter fulvigenes*
DI-38	GenBank: MH119682			*Psychrobacter pulmonis*
		B_K218 (n = 2)	GenBank: MH128621	*Psychrobacter maritimus*
JZ RK-65	GenBank: MH119678			*Raoultella terrigena*
		C_K100 (n = 2)	GenBank: MH128620	*Raoultella ornithinolytica*
		K82	GenBank: MH128630	*Rhodanobacter glycinis*
JZ R-160	GenBank: MH119715			*Rhodococcus fascians*
JZ R-4	GenBank: MH119732			*Rothia endophytica*
JZ RK-38 (n = 2)	GenBank: MH119683			*Serratia liquefaciens*
		C_K62	GenBank: MH128622	*Serratia marcescens*
JZ RK-98	GenBank: MH119741			*Sphingobacterium faecium*
JZ RK-46	GenBank: MH119742			*Sphingobacterium kitahiroshimense*
		B_K122	GenBank: MH128684	*Sphingobacterium* sp.
JZ RK-126	GenBank: MH119687			*Stenotrophomonas lactitubi*
DI-47	GenBank: MH119688			*Stenotrophomonas terrae*
JZ R-73 (n = 2)	GenBank: MH119698	K354	GenBank: MH128645	*Staphylococcus aureus*
JZ R-3	GenBank: MH119699			*Staphylococcus epidermidis*
JZ RK-17 (n = 3)	GenBank: MH119700			*Staphylococcus equorum*
JZ R-86	GenBank: MH119701			*Staphylococcus haemolyticus*
JZ R-45	GenBank: MH119702	C_K91	GenBank: MH128646	*Staphylococcus sciuri*
JZ R-52	GenBank: MH119703			*Staphylococcus cohnii*
JZ R-63	GenBank: MH119704			*Staphylococcus vitulinus*
JZ R-15 (n = 3)	GenBank: MH119705			*Staphylococcus xylosus*
JZ R-106	GenBank: MH119690	B_K9	GenBank: MH128633	*Streptococcus parasuis*
JZ R-154	GenBank: MH119691	B_K403 (n = 18)	GenBank: MH128634	*Streptococcus uberis*
JZ R-97 (n = 4)	GenBank: MH119692	K725 (n = 20)	GenBank: MH128635	*Streptococcus parauberis*
JZ R-75	GenBank: MH119693	K346 (n = 5)	GenBank: MH128636	*Streptococcus dysgalactiae*
		K286 (n = 6)	GenBank: MH128637	*Streptococcus macedonicus*
		B_K528 (n = 11)	GenBank: MH128638	*Streptococcus lutetiensis*
		C_K66 (n = 2)	GenBank: MH128639	*Streptococcus thermophilus*

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Table 1 (*continued*)

Isolates	Acc.-No.	Clones	Acc.-No.	Taxon
		B_K225	GenBank: MH128669	*Trichococcus pasteurii*
		B_K248 (n = 5)	GenBank: MH128626	*Yersinia frederiksenii*
		C_K113 (n = 2)	GenBank: MH128627	*Yersinia* sp.
		B_K50	GenBank: MH128631	*Zoogloea oryzae*

wells with 200 µL TSB served as negative controls. Tests were performed in quadruplicate and incubated at 30 °C for 24 h and at 10 °C and 4 °C for 7d. The grown cultures were discarded and biofilms formed in the wells were stained with 250 µL crystal violet (4 g/L in 20% methanol) for 5 min and were subsequently washed under running water to remove non-adhering biomass. The remaining crystal violet from the adhering biomass was dissolved in 300 µL ethanol for 1 h. Absorption was measured at 550 nm ($\mathbf{A_{550nm}}$) using a microplate spectrophotometer (Epoch BioTek, USA). Isolates were considered positive for biofilm formation if ΔA_{550nm} was above 0.1.

2.9. Statistical analysis

Correlations of bacterial counts and diversity indices were analyzed by Pearson product-moment correlation coefficient. *P*-values below 0.05 were considered significant. Normal distribution of data was confirmed by Shapiro-Wilk test.

3. Results and discussion

3.1. Influence of preservation on bacterial count

Raw milk, sampled from the bulk tank for routine analyses of milk quality by the local authorities, is treated immediately with a preservative agent to maintain the properties of milk up until analysis (Barcina et al., 1987). The milk samples analyzed in this study had been conserved either by azidiol or by boric acid.

