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Redefining the effect of salt on thermophilic starter cell viability, culturability and metabolic activity in cheese

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ABSTRACT

This study investigated the differential effect of salt concentration in the outside and inside layers of brine salted cheeses on viability, culturability and enzyme activity of starter bacteria. The high-salt environment of the outside layer caused a sharp decrease in *L. helveticus* viability as measured by traditional plate counts. Remarkably, this was associated with lower release of intracellular enzymes (LDH), reduced levels of proteolysis and larger membrane integrity as measured by flow cytometry (FC) following classical Live/Dead staining. FC analysis of light scattering properties highlighted a significant reduction in size and granularity of the microbiota located in the cheese surface, suggestive of cell shrinkage and condensation of internal macromolecules probably due to hyperosmotic stress. The microbiota of the cheese surface were found to experience greater oxidative stress, as measured by FC analysis of the total levels of reactive oxygen species, compared to that of the interior layer. These results lead us to postulate that the physiology and health status of the microbiota were significantly different in the outer and inner layers of the cheese. The hyperosmotic environment of the outer layer resulted in reduced cell lysis, as measurable by assays based upon membrane integrity, but rather triggered cell death via mechanisms involving cell shrinkage and ROS-mediated damage of vital intracellular components. This study challenges the current thinking on how salt controls microbial activity in ripening cheese, especially in cheeses which are brine salted as local variations in biochemical ripening indices can differ significantly from the outside to the inside of a ripening cheese.

1. Introduction

Salt has been used as a food preservative since prehistoric times and has worked especially well, combined with fermentation and dehydration, as is the case with cheese production (Guinee and Fox, 2004). Salt content directly influences cheese flavour, provides sodium, essential for control of blood pressure and healthy cell function within the body, and crucially acts as a preservative. Salt lowers the water activity within the cheese matrix and subsequently controls microbial growth, enzyme activity, extent of protein hydration and aggregation along with rheological and cooking properties of cheese (Guinee, 2004; Guinee and Fox, 2004). Brine salted cheeses differ from their dry salted counterparts in that salt uptake is gradual and salt equilibrium occurs over a period of days/weeks/months (Sutherland, 2002), unlike dry salted cheeses where salt is relatively evenly distributed during the salting process (Fox et al., 2000).

The diffusion coefficient (D^*) of salt in brine salted cheeses has been investigated previously (Floury et al., 2010; Floury et al., 2012; Gomes et al., 1998; Guinee, 2004; Lee et al., 1980; Pajonk et al., 2003; Payne and Morison, 1999; Turhan and Kaletunc, 1992) with the D^* generally estimated at ~0.2 cm^2/day, but ranges from 0.1 to 0.45 cm^2/day, depending on curd temperature and moisture composition and brining conditions. Brining of cheese results in a net movement of Na^+ and Cl^- from the brine into the outer layer of the cheese block due to osmotic pressure (Guinee, 2004; Turhan and Gunasekaran, 1999). This osmotic pressure and high salt concentration of the brine (15–23% NaCl) which initially locates at the outer layer of the cheese is of interest in relation to its effect on cell viability, NSLAB growth, enzyme activity and possible development of ripening hotspots due to localised variations in salt in moisture levels (Fox et al., 2000).

Salt has long been associated with the control of lactose

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metabolism, curd pH and starter bacteria growth and rate of lysis (Wilkinson, Guinee and Fox, 1994a; Wilkinson, Guinee, O'Callaghan and Fox, 1994b; Yanachkina et al., 2016). The subsequent autolysis of bacterial cells and release of intracellular enzymes is heavily involved in the development of cheese matrix structure and flavour (Doolan and Wilkinson, 2009; Rulikowska et al., 2013; Sheehan et al., 2006; Thomas and Pearce, 1981; Wilkinson et al., 1994a; Wilkinson et al., 1994b). The effect salt concentration, resulting from brine salting of cheese, has on these crucial intracellular enzymes and their functions has yet to be fully investigated.

Flow cytometry (FC) enables the assessment of various structural and functional cell properties, often leading to a deeper characterization of the physiological heterogeneity of a microbial population (Díaz et al., 2010). In combination with fluorescent probes sensitive to the integrity of the cell membrane, FC has been used for the identification of cell viability and predicting levels of cell lysis during cheese ripening (Bunthof et al., 2001; Comas-Riu and Rius, 2009; Doolan and Wilkinson, 2009; Doolan et al., 2014; Sheehan, O'Loughlin, O'Cuinn, FitzGerald and Wilkinson, 2005; Sheehan, O'Cuinn, FitzGerald and Wilkinson, 2009). The gap between FC results and viable cell counts via traditional plating on various selective agars has long been a major issue with suggested overestimations of cell count levels in comparison to counts using FC (Bunthof and Abee, 2002).

In cheese manufacture, dry or brine salting is used to control the proliferation of spoilage and undesired microbiota but will obviously impact on the physiology of starter bacteria as well. Transcriptomic studies have shown the induction of various oxidative stress response genes and enzymic antioxidants in bacteria exposed to salt stress (den Besten et al., 2009; Tsuzuki et al., 2011), thereby pointing to the potential generation of reactive oxygen species (ROS). When ROS accumulate to levels exceeding the scavenging capacity of the bacterial cell, a state of oxidative stress is generated where excess ROS break nucleic acids, carbonylate proteins, peroxidate lipids, inhibit enzymes and ultimately lead to cell death. Consequently, FC is utilised in this study to compare total cell numbers with traditional plate count methods, along with assessing bacterial cell membrane integrity, morphology and reactive oxygen species (ROS) levels in order to determine how bacteria react to environmental stressors (i.e. salt).

