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Application of ruthenium red and colloidal gold-labeled lectin for the visualization of bacterial exopolysaccharides in Cheddar cheese matrix using transmission electron microscopy

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Abstract

The objective of the present study was to develop a methodology for direct observation of capsular and ropy strains and their exopolysaccharides (EPS) in a Cheddar cheese matrix. Cheddar cheeses with 50% reduced fat were made from milk containing 1.7% fat using mixed starter culture containing either capsule-forming *Lactococcus lactis* subsp. *cremoris* (SMQ-461) or ropy *L. lactis* subsp. *cremoris* (JRF-1) strains. Control cheese was made using the EPS-negative *L. lactis* subsp. *cremoris* (RBL132) strain. Following cheese pressing, samples were taken from each cheese treatment and examined by transmission electron microscopy (TEM). Samples were divided into two series: the first was prepared following the conventional methods (involving fixation, post fixation, dehydration and embedding in resin) and the second with added ruthenium red at 0.15% (w/v) during the fixation, post fixation and washing procedures. Gold-labeled lectin was also used for the visualization and localization of EPS in cheese matrix. Electron micrographs showed that ruthenium red makes it possible to visualize and enhance the resolution of the EPS in a Cheddar matrix compared with the conventional method. The EPS layer of the capsular strain appeared regular and evenly distributed around the cell, whereas the cell-associated EPS layer produced by the ropy strain was longer, more irregular (having a filamentous structure) and unevenly surrounded the cell. EPS released from the ropy strain appeared to form a network-like structure located principally in whey pockets and appeared to interact with the casein matrix and fat globule membrane. Labeling EPS by lectin conjugated to colloidal gold could only be performed with conventional preparation of cheese samples and appeared to react only with the cell surface rather than with liberated EPS. Besides their ability to bind water and increase cheese yield, capsular and ropy strains used in this study appear to have potential autolytic characteristics, which may have an impact on cheese proteolysis, texture and flavor quality.

Keywords: Exopolysaccharides; Cheese; Transmission electron microscopy; Lectin labeling; Ruthenium red; *Lactococcus*

1. Introduction

Lactic acid bacteria (LAB) are extensively used in the production of a wide variety of fermented foods and beverages, including dairy products such as cheeses and yogurt, meat, vegetables and wine. LAB contribute to flavor, texture and keeping quality of these products. In addition, some of these bacteria have been shown to exert several health benefits and are known as probiotics "for life organisms".

As for many bacteria, LAB can produce exopolysaccharides (EPS) which are believed to play a role in the protection of the microbial cells against desiccation, phagocytosis, phage attack, antibiotics and toxic compounds (van Kranenburg, Boels, Kleerebezem, & de Vos, 1999). The EPS are either excreted in the growth

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medium as slime (ropy) or remain attached to the bacterial cell wall, thus forming a capsular EPS (Cerning, 1990). These substances are believed to have both technological and health benefits. In the dairy industry, EPS produced by LAB enhance water retention and reduce whey separation in low-fat dairy products such as yogurt, frozen yogurt and cheese (Abd El-Rahman, Hassan, & Kheadr, 2000; Hassan, Corredig, & Frank, 2002a; Perry, McMahon, & Oberg, 1997). Meanwhile, EPS may confer various health benefits as they are considered as prebiotics (non-digestible food fraction) and appeared to have anti-tumoral, immunostimulating, cholesterol lowering (Gibson & Roberfroid, 1995) and antimutagenic effects (Sreekumar & Hosono, 1998).

