International Journal of Food Microbiology 211 (2015) 57–65

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Biodiversity of refrigerated raw milk microbiota and their enzymatic spoilage potential

Mario von Neubeck [a], Claudia Baur [b], Manuel Krewinkel [b], Marina Stoeckel [c], Bertolt Kranz [b], Timo Stressler [b], Lutz Fischer [b], Jörg Hinrichs [c], Siegfried Scherer [a], Mareike Wenning [a,*]

[a] Lehrstuhl für Mikrobielle Ökologie, Zentralinstitut für Ernährungs- und Lebensmittelforschung (ZIEL), Wissenschaftszentrum Weihenstephan, Technische Universität München, Weihenstephaner Berg 3, 85354 Freising, Germany
[b] University of Hohenheim, Institute of Food Science and Biotechnology, Department of Biotechnology and Enzyme Science, Garbenstr. 25, 70599 Stuttgart, Germany
[c] University of Hohenheim, Institute of Food Science and Biotechnology, Department of Soft Matter Science and Dairy Technology, Garbenstrasse 21, 70599 Stuttgart, Germany

ARTICLE INFO

Article history:
Received 9 February 2015
Received in revised form 23 June 2015
Accepted 2 July 2015
Available online 7 July 2015

Keywords:
Raw milk
Microbiota
Peptidase
Lipase
Enzyme activity
Pseudomonas

ABSTRACT

The refrigerated storage of raw milk selects for psychrotolerant microorganisms, many of which produce peptidases and lipases. Some of these enzymes are heat resistant and are not sufficiently inactivated by pasteurisation or even ultra-high temperature (UHT) treatment. In the current study, 20 different raw cow's milk samples from single farms and dairy bulk tanks were analysed close to delivery to the dairies or close to processing in the dairy for their cultivable microbiota as well as the lipolytic and proteolytic potential of the isolated microorganisms. Altogether, 2906 isolates have been identified and assigned to 169 species and 61 genera. *Pseudomonas*, *Lactococcus* and *Acinetobacter* were the most abundant genera making up 62% of all isolates, whereas 46 genera had an abundance of < 1% and represent only 6.6%. Of all isolates, 18% belong to hitherto unknown species, indicating that a large fraction of the milk microbiota is still unexplored. The potential of the isolates to produce lipases or peptidases followed in many cases a genus or group specific pattern. All isolates identified as members of the genus *Pseudomonas* exhibited mainly lipolytic and proteolytic activity or solely proteolytic activity. On the other hand, nearly all isolates of the genus *Acinetobacter* were lipolytic but not proteolytic. Only 37% of all tested lactic acid bacteria (LAB) showed enzymatic activity at 6 °C and the type of activity was proteolytic in 97% of these cases.

1. Introduction

Raw milk is rich in nutrients and neutral in pH and therefore offers ideal conditions for growth of many microorganisms. The raw milk microbiota is composed of microorganisms introduced by recontamination originating from the cowshed, the feed, bedding material, the teat surface and apex or the dairy equipment (Cousin, 1982; Vacheyroum et al., 2011). Therefore, a huge variety of species belonging to the domains of bacteria and fungi can be found (for review see Quigley et al., 2011, 2013b; Samaržija et al., 2012). In general, a high number of lactic acid bacteria like *Lactococcus*, *Streptococcus* and *Leuconostoc* can be found in fresh milk besides several other Gram-positives like *Bacillus*, *Microbacterium*, *Micrococcus* and *Staphylococcus*. Gram-negative bacteria like *Pseudomonas*, *Aeromonas*, *Acinetobacter*, *Stenotrophomonas* and *Chryseobacterium* as well as several Enterobacteriaceae such as *Enterobacter*, *Hafnia* and *Klebsiella* are also frequently found in raw milk. The same is true for a few yeast genera like *Candida*, *Kluyveromyces* and *Pichia* (Delavenne et al., 2011; Fleet, 1990; Quigley et al., 2011).

In Germany, raw milk is mostly not directly processed after milking and is therefore stored and held refrigerated until it is delivered to the dairy. This may last up to three or four days depending on the milk collection intervals and trading distances. In the dairy, an additional storage until processing is possible. During storage, the cold temperature selects for psychrotolerant microorganisms, which can outgrow those that can only persist (Fricker et al., 2011; Lafarge et al., 2004; Rasolofo et al., 2010). A huge number of psychrotolerant isolates has been identified as members of the genus *Pseudomonas* (Eneroth et al., 1998; Hantsis-Zacharov and Halpern, 2007; Martins et al., 2006), making it one of the most important genera in the dairy environment.