To the authors knowledge, no study to date has investigated the effect of salt gradients in brine salted cheeses on bacterial viability and subsequent enzymatic activity within the cheese matrix. The objectives of this study were to (i) establish differing salt concentrations and cheese compositions at localised level (ii) establish the influence of varying salt concentrations on microbial activity (iii) establish the influence of varying salt concentrations on microbial metabolic activity and (iv) evaluate the use of FC and fluorescent probes in comparison to plate counts in the study of cheese microbiology.

2. Materials and methods

2.1. Starter strains

Thermophilic starter cultures typically used in Swiss-type cheese manufacture (Scott, 1981), i.e., *L. helveticus* LHB02 (LH) and *S. thermophilus* TH3 (ST) were sourced (Chr. Hansen Ltd, Little Island, Co. Cork, Ireland) as individual frozen DVS concentrates and stored at −80 °C until cheese manufacture.

2.2. Cheese manufacture and sampling procedure

Three replicate cheesemaking trials, each consisting of four vats of 380 kg cheese milk were undertaken over a 6 month period. Raw milks were obtained from a local dairy company, standardised to a protein to fat ratio of 1.01:1, held overnight at < 10 °C, pasteurized at 72 °C for 15 s, and pumped at 36 °C into cylindrical, jacketed, stainless steel vats (500 L) with automated variable speed cutting and stirring equipment (APV) Schweiz AG, Worb, Switzerland).

Calcium Chloride ($CaCl_2$) was added at 0.022% (v/v). The milks were inoculated with 1 of 3 starter blends.

LH: 0. 015% w/v *Lactobacillus helveticus* (LHB02)
ST: 0.015% w/v Streptococcus thermophilus (TH3)
ST + LH. 0.01% w/v Streptococcus thermophilus (TH3) & 0.005% w/v Lactobacillus helveticus (LHB02)

After a 60 min ripening period, a *Cryphonectria parasitica* rennet (Thermolase (625 IMCU/ml), Chr. Hansen Ltd., Little Island, Co. Cork, Ireland), diluted ~1:10 with de-ionised water, was added at a level of 3.7 mL 100 kg^{-1} milk. A coagulation period of ~30 min was allowed prior to the mechanical cutting of the coagulum. After a 5 min healing period, the curd/whey mixture was stirred and cooked by steam injection into the jacket of the vat. Curds were cooked at a rate of 0.5 °C min^{-1} from 36 to 45 °C and at 1 °C min^{-1} from 45 °C to maximum scald (50 °C).

Curds were pitched at pH 6.3, pre-pressed under whey at 5.4 kPa for 5min after which the curd was placed in 10 kg round moulds and pressed under increasing pressure up to 6 kPa. At pH 5.3, the cheeses were placed for 60 h in either a saturated brine solution (22% w/w NaCl, 0.2% w/w Ca, pH 5.2) or in a brine solution containing 18% w/w NaCl, 0.2% w/w Ca at pH 5.2 solution and at 18–20 °C (controlled via calcium chloride addition). On removal from the brine, all cheeses were vacuum packed and ripened at 8 °C for 26 d (outlined in Supplementary Table 1). Cheeses were sampled by removing a wedge aseptically from each wheel. Each wedge was separated into 2 samples based on location. The outer 2.5 cm was removed from the top, bottom and edge of the cheese wedge. The outer most 1.5 cm was grated and used as the outside sample point, while a similar quantity of the inner portion (w/w) of the cheese was grated and represented the inside portion.

2.3. Enumeration of starter and non-starter bacteria

During cheese manufacture, curd samples were removed aseptically pre and post brining. Further samples were acquired aseptically at 4, 7, 11, 18 and 26 d of ripening. Samples were placed in a sterile stomacher bag, diluted 1:10 with sterile 2% (w/v) trisodium citrate and homogenized in a stomacher (Stomacher, Lab-Blender 400, Seward, Thetford, Norfolk, UK) for 10 min. Further serial dilutions were prepared as required using maximum recovery diluent (MRD).

Viable *S. thermophilus* cells were enumerated on lactose-M17 agar after aerobic incubation at 42 °C for 3 d (Terzaghi and Sandine, 1975), *L. helveticus* cells were enumerated on MRS agar pH 5.4 after anaerobic incubation for 3 d at 42 °C (IDF, 1988a), Enterococci cells were enumerated on kanamycin aesculin azide (KAA) agar aerobically at 37 °C for 24 h s and non-starter lactic acid bacteria (NSLAB) were enumerated on LBS agar with an LBS agar overlay at 30 °C for 5 d (Rogosa et al., 1951).

2.4. Cheese composition

Grated cheese samples were analyzed at 0, 4, 7, 11, 18 and 26 d of ripening for total salt (IDF, 1988b), protein (IDF, 1993), moisture and fat via nuclear magnetic resonance (NMR) (Fast Trac analysis system, CEM Microwave technology Ltd., Dublin, Ireland). Cheese pH was measured by preparing a cheese slurry from 20 g of grated cheese combined with 12 g of H_2O (45–55 °C).