Preservation with azidiol or boric acid had no significant bactericidal effect on the SPC of raw milk stored at 4 °C up until 24 h. After 24 h, the SPC was 96.7% (standard deviation SD 12.3%) of the initial, unpreserved SPC. Only after 4d of storage, the SPC decreased to 89.2% (SD 7.3%) of the initial SPC. This confirmed earlier studies made by Gill (1932) and Amores et al. (2010), who did not find significant negative effects of the preservative agents azidiol or boric acid on the cultivation of bacteria from raw milk stored at refrigeration within 48 h. Gill (1932) recommended the use of boric acid as preservative if raw milk samples must be sent to the laboratory for analysis. Other studies confirmed that the use of preservatives did not have a negative effect on culture-independent, chemical or cytological analyses of refrigerated raw milk (Amores et al., 2011; Barcina et al., 1987; Llopis et al., 2013).

3.2. Bacterial counts of raw milk samples

The mean SPC of 48 raw milk samples at 30 °C was 5.8 log cfu/mL (range from 4.4 to 6.8 log cfu/mL) and the psychrotrophic count at 10 °C was 5.3 log cfu/mL (0–6.5 log cfu/mL). This was in accord with the results of the state control association LKV North Rhine-Westphalia and the institute for milk analyses IfM Verden, where all samples had been analyzed for TBC by flow cytometry. These data reported 5.7 log cfu/mL (5.0–7.0 log cfu/mL) as mean TBC for the 48 milk samples analyzed. The average ratio of SPC to TBC was 98.7%, with a range of 80.2–121.8%. The psychrotrophic count was generally lower than the SPC. On the average, the psychrotrophic count represented 87.4% of the SPC, with a range of 0–113.3%.

3.3. Identification of raw milk associated microbiota

3.3.1. Isolates

382 bacterial isolates were obtained from 48 raw milk samples. 226 isolates from cultivations at 30 °C and 157 isolates from cultivations at 10 °C. Isolates were divided into 18 groups and subgroups according to their FA patterns, morphological features, gram-staining behavior and oxidase activity. Full-length data of FA groups and subgroups is given as supplemental material (Table S1) in the online version of this article. 176 representative isolates were identified using 16S rRNA gene sequences. A total number of 89 different bacterial species were isolated from raw milk. A complete list of the identified strains and accession numbers of representative full or partial 16S rRNA gene sequences are given in Table 1. Isolates with 16S rRNA gene sequence similarity values above the threshold of 98.7% (Kim et al., 2014) were assigned to their closest related type strains. Otherwise, the isolates were assigned to the next related genera, indicating a new and possibly not yet described species.

3.3.2. Clone sequences

486 clone sequences were obtained from the analyzed raw milk samples. Among them, 98 OTUs (sequence similarity > 99%) with 600–1,500 bp were identified. This allows a higher taxonomic resolution and identification to species level, compared to data from other sequencing platforms like Illumina, which provides sequence lengths of about 300 bp. Representative clones of each OTU and accession numbers of partial or full 16S rRNA gene sequences are given in Table 1. An average coverage of 73.3% was calculated for the 48 raw milk samples included in this study. 40 raw milk samples had a coverage > 60%, including 15 samples with a coverage of 100%.

3.4. Diversity of raw milk samples

Diversity indices (H and J) were calculated to measure the bacterial diversity of the analyzed raw milk samples. J (between 0 and 1) indicates the evenness of species abundance in a given community and low evenness values indicate the presence of a single dominating species (Magurran, 1988). Based on this, the raw milk samples were assigned into two different groups according to their evenness values in this study: $J < 0.5$ or $J > 0.5$. $J < 0.5$ indicates the presence of one dominating bacterial taxon and $J > 0.5$ indicates the presence of a diverse microbial community without dominating taxa. Average H for samples with $J > 0.5$ was 1.30 (0.41–2.43) and average J was 0.82 (0.59–1.00). For samples with $J < 0.5$, average H was 0.28 (0.00–1.06) and average J was 0.20 (0.00–0.49).