Many organisms are known to produce peptidases and lipases some of which are able to withstand pasteurisation or even UHT-treatment (Chen et al., 2003; Marchand et al., 2009; Sørhaug and Stepaniak, 1997). In the cheese industry, moderate concentrations of these enzymes may contribute to aroma development and therefore may have a beneficial effect, but in many other products a residual activity of microbial enzymes can lead to off-flavours and other quality defects before products reach the expiry date. This problem is even more important for dairy products with a long shelf life like UHT milk or milk powder. Proteolytic enzymes can lead to an increase in viscosity, evoke a bitter flavour and cause gelation (Datta and Deeth, 2003), while milk fat hydrolysis due to lipolytic enzymes can result

* Corresponding author.
E-mail address: mareike.wenning@wzw.tum.de (M. Wenning).

http://dx.doi.org/10.1016/j.ijfoodmicro.2015.07.001
0168-1605/

in rancidity (Deeth and Fitz-Gerald, 2006). As the enzymes are difficult to inactivate by technological means, it is necessary to reduce the risk for enzyme production in the raw material. Therefore, knowledge is needed about which microorganisms are present in raw milk close to processing and which enzymes they are able to produce.

Thus, the aim of the current study was to analyse the microbiota of raw milk samples at the end of cold storage at either the farm or dairy and what enzymatic potential these organisms displayed in order to evaluate those groups with the highest relevance to potential quality defects in the resulting products. 20 raw milk samples from single farms and dairy silo tanks were analysed for their microbiota. To capture not only the most dominant species, 150 colonies were identified for each sample. After identification of the almost 3000 isolates approx. 1000 representative isolates were tested for their potential to produce lipases and peptidases.

2. Materials and methods

2.1. Isolation of microorganisms from raw cow's milk

In total, 20 raw milk samples from 10 different single farms and 10 different dairies were analysed. The samples were completely independent, as each sample was collected at a different day and each farm or dairy was sampled only once. The farms were located in the south-west of Germany and housed between 10 and 110 cows (47 on average). The milk collection frequency was between 2–3 days and the storage temperature was approx. 4–5 °C. Farm samples were taken between August 2011 and May 2012 aseptically at the day of milk collection and shipped to the laboratory on ice overnight. Consequently, at the time of analysis milk was 3–4 days old. Nine of ten dairy plants were located across Germany and one in Slovakia. Samples were taken aseptically over a period of 5 months between February and June 2012. All samples were shipped to the laboratory on ice overnight and analysed immediately after arrival. Storage time of the dairy samples is unknown, because bulk tank milk is a mixture of different milk deliveries to the dairy and silo tanks undergo a permanent process of filling and depleting.

From each raw milk sample decimal dilutions with sterile Ringer solution were plated on plate count agar supplemented with 1% skim milk (PCM agar). Every dilution step was plated three times in triplicates and incubated at 30 °C for five days, 15 °C for seven days and 6 °C for ten days. After incubation, total plate counts were calculated and for each sample 150 colonies were randomly collected from plates of the same dilution incubated at 15 °C and showing approx. 50 to 150 colonies per plate. Isolates were streaked on PCM agar and incubated for 3 days at 30 °C. None of the isolates failed in growth at 30 °C.

2.2. Fourier transform infrared (FTIR) spectroscopy

The microbial isolates were differentiated into yeasts, aerobic sporeformers, lactic acid bacteria (LAB) and aerobic non-sporeforming bacteria by microscopy and catalase test and were subsequently identified by FTIR spectroscopy (Kummerle et al., 1998; Wenning et al., 2008, 2010, 2014). Briefly, the growth medium was inoculated with cells, which were spread with a drigalski spatula leading to a confluent lawn of microorganisms after growth for 24 ± 0.5 h. Yeasts were incubated at 27 °C on YGC agar, lactic acid bacteria at 34 °C on APT agar, aerobic sporeformers at 25 °C and aerobic non-sporefomers at 30 °C, both on TS agar. After incubation, one loop full of cells was suspended in 100 μl of sterile water and 25 μl of this suspension were applied to an IR transparent ZnSe sample holder and dried for 45 min at 40 °C. IR spectra were measured using a Tensor 27 spectrometer coupled to the HTS-XT device for high throughput measurements (both Bruker Optics, Germany) and evaluated according to the methods previously described (Oberreuter et al., 2002; Wenning et al., 2014) using the OPUS 6.5 software (Bruker Optics, Germany). Isolated microorganisms were identified by matching their FTIR spectra to in-house FTIR reference libraries containing approximately 8.000 spectra of 240 genera and 1000 species.

