Short communication: Presence of *Lactococcus* and lactococcal exopolysaccharide operons on the leaves of *Pinguicula vulgaris* supports the traditional source of bacteria present in Scandinavian ropy fermented milk

Davide Porcellato,¹ Malena Tranvåg, and Judith Narvhus

Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, PO Box 5003, N-1432 Ås, Norway

ABSTRACT

Some traditional Scandinavian fermented milk products have a pronounced ropy consistency due to the presence of exopolysaccharide-producing strains of *Lactococcus lactis* ssp. *cremoris*. Norwegian food folklore describes how leaves from the carnivorous plant *Pinguicula vulgaris* (common butterwort) may be added to milk to initiate the fermentation of the traditional fermented milk product *tettemelk*. However, scientific confirmation of the link between the plant and the milk product has not been previously published. In the present study, the microbiome on 20 samples of *P. vulgaris* leaves collected from 5 different rural geographical locations in Norway and from 4 samples of commercial *tettemelk* was analyzed using high-throughput sequencing methods. The leaf microbiota of *P. vulgaris* was dominated by *Proteobacteria* and *Firmicutes* and the genus *Lactococcus* was demonstrated in all leaf samples. In addition, DNA extracted from the leaf microbiome contained genes identical to those responsible for exopolysaccharide production in *Lactococcus*. These results confirm the traditional use of *P. vulgaris* as a source of bacteria for the Norwegian ropy fermented milk product *tettemelk* and indicate that *P. vulgaris* microbiomes can be a potential source of lactic acid bacteria with interesting dairy technological features.

Key words: *Lactococcus*, exopolysaccharide, *tettemelk*, *Pinguicula vulgaris*

Short Communication

A type of traditional fermented milk, generically known as “ropy milks,” is consumed in northern Scandinavian countries. In Norway, food folklore describes the use of the leaves of *Pinguicula vulgaris* (common but-

terwort, known as *tettegras* in Norway) to initiate the fermentation of a ropy (or slimy) fermented milk, known as *tettemelk*. The methodology for this is not described in detail but apparently involves several subcultures of the original leaf and milk mixture before a satisfactory *tettemelk* is obtained. However, many attempts to produce this traditional product from the leaves have failed, thus leading to doubt that this is actually a correct interpretation of earlier practices (Narvhus, 2014). *Tettemelk* is characterized by an extremely viscous consistency, a mild aromatic flavor, and resistance to syneresis. Exopolysaccharide (EPS)-producing strains of *Lactococcus lactis* ssp. *cremoris* have been isolated from *tettemelk* and similar Scandinavian fermented milk products, but unequivocal proof that the bacterium producing the ropy consistency is present on the leaves of *P. vulgaris* has not been obtained (Narvhus, 2014). Using an artisan starter culture collected from neighboring farms in the Røros district, *tettemelk* has been commercially produced by Rørosmeieriet since 1996. The culture is propagated and stored at the dairy, and the culture itself is not commercially available. Production of EPS is a key property for the production of several types of fermented milk, and EPS-producing lactic acid bacteria are widely used by dairy companies to improve the consistency of fermented milk products.

The aim of this study was to investigate the possible link between *P. vulgaris* and *tettemelk*. We analyzed the microbiome on the *P. vulgaris* leaves and *tettemelk* using massive parallel sequencing and targeting specific lactococcal genes involved in the production of exopolysaccharides.

Twenty samples of *P. vulgaris* leaves were collected from 5 rural geographical locations in Norway (Lykkja, Hardanger, Damtjern, Vega, and Røros) and 4 cartons of commercial ropy fermented milk (*tettemelk*) were collected. Leaf samples were homogenized for 2 min in Ringer's solution (Oxoid, Basingstoke, UK) using an Omni mixer (Omni International, Waterbury, CT). The DNA was extracted from leaf homogenates and commer-

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¹Corresponding author: davide.porcellato@nmbu.no

Figure 1. Relative abundance of the bacterial 16S rRNA sequences obtained from *Pinguicula vulgaris* leaves and traditional fermented milk. (A) Percentage abundance of bacterial phyla in all the samples. Numbers over the barplot indicate the percentage of *Firmicutes* in each sample. The 4 samples of fermented milk (*tettemelk*) contained 100% *Firmicutes*. (B) Boxplot of the 6 phyla identified in leaves and fermented milk microbiomes. (C) Percentage abundance of genus within the *Firmicutes* phyla for all the samples of leaves and fermented milk. * indicates the presence of lactococcal exopolysaccharide operons in the sample. (D) Boxplot of the 10 most abundant genera within the *Firmicutes* phylum. OTU = operational taxonomic unit. Color version available online.