Data from earlier studies suggested that high TBC raw milk from the bulk tank shows mainly single dominating bacteria, especially psychrotrophic bacteria, which are well adapted to the cold environment (Holm et al., 2004; Rodrigues et al., 2017). This could not be confirmed in this study. Actually, only the minority (24%) of raw milk samples showed a colonization with single dominating bacterial taxa and the majority was colonized by a diverse microbial community ($J > 0.5$). Additionally, correlation analysis revealed no significant correlation between SPC and J ($r = -0.015$, $p > 0.2$) or SPC and H ($r = -0.165$, $p > 0.2$). Figs. 1 and 2 show the relationship between these variables.

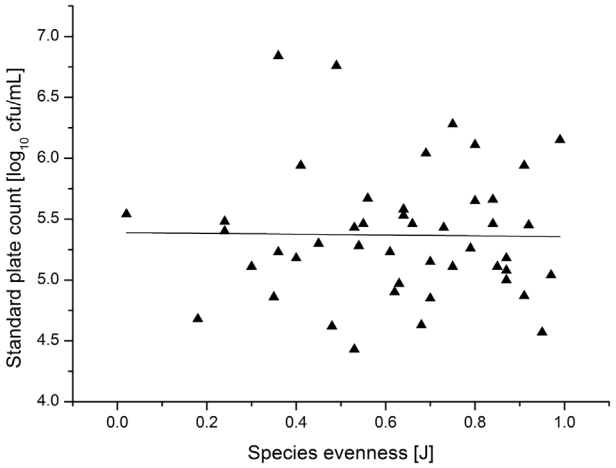

Fig. 1. Relationship between species evenness (J) and standard plate count (cfu/mL) among raw milk samples from the bulk tank ($r = -0.015$, $p > 0.2$). The line represents a linear fit of the data.

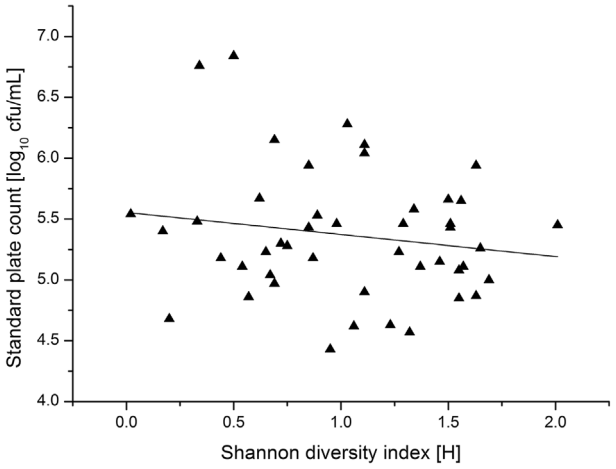

Fig. 2. Relationship between Shannon diversity index (H) and standard plate count (cfu/mL) among raw milk samples from the bulk tank ($r = -0.165$, $p > 0.2$). The line represents a linear fit of the data.

3.5. Dominating microbiota of raw milk samples

Table 2 shows percentages of samples with J < 0.5 and the corresponding dominating bacterial taxa. *Acinetobacter* spp., *Streptococcus* spp. and *Pseudomonas* spp. were the most prevalent dominating genera, but also *E. coli*, *Bacillus* sp., *Lactococcus* sp. and *Staphylococcus* sp. were detected. This shows that both psychrotrophic bacteria, with high growth rates at low temperatures, and mesophilic bacteria, which are not able to grow and can only persist at refrigeration, are able to represent the single dominating organism of high TBC raw milk. This can be explained by an already high environmental contamination of raw milk with non-psychrotrophic bacteria before storage in the bulk tank.

Table 2
Percentages of raw milk samples with J < 0.5 and the corresponding dominating bacterial taxa, as revealed by the culture-independent and -dependent approach.

Taxon	Culture-independent	Culture-dependent	
		30 °C	10 °C
	% of samples with J < 0.5	% of samples with J < 0.5	% of samples with J < 0.5
Acinetobacter spp.	10	2	15
Streptococcus spp.	10	8	0
Pseudomonas spp.	0	10	8
E. coli	4	2	4
Bacillus persicus	0	2	0
Lactococcus garviae	0	0	2
Staphylococcus equorum	0	0	2
Total	**24**	**24**	**31**

Table 3
Relative abundance of bacterial genera in 48 raw milk samples from the bulk tank with microbial load ≥100,000 cfu/mL identified by culture-dependent and –independent methods.