After FTIR measurement, the spectra of all isolates were compared for each milk sample by hierarchical cluster analysis (HCA). As FTIR spectroscopy is discriminative down to the strain level (Wenning et al., 2014), it can be used to reduce the number of isolates for further analysis by sorting out clonal isolates. HCA using the clustering algorithm Average Linkage (embedded in the OPUS 6.5 software package) was calculated based on vector normalised first derivatives of FTIR spectra in the spectral regions 3000 to 2800, 1800 to 1500, 1500 to 1200, 1200 to 900 and 900 to 700 cm^{-1}. A cluster was defined as a group of spectra having spectral distances of around 0.3 or less. For each cluster, one or more isolates (depending on the cluster size) were selected and used for further investigations.

2.3. Gene sequence analysis

All representative isolates selected by HCA were additionally identified by gene sequence analysis. The 16S rRNA gene was used for identification of bacteria and its counterpart 26S rRNA gene was used for the yeast isolates. Since the 16S rRNA gene is not discriminatory enough for identification of closely related species of some genera, isolates of two of the most abundant genera were additionally identified by using sequencing of housekeeping genes. The sigma 70 subunit gene (*rpoD*) of the RNA polymerase was used for identification of *Pseudomonas* isolates (Mulet et al., 2009, 2010) and the gene for the β-subunit (*rpoB*) was used for identification of *Staphylococcus* isolates (Mellmann et al., 2006).

DNA of bacteria and yeasts was extracted by mechanical cell lysis (2*45 s at 6.5 m/s FastPrep24 from MP Biomedicals, LLC.) with zirconia/silica beads (BioSpec Products, Inc.) of 0.1 mm diameter for bacterial and 0.5 mm diameter for yeast isolates. Heating to 95 °C for inactivation of desoxyribonucleases was followed by a cooling step (5 min on ice) prior to centrifugation at 13,000 rpm for 5 min and storage of the DNA containing supernatant at −20 °C. Each PCR reaction contained 2.5 μl (10×) reaction buffer (Thermo Scientific), 2.5 μl (2 mM) desoxynucleosid triphosphate mix (dNTPs), 1.5 μl $MgCl_2$ (25 mM), 0.25 μl (50 mM) of each of the primers, 0.15 μl Thermo-Start Taq DNA Polymerase (Thermo Scientific) and 16.85 μl sterile aqua dest. to a final volume of 25 μl. Primers were: 16S_27f (5′-agagtttgatcctggctca-3′) and 16S_1492r (5′-cggctaccttgttacgac-3′) resulting in the almost complete 16S rRNA gene fragment, PsEG30F (5′-atygaaatcgccaarcg-3′) and PsEG790R (5′-cggttgatktccttga-3′) leading to a partial *rpoD* gene sequence of ~750 bp (Mulet et al., 2009), 1418 (5′-caattcatggaccaagc-3′) and 3554 (5′-ccgtcccaagtcatgaaac-3′) giving a partial *rpoB* gene sequence of ~900 bp (Mellmann et al., 2006) and NL-1 (5′-gcatatcaataagcggaggaaaag-3′) and NL-4 (5′-ggtccgtgtttcaagacgg-3′) resulting in a partial 26S gene sequence of ~500 bp (Kurtzman and Robnett, 2003).

Amplification by PCR was performed using a Biometra® T 3000 thermocycler under the following conditions: initial denaturation at 95 °C for 15 min followed by 35 cycles of denaturation at 95 °C for 20 s, annealing at appropriate temperature (52 °C for 16S primers, 45 °C for *rpoD* primers, 48 °C for *rpoB* primers and 55 °C for 26S primers) for 40 s and elongation at 72 °C for 100 s using the 16S primers, 70 s using *rpoD* and *rpoB* primers and 45 s using 26S primers. The last cycle was followed by a final elongation step at 72 °C for 5 min.

PCR products were sequenced at LGC Genomics GmbH (Germany, Berlin) using the primer 16S_926r (5′-ccgtcaattcctttgagttt-3′) for the first two-thirds of the 16S rRNA gene, for the three other genes the corresponding forward PCR primer was used. The 26S rRNA, *rpoB* and *rpoD* gene sequences were checked for similarity to type strains using the BLAST algorithm (Altschul et al., 1990) and 16S rRNA gene sequences were identified using the EzTaxon-e server (Kim et al., 2012).

2.4. Phylogenetic analysis of potential novel genera and species

Nucleotide sequences of isolates showing similarity values <98% for the 16S gene (Kim et al., 2014) and <97% for the rpoD gene (Sanchez et al., 2014) to known species were aligned with corresponding