In the cheese industry, starter cultures participate in cheese-making by fermenting lactose to lactic acid, which aids in separation of whey from cheese curd during cheese manufacture (IDF, 1980). Starters also contribute to the development of cheese flavor and texture through the secretion of several proteinases and peptidases during the ripening process. In addition to their enzymatic compartment, EPS produced by the starter can modify cheese structure and functional properties. The majority of cheese starter cultures are composed of mixtures of mesophilic and/or thermophilic LAB strains. The role of thermophilic LAB-producing EPS, particularly those belonging to the genera *Streptococcus* and *Lactobacillus*, on cheese functional properties has been studied for the production of low-fat Mozzarella cheese (Perry et al., 1997; Perry, McMahon, & Oberg, 1998; Low et al., 1998; Petersen, Dave, McMahon, Oberg, & Broadbent, 2000). Mesophilic strains, especially those belonging to *Lactococcus* sp. are extensively used for the production of semi-hard and some hard cheeses. In contrast to thermophilic starters, the impact of EPS-producing mesophilic starters, principally lactococci, on cheese properties is not known.

Few protocols have been developed to permit direct microscopic observations of bacterial EPS in dairy products. Confocal-scanning laser microscopy (CSLM) has been used to visualize EPS-forming bacteria in yogurt samples and to follow acid development and the structural properties of the gel formed during yogurt incubation (Hassan, Frank, Farmer, Schmidt, & Shalabi, 1995a, b). The use of lectin conjugates in combination with CSLM could provide more details and high resolution during direct visualization of bacterial EPS in dairy products. Hassan, Frank and Qvist (2002b) developed a rapid and simple method for visualizing EPS produced by capsular and ropy dairy starter cultures in both Feta cheese as well as set and stirred fermented milks. This method involved staining EPS with either wheat germ agglutinin labeled with Alexa fluor 488 or concanavalin A488 prior to observation by CSLM. Cryo-scanning electron microscopy was also used for visualizing bacterial EPS in Feta and Karish cheeses and to determine their contribution to the microstructure of these cheeses (Hassan, Frank, & El Soda, 2003).

Most of the methods dealing with direct microscopic observations of EPS in dairy products have been directed toward products containing high moisture. These methods may not be applicable to moderate and low-moisture-containing products such as semi-hard and hard cheeses. In an attempt to address this issue, the present study was designed to develop a transmission electron microscopy methodology that could improve the visualization and characterization of capsular and ropy strains of *L. lactis* subsp. *cremoris*, as well as their EPS distribution and interaction with reduced-fat Cheddar cheese matrix.

2. Materials and methods

2.1. Bacteria and growth conditions

Exopolysaccharide non-producing strains *L. lactis* subsp. *lactis* RBL259 (*L. lactis* RBL259), *L. lactis* subsp. *lactis* RBL133 (*L. lactis* RBL133) and *L. lactis* subsp. *cremoris* RBL132 (*L. cremoris* RBL132) were obtained from the Canadian Research Network on Lactic Acid Bacteria (NLAB) culture collection (Universitý Laval, Quebec, PQ, Canada). EPS capsule-forming *L. lactis* subsp. *cremoris* SMQ-461, a raw milk isolate, was provided by Dr. S. Moineau, Department of Biochemistry and Microbiology, University Laval, Quebec, PQ, Canada. EPS ropy-forming strain *L. lactis* subsp. *cremoris* JRF-1 (*L. cremoris* JRF-1), isolated from retail cultured buttermilk, was provided by Dr. J. Frank and Dr. A. Hassan, Department of Food Science and Technology, University of Georgia, Athens, Georgia, USA. All strains were phenotypically characterized and genetically identified as described by Dabour, Kheadr, Fliss, and LaPointe (in press).

Pure bacterial cultures were all maintained in 20% glycerol stock at −80 °C. They were cultivated in M17 broth medium at pH 7.1 (Quelab, Montreal, PQ, Canada) supplemented with 0.5% (w/v) glucose and incubated overnight at 30 °C (Terzaghi & Sandine, 1975). Prior to begin the experiments, each bacterial strain was subcultured at least three times (1%, v/v) in sterilized reconstituted skim milk (11% total solids) at 24 h intervals.

2.2. Cheese-making procedure

A cheese starter mixed culture composed of EPS-non-producing *L. lactis* (RBL259); *L. lactis* (RBL133) and *L. cremoris* (RBL132) at a ratio of 0.67:0.33:1.0 (v/v/v),