cial samples as previously described (Porcellato et al., 2014), and amplification of the V3 and the V4 regions of the bacterial 16S rRNA gene was performed using the universal primer Uni340F (5'-CCTACGGGRBG-CASCAG-3') and Bac806R (5'-GGACTACYVGGG-TATCTAAT-3'; Takai and Horikoshi 2000). Unique 6-bp barcodes were used to identify each library. Massive parallel sequencing was performed on an Illumina Miseq (Illumina, San Diego, CA). Raw files were demultiplexed, and forward and reverse reads were merged using Qiime 1.9.0 (Caporaso et al., 2010). Reads were quality filtered using Usearch 8.0 (Edgar, 2010) and operational taxonomic units (OTU) were picked using the UPARSE OTU algorithm (Edgar, 2013) clustered at 97% pairwise identity. Taxonomy was assigned to each OTU representative sequence after aligning each sequence against the Silva database (Quast et al., 2013) using the blastn algorithm (Camacho et al., 2009). The 20 best hits for each OTU representative sequence were extracted, and the hits with lowest e-value and highest identity were used to assign the taxonomy.

The microbiota composition obtained from the 20 leaf samples and from the fermented milk consisted of 166 OTU clustered at 97% similarity. Permutational multivariate ANOVA using Unifrac distance matrix was used

to compare microbial communities of groups of samples. The fermented milk samples showed a simple microbiota consisting of approximately 90% *Lactococcus* and 10% *Leuconostoc* (Figure 1), whereas the average OTU richness of the leaves was 50 ± 21 OTU. The phyla present on the leaves in greatest abundance were *Proteobacteria* (88% of all the reads) and *Firmicutes* (10%; Figures 1A and 1B). Among the *Firmicutes*, the genus *Lactococcus* was the most abundant OTU detected (75% of the reads assigned to *Firmicutes*) and was detected in all the samples, with abundance varying from 0.2 to 51% of the total reads (Figure 1). Other abundant OTU of the phylum *Firmicutes* on the leaf samples were *Carnobacterium* (8.6%), *Streptococcus* (7.4% and present in all the samples), *Bacillus* (2.6%), and *Staphylococcus* (1.4%) as shown in Figures 1C and 1D. The microbiota of the fermented milk comprised 2 genera of the phylum *Firmicutes* found on the leaves, namely *Lactococcus* and *Leuconostoc* (Figures 1A and 1C). *Leuconostoc* was detected in 10 leaf samples and in low abundance (from 0.01 to 0.16%). Microbiota composition was also compared using nonmetric multidimensional scaling. Clear separation on the NMDS plot was detected for the leaf samples and the fermented milk (Figure 2). However, this result was not unexpected since the development

Figure 2. Nonmetric multidimensional scaling (NMDS) plot using unweighted Unifrac-distance matrix for all the 16S amplicon libraries of leaf and fermented milk samples. Samples are grouped by place and category. Color version available online.

of a good *tettemelk* culture from the leaves is reputed to require several subcultures in milk. Considering the mixed microbiota on the leaves, this subculturing could lead to the gradual domination of acid-forming lactic acid bacteria over the acid-sensitive *Proteobacteria*.

To investigate whether *Lactococcus* EPS genes were present on the leaf samples and fermented milk, new primers targeting the lactococcal EPS operon were designed. Ten *Lactococcus* EPS operons (downloaded from National Center for Biotechnology Information, GenBank accession number CP009472, CP003132, AF142639.1, EF192212, AF036485, AE005176, CP006766, CP009054, AY741550, and HQ665557) and 3 operons obtained from genome sequences of laboratory strains of *Lactococcus lactis* ssp. *cremoris* were used to design EPS operon primers, which amplify a large fragment (>1,000 bp). The primers used in this study were EPS380F (5'-AGAAAGGGAATTGGGT-TACCCT-3') and EPS1623R (5'-TGAGGCTGTGTA-AACTGGGG-3'). The PCR products were separated by gel electrophoresis and resulting bands were sequenced and identified as equivalent to the *Lactococcus lactis* ssp. *cremoris* EPS operon. Fourteen of the 20 leaf samples were positive for EPS operons as well as all the 4 samples of fermented milk (indicated by a star above the columns representing each sample in Figure 1C).

Although lactococci have been isolated from a variety of plant environments, *Lactococcus lactis* ssp. *cremoris* is rarely found outside the dairy environment. It has been suggested that industrial dairy strains are descended from lactococci found on plants and have undergone adaptation to the dairy environment (Cavanagh et al., 2015). In this study, lactococci were demonstrated to be part of the microbiome in all 20 leaf homogenates and 14 of these homogenates showed the presence of *Lactococcus* and the lactococcal EPS operon. Thus, we have shown that the leaves of *Pinguicula vulgaris* are a

source of EPS-producing lactococci, thereby confirming the traditional use of these leaves to make *tettemelk*, as described in Norwegian food folklore.

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