Taxon	% of clone sequences (n = 436)	% of isolates (n = 382)
Acinetobacter spp.	31	20
Streptococcus spp.	14	4
Pseudomonas spp.	12	25
Lactococcus spp.	7	9
Escherichia spp.	7	2
Chryseobacterium spp.	6	5
Carnobacterium spp.	2	4
Hafnia spp.	2	3
Staphylococcus spp.	< 1	5
Arthrobacter spp.	< 1	4
Bacillus spp.	–	3
Corynebacterium spp.	–	2
Other[a]	18	14

[a] Abundance ≤1%.

The genera and species detected in samples with J > 0.5 were similar to those detected in samples with J < 0.5. *Acinetobacter* and *Pseudomonas* were the predominant genera, but compared to the J < 0.5 group, the relative abundance of *E. coli* and *Streptococcus* spp. decreased significantly and the genera *Lactococcus*, *Chryseobacterium*, *Arthrobacter* and *Carnobacterium* emerged. Table 3 shows the relative abundance of bacterial genera in all raw milk samples, identified by the culture-dependent and -independent approach.

Compared to 30 °C, the culturable microbiota of the analyzed samples at 10 °C differed especially in relation to the psychrotrophic genera *Acinetobacter* and *Pseudomonas*. Here, the relative abundance of the two genera was higher than at 30 °C (54% at 10 °C, compared to 40% at 30 °C). In contrast to this, the genera *Streptococcus*, *Bacillus* and *Staphylococcus* decreased.

Streptococcus species dominated the microbiota of 8–10% of the raw milk samples from the bulk tank (Table 2), although the genus is considered mesophilic and most of the strains are not able to grow at temperatures below 10 °C. Additionally, the abundance of *Streptococcus* spp. decreased significantly with rising evenness of the raw milk samples. In samples with J > 0.5 only few *Streptococcus* strains with minor abundance were detected. Identification of the dominant species revealed *S. uberis*, *S. parauberis*, *S. macedonicus* and *S. lutetiensis*. All of these species were associated with diseases in animals or humans (Abdulamir et al., 2011; Jeffrey and Wilson, 1987; Jin et al., 2013) and especially *S. uberis* is highly related to clinical mastitis in cows (Djabri et al., 2002; Minst et al., 2012). *Streptococcus* spp. has been detected in raw milk samples with elevated bacterial counts before (Hayes et al., 2001; Holm et al., 2004; Rodrigues et al., 2017) and it presumably originates from the cow's udder microbiota. Unidentified clinical or subclinical mastitis of the cow may lead to elevated bacterial counts of raw milk. This was also shown by Jeffrey and Wilson (1987), who detected mastitis related bacteria in almost 50% of the bulk tank raw milk samples with high TBC.

As it was suggested in various studies before, psychrotrophic genera (*Acinetobacter* and *Pseudomonas*) were the most prevalent in raw milk from the bulk tank. Nevertheless, percentages of strains of the genus *Pseudomonas* were much lower than expected. In contrast, the presence of the genus *Acinetobacter* was higher (Table 3). Both genera are found

Table 4
Relative abundance of lipolytic- and proteolytic activity and biofilm formation of isolated taxa at different temperatures. Numbers represent percentages of strains, which showed growth and activity in the respective test related to the total number of strains (n).

Taxon	Lipolytic activity [a] (%)			Proteolytic activity [b] (%)			Biofilm formation [c] (%)		
	30 °C	10 °C	4 °C	30 °C	10 °C	4 °C	30 °C	10 °C	4 °C
Acinetobacter spp. (n = 33)	97	90	70	3	3	3	91	94	73
Pseudomonas spp. (n = 29)	67	85	85	37	74	67	83	93	90
Staphylococcus spp. (n = 12)	85	69	54	62	15	0	100	33	0
Streptococcus spp. (n = 6)	0	0	0	100	33	17	20	20	0
Lactococcus spp. (n = 11)	20	10	0	90	60	30	55	36	18
Chryseobacterium spp. (n = 12)	18	9	0	55	18	9	17	58	33
Carnobacterium spp. (n = 7)	14	29	0	100	86	86	71	43	29
Bacillus spp. (n = 6)	88	50	38	38	38	0	83	33	17
Arthrobacter spp. (n = 5)	50	100	75	25	50	0	60	80	20
Corynebacterium spp. (n = 5)	0	0	0	0	0	0	80	40	20
Escherichia coli (n = 3)	0	0	0	33	100	0	100	100	0
Other (n = 32)	55	36	27	27	18	0	88	66	44
Total	**59**	**55**	**44**	**41**	**34**	**21**	**77**	**68**	**47**

