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Cheese brines from Danish dairies reveal a complex microbiota comprising several halotolerant bacteria and yeasts

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ABSTRACT

The Danish Danbo cheese is a surface ripened semi-hard cheese, which before ripening is submerged in brine for up to 24 h. The brining is required in order to obtain the structural and organoleptic properties of the cheeses. Likewise, the content of NaCl in the cheese will influence especially the surface microbiota being of significant importance for flavour development and prevention of microbial spoilage. Even though the microbiota on cheese surfaces have been studied extensively, limited knowledge is available on the occurrence of microorganisms in cheese brine. The aim of the present study was to investigate by both culture-dependent and -independent techniques the brine microbiota in four Danish dairies producing Danbo cheese. The pH of the brines varied from 5.1 to 5.6 with a dry matter content from 20 to 27% (w/w). The content of lactate varied from 4.1 to 10.8 g/L and free amino acids from 65 to 224 mg/L. Bacteria were isolated on five different media with NaCl contents of 0.85–23.0% (w/v) NaCl. The highest count of 6.3 log CFU/mL was obtained on TSA added 4% (w/v) NaCl. For yeasts, the highest count was 3.7 log CFU/mL on MYGP added 8% (w/v) NaCl. A total of 31 bacterial and eight eukaryotic species were isolated including several halotolerant and/or halophilic species. Among bacteria, counts of ≥6.0 log CFU/mL were obtained for *Tetragenococcus muriaticus* and *Psychrobacter celer*, while counts between ≥4.5 and < 6.0 log CFU/mL were obtained for *Lactococcus lactis*, *Staphylococcus equorum*, *Staphylococcus hominis*, *Chromohalobacter beijerinckii*, *Chromohalobacter japonicus* and *Microbacterium maritypicum*. Among yeasts, counts of ≥3.5 log CFU/mL were only obtained for *Debaryomyces hansenii*. By amplicon-based high-throughput sequencing of 16S rRNA gene and ITS2 regions for bacteria and eukaryotes respectively, brines from the same dairy clustered together indicating the uniqueness of the dairy brine microbiota. To a great extent the results obtained by amplicon sequencing fitted with the culture-dependent technique though each of the two methodologies identified unique genera/species. Dairy brine handling procedures as e.g. microfiltration were found to influence the brine microbiota. The current study proves the occurrence of a specific dairy brine microbiota including several halotolerant and/or halophilic species most likely of sea salt origin. The importance of these species during especially the initial stages of cheese ripening and their influence on cheese quality and safety need to be investigated. Likewise, optimised brine handling procedures and microbial cultures are required to ensure an optimal brine microbiota.

1. Introduction

Worldwide, there are > 1000 varieties of cheese produced on artisanal or industrial scale (Irlinger et al., 2015). The predominant proportion of these cheeses is salted by immersion in brines with a NaCl content of 18 to 24% (w/v) (Søndergaard et al., 2015). The brining has an important effect on the structure and flavour of the cheeses and is significant in regulation of the microbiota on the cheese surface (Gori et al., 2012, 2013; Irlinger et al., 2015; Jaeger et al., 2002; Ryssel et al., 2015; Sørensen et al., 2011). Cheese brines are normally kept at relatively low temperatures e.g. around 10 °C (Larson et al., 1999). Different brining systems exist, which generally are either static or a raceway system. In a static system, the cheeses are immersed into a brine vat, while in a raceway system the cheeses are slowly conveyed through the brine vat. In both systems, NaCl may be added directly to the vat or via brine circulation from a brine supply. Accumulated cheese particles

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can for both systems be removed by filters or membrane units. To reduce the microbial load, cheese brines may occasionally be pasteurized (Larson et al., 1999) or microfiltrated (Ottosen and Køningsfeldt, 1999; Skrzypek and Burger, 2010). However, some dairies may prefer to keep the indigenous microbial load in the brine, considering the brine a natural inoculant and might therefore not want to pasteurize or microfiltrate the brine or do it infrequently (Bockelmann, 2002; Ingham et al., 2000).

The microbiota on the cheese surfaces is complex and characterized by a succession of different microorganisms which highly influence the organoleptic and structural properties of the cheeses (Irlinger and Mounier, 2009). Establishment of the microbial consortium is often promoted by smearing of the cheeses (Gori et al., 2013; Petersen et al., 2002) and ripening of the cheeses at controlled temperatures (~14°) and high relative humidity (> 95%) (Cogan et al., 2014). Several studies have characterized the interior and surface cheese microbiota as well as the microbiota of the dairy processing environment by both culture-dependent and/or -independent techniques (Bokulich and Mills, 2013; Calasso et al., 2016; Gori et al., 2013; Mounier et al., 2006; Ryssel et al., 2015; Schirmer et al., 2013; Stellato et al., 2015).

The culture-dependent methodologies require isolation on media such as milk plate agar, plate count agar, tryptic soy agar, de Man, Rogosa and Sharpe (MRS) agar or M17 agar (Gori et al., 2013; Mounier et al., 2006; Schirmer et al., 2013). However, none of these media are specific for isolation of halotolerant or halophilic microorganisms and only very few authors focus specifically on this group of microorganisms. Ishikawa et al. (2007) used 7% NaCl, glucose, yeast extract, peptone and fish extract agar (GYPF) to address the presence of halophilic and alkaliphilic lactic acid bacteria (LAB) in various cheeses, whereas Henriet et al. (2014) used modified growth medium (MGM) added 23% NaCl to isolate halophilic microorganisms from food-grade salt samples. A more comprehensive overview on both viable and dead microorganisms can be obtained by culture-independent techniques and recent publications have identified several halophilic microorganism during dairy processing by either pyrosequencing or Illumina sequencing (Bokulich and Mills, 2013; Calasso et al., 2016; Marino et al., 2017; Ryssel et al., 2015; Stellato et al., 2015).