[a] On tributyrin agar.
[b] On skim milk agar.
[c] Tested in polystyrene microtiter plates.

ubiquitous in the environment and contamination may result from the environment in the stable, e.g. hay or air, and ineffective cleaning processes (Vacheyrou et al., 2011). *Pseudomonas* spp. are considered the most prevalent psychrotrophic contaminant of raw milk (Ercolini et al., 2009; Quigley et al., 2013), but significant differences have been observed between the culture-dependent and -independent approach in this study. *Pseudomonas* spp. were not identified as dominating taxa of any sample using the culture-independent approach and almost all *Pseudomonas* clones came from samples with J > 0.5, although it dominated 8–10% of the culturable microbiota at 30 °C and 10 °C (Table 2). Additionally, overall percentages of *Pseudomonas* spp. among the clone sequences were only 12%, following *Acinetobacter* and *Streptococcus* (Table 3). A recently published study did not identify *Pseudomonas* as one of the most prevalent genera in raw milk with elevated bacterial counts using a culture-independent approach as well (Rodrigues et al., 2017). This shows the importance of both culture-dependent and –independent methods in order to assess the influence of *Pseudomonas* spp. or other bacterial taxa in raw milk. Most of the *Pseudomonas* isolates and clone sequences belonged to *P. azotoformans*, *P. fragi* or *P. fluorescens*. These species have been associated with raw milk before and particularly *P. fragi* and *P. fluorescens* were identified as predominant *Pseudomonas* species in refrigerated raw milk and the dairy processing environment (De Jonghe et al., 2011; Stellato et al., 2017). However, these species belong to groups of closely related species, including *P. lundensis*, *P. extremaustralis*, *P. synxantha* or *P. psychrophila*, which have been associated with raw milk before (Fricker et al., 2011; Hantsis-Zacharov and Halpern, 2007; Von Neubeck et al., 2015; Weber et al., 2014). Here, the identification to species level by analysis of their 16S rRNA sequences is not reliable because sequence similarity values are above 99% between these species. Sequencing of housekeeping genes like *rpoD* or *gyrB* and sequencing of the internally transcribed 16S–23S rDNA spacer (ITS1) region were suggested to discriminate closely related *Pseudomonas* species (Guasp et al., 2000; Yamamoto et al., 2000).

Acinetobacter is a known psychrotrophic genera, but it was not yet in focus of many studies. It has been detected as main component among the psychrotrophic bacteria of raw cow's milk before (Von Neubeck et al., 2015; Vithanage et al., 2014), but the proportions were lower compared to the results from our analyses. Species identification revealed *A. johnsonii* or *A. albensis* as dominating *Acinetobacter* species in raw milk samples with J < 0.5. *A. johnsonii* and *A. albensis* were the most prevalent species among the 19 different *Acinetobacter* species identified in this study and were recovered from various different farms and from both geographic regions. Approximately 80% of the isolates and 70% of the clone sequences were assigned to the species *A. johnsonii* and *A. albensis*. *A. johnsonii* was frequently isolated from raw milk (Fricker et al., 2011; Hantsis-Zacharov and Halpern, 2007; Weber et al., 2014) but *A. albensis* was just recently described from soil and water samples (Krizova et al., 2015) and was not associated with raw milk before. Additionally, several clone sequences could not be assigned to any of the known *Acinetobacter* species (sequence similarity with type strains < 98.7%; Table 1). This shows that the knowledge about the diversity of *Acinetobacter* spp. from raw milk is still fragmentary and that further analyses are required.