The Danish semi-hard Danbo cheese has recently been registered by the European Commission as an EU protected geographical indication (PGI) (Anonymous, 2012, 2017). Danbo cheese is characterized by a surface ripening by adventitious bacteria and yeasts applied using commercial smear bacteria and/or yeasts or by applying a smear from ripened cheeses, the latter often referred to as “old-young smearing” (Gori et al., 2013). The microbial consortium and its development throughout ripening on the surface of a Danbo cheese have previously been investigated (Gori et al., 2013; Petersen et al., 2002; Ryssel et al., 2015). In the fresh cheese prior to smearing, the bacterial community was dominated by *Lactococcus* spp. and the eukaryotic community dominated by *Debaryomyces* spp., *Trichosporon* spp. and some unclassified yeasts (Ryssel et al., 2015). The relative amount of *Lactococcus* spp. after brining of the cheeses declined while *Staphylococcus* spp. increased and became dominant after 6 days of ripening. After four weeks of ripening *Corynebacterium* spp. became dominant, whereas the relative amount of *Staphylococcus* spp. declined. Other identified genera included *Brevibacterium*, *Clostridiisalibacter*, *Pseudoclavibacter*, *Alkalibacterium* and *Marinilactibacillus*. Shortly after smearing and throughout the ripening, *Debaryomyces* spp. became the dominating yeasts on the cheese surface (Ryssel et al., 2015). *D. hansenii* is important during cheese ripening as it is responsible for the increase in pH due to assimilation of lactate and production of alkaline compounds such as ammonia. The increased pH of the cheese surface enables the growth of less acid tolerant coryneform bacteria (Gori et al., 2007; Petersen et al., 2002).

Less abundant bacteria such as *Brachybacterium alimentarium*, *Leucobacter albus*, *Microbacterium gubbeenense*, *Halomonas* spp., *Agrococcus* spp., *Alcaligenes faecalis*, *Proteus vulgaris*, *Micrococcus* spp., *Bavariicoccus seileri*, *Arthrobacter* spp., *Vibrio* sp., *Bacillus* sp. and *Alkalibacterium* spp. have been reported to occur on the surface of Danbo and/or other surface ripened cheeses (Feurer et al., 2004; Gori et al., 2013; Ishikawa et al., 2007; Maoz et al., 2003; Mounier et al., 2005; Wolfe et al., 2014). By high-throughput sequencing (HTS) of the microbiota from a high number of cheese rinds, Wolfe et al. (2014) concluded that on average at least 60% of the bacteria and 25% of the eukaryotes found on cheese surfaces were not part of starter cultures and therefore originated from environmental sources. Even though not investigated thoroughly, it has been suggested that the cheese brine could be an important reservoir for microorganisms found on the cheese surface (Beresford et al., 2001; Mounier et al., 2005). This is supported by the fact that several studies have found marine halophilic bacteria on surface ripened cheeses (Gori et al., 2013; Ishikawa et al., 2007; Ryssel et al., 2015). Authors have found *Debaryomyces hansenii* and *Staphylococcus* spp. to be the predominant microorganisms in cheese brines occurring at levels up 10^4–10^5 CFU/mL (Bockelmann and Hoppe-Seyler, 2001; Mounier et al., 2006). Recently a wide bacterial community was identified in brines used for cheese-making in Italy as investigated by HTS and plate counting (Marino et al., 2017). However, despite the fact that brining is an important step in the production of a huge variety of cheeses, few detailed studies are available and limited focus has been placed on identification of eukaryotes in cheese brines.

The aim of the present study was to investigate, by both culture-dependent and -independent techniques, bacteria and yeasts in brines from Danish dairies producing surface ripened cheeses of the Danbo type. The dairies were selected in order to represent a variety of brine handling procedures. The investigations included media optimisation to ensure growth of halotolerant and/or halophilic microorganisms as well as high-throughput amplicon sequencing of both bacteria and eukaryotes. Knowledge on the brine microbiota could lead to a more comprehensive understanding of the establishment of the microbiota at the cheese surfaces. New procedures should be developed for brine handling including use of specifically adapted microbial cultures to control the brine microbiota and thereby the microbiota on the cheese surfaces in the early ripening period.

2. Materials and methods

2.1. Dairies and brine sampling

Samples of brines from four different Danish dairies (Dairy A, B, C and D) were collected (Table 1). All dairies produce cheeses of the Danbo type from pasteurized conventional or organic milk, using DL-starter cultures. The temperature of all brines investigated was kept at approximately 15 °C. All dairies use Suprasel Fine Salt© (Akzo Nobel Industrial Chemicals, Mariager, Denmark), which is a fine pure dried vacuum salt of food grade quality. Suprasel Fine Salt© originates and is produced at sites in Denmark or the Netherlands. In rare cases Suprasel Fine Salt© may be replaced by other salts (e.g. sea salt or Himalayan salt). Dairies producing cheese from both conventional and organic milk use two separated brine systems. Subsequent to brining, all dairies use the “old-young smearing” approach to inoculate the cheeses with microorganisms from the smear.

Representative samples were taken from the upper layer of the brine and collected in sterile 50 mL Conical Base Tubes (Sarstedt AG & Co, Numbrecht, Germany). Dairy A has 35 vats without circulation, two of these were investigated. In the larger dairies (Dairy B–D) only one vat exists for each of the production lines. For these dairies, samples were taken after circulation of the brine.

2.2. Chemical characterization

2.2.1. pH and total solids content

A calibrated PHM240 pH/ion-Meter (Radiometer Analytical Sas, Villeurbanne Cedex, France) was used for pH measurements. Total solid