The amount of Enterobacterales among the raw milk samples was relatively low, except for two samples with high counts of *Escherichia coli*. Among all samples, 9% of all bacterial isolates and 13% of the clone sequences were assigned to species of the order Enterobacterales (Table 1), *Escherichia coli* and *Hafnia* spp. were the most prevalent (Table 3). *E. coli* was associated with high bacterial counts in bulk tank raw milk before (Hayes et al., 2001) and is associated with bovine mastitis as well (Goldstone et al., 2016). Although it is a common compound of bovine feces, Kagkli et al. (2007) implied that raw milk is only rarely contaminated with *E. coli* from bovine feces.

3.6. Lipolytic and proteolytic activity

To obtain information about the spoilage potential of the raw milk microbiota, the isolation of bacterial strains is indispensable. The prevalence of lipolytic and proteolytic activity among isolates is shown in Table 4. Approximately half of the isolates showed lipolytic or proteolytic activity at 30 °C, 10 °C or 4 °C. This was in the range with findings of recent studies (Baur et al., 2015; Ribeiro Júnior et al., 2018; Vithanage et al., 2014). The activity of the *Staphylococcus*, *Streptococcus* and *Lactococcus* isolates decreased with lower growth temperature, but most of the *Acinetobacter* and *Pseudomonas* isolates showed consistent lipolytic or proteolytic activity at 10 °C and 4 °C as well. Additionally, some of the *Pseudomonas* isolates were only active at cold temperatures, particularly with regard to proteolytic activity, but not at 30 °C. Whereas isolates of the genera *Acinetobacter*, *Streptococcus* and *Lactococcus* predominantly showed either lipolytic or proteolytic activity, *Pseudomonas* isolates were able to perform both lipolytic and proteolytic activity.

Especially *Pseudomonas* spp. are known for their enzymatic activity (Hantsis-Zacharov and Halpern, 2007; Vithanage et al., 2014; Von Neubeck et al., 2015). In addition, we detected lipolytic or proteolytic activity at cold temperatures for several genera of minor abundance, e.g. *Arthrobacter* spp. and *Carnobacterium* spp. Vithanage et al. (2014)

assessed the extracellular protease activity of the psychrotrophic bacterial composition of raw milk and showed that half of the isolates were able to perform proteolytic activity. Among them, the *P. fluorescens* group, *Hafnia alvei* and *P. fragi* were the most common taxa. Additionally, a quarter of the proteases were considered as heat-resistant (Vithanage et al., 2014).

3.7. Biofilm formation capacity

Biofilm formation of microorganisms in the storage tank or the milking equipment leads to increased contamination of raw milk because biofilm-associated organisms show high levels of resistance against cleaning and disinfection (Marchand et al., 2012). 77% of the tested isolates in this study were able to produce biofilms in polystyrene microtiter plates (Table 4). Biofilm formation was less expressed at lower temperatures, but 47% of the isolates still produced biofilms at 4 °C. The genus *Pseudomonas* is well known for its ability to produce biofilms, but isolates of other genera, especially *Acinetobacter*, but also *Carnobacterium* and *Arthrobacter,* consistently produced biofilms at 10 °C or 4 °C (Table 4). The isolates of these genera represented the most prevalent bacteria among the high TBC raw milk samples (Table 3) and this confirms that elevated bacterial counts can result from biofilms and poor hygiene in the milk processing environments.

4. Conclusion

Contrary to other reports, psychrotrophic bacterial taxa did not always dominate raw milk from the bulk tank with high TBC. The dominating bacteria of high TBC raw milk were either psychrotrophic and related to poor hygiene and food spoilage or were mesophilic and potential mastitis pathogens. Additionally, only the minority of the analyzed raw milk samples with high bacterial counts were dominated by a single bacterial species and a relationship between bacterial diversity and bacterial counts was not found. The same psychrotrophic genera and species were recovered from raw milk samples with high or low diversity. This leads to the conclusion that dominance and outgrowth under refrigeration is not specific for single psychrotrophic species but may be strain specific and may depend on the source of contamination. The coexistence of several bacterial genera in raw milk samples with high TBC indicates the presence of synergistic cooperation between the taxa detected.

Most of the organisms were well adapted to cold temperatures and showed unchanged or even increased enzymatic activity at 10 °C or 4 °C. This was detected as well among genera that are not yet in focus as food spoilage microorganisms, e.g. *Carnobacterium* or *Arthrobacter*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2018.10.019.

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