The Cheese matrix-physicochemical and microbial considerations for probiotic delivery

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Overview of Presentation

- The Cheese matrix: -physicochemical and microbial considerations for probiotic delivery- It's a multidisciplinary issue…….
- Ref: Hickey *et al.,* 2015a,b O'Sullivan *et al.,* 2013.
- A personal perspective with international published research Interspersed with research results from my group

- Specifics:
  - Focus more on the environment for probiotic incorporation
  - Cheese manufacture process
  - Bacterial incorporation into cheese
  - Location and localisation
  - Interaction with the cheese matrix
  - Recent research on probiotics in cheese

- Conclusions / Observations

Publications of potential interest……

frontiers in MICROBIOLOGY

REVIEW ARTICLE
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Nucleic acid-based approaches to investigate microbial-related cheese quality defects

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The microbial profile of cheese is a primary determinant of cheese quality. Microorganisms can contribute to aroma and taste defects, form biogenic amines, cause gas and secondary fermentation defects, and can contribute to cheese pinking and mineral deposition issues. These defects may be as a result of seasonality and the variability in the composition of the milk supplied, variations in cheese processing parameters, as well as the nature and number of the non-starter microorganisms which come from the milk or other environmental sources. Such defects can be responsible for production and product recall costs and thus represent a significant economic burden for the dairy industry worldwide. Traditional non-molecular approaches are often considered biased and have inherently slow turnaround times. Molecular techniques can provide early and rapid detection of defects that result from the presence of specific spoilage microbes and, ultimately, assist in enhancing cheese quality and reducing costs. Here we review the DNA-based methods that are available to detect/quantify spoilage bacteria, and relevant metabolic pathways in cheeses and, in the process, highlight how these strategies can be employed to improve cheese quality and reduce the associated economic burden on cheese processors.

Keywords: molecular methods, cheese quality defects, microbial defects

INTRODUCTION

There are approximately 1000 varieties of cheeses, corresponding to nine different cheese families (Cheddar, Dutch, Swiss, Iberian, Italian, Balkan, Middle Eastern, Mould-ripened, and Smear-ripened) produced worldwide (Sandine and Elliker, 1970; Fox and McSweeney, 2004; Fox et al., 2004). Cheese is one of the most traded dairy products in the world with EU production of more than 8.4 million tons in 2011 (www.eurostat.eu). This generates huge revenues for leading cheese exporting economies. The primary ingredients of cheese are milk, rennet, and salt. However, it is microbial interactions with these major ingredients which allows for the production of the different varieties. These microbial populations are also the least controllable factor in cheese production (Fox, 2000; Iany and Barbier, 2008).

Microbial populations in cheese can be split into two distinct groups i.e., starter and non-starter microorganisms. Generally, starter and non-starter populations exhibit an inverse numerical relationship, with starter culture populations dominating during early cheese manufacture, but decreasing in number throughout the ripening process to be eventually replaced by the secondary microbiota. The starter microbiota cause rapid acidification via the production of lactic acid and produce enzymes that are important for flavor development during ripening (Leroy and De Vuyst, 2004). The most commonly used starter cultures are from the genera *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc*, and *Enterococcus* (Beresford et al., 2001) and are used as either pure or mixed cultures (McSweeney, 2007). Non-starter/secondary organisms are primarily bacteria but can also include yeasts, molds, and filamentous fungi (Fox, 2000). Secondary, or initially subdominant microbiota, and in particular non-starter lactic acid bacteria (NSLABs), can play a key role in ripening and flavor development, for example, propionic acid bacteria (PAB) and/or smear cultures (including *Brevibacterium linens*). However, they can also be associated with the occurrence of defects. NSLAB are adventitious bacteria that gain access to cheese via the ingredients used and/or the production and ripening environment. They occur as heterogeneous populations with cell densities exceeding 10^6 cfu/g cheese during the ripening process (Sweartngen et al., 2001). They primarily consist of facultatively heterofermentative (mesophilic) lactobacilli (FHLs) as well as Pediococci, Enterococci, and Leuconostocs (Beresford et al., 2001; Beresford and Williams, 2004). FHLs are Gram-positive non-motile bacteria capable of growth at pH ranging from 5.5 to 6.2, in 4–6% salt and temperatures from 2°C to 54°C (Lynch et al., 1996). It is the relationship between these non-starter microbes and the physical features of the cheese (salt, pH, and moisture) that can lead to specific (un)desirable characteristics (Ndoye et al., 2011).

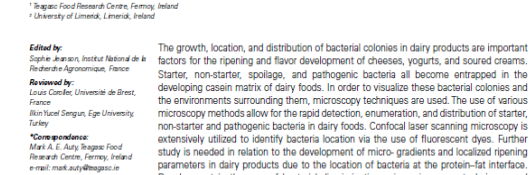
Defects caused by microorganisms that affect the quality of cheese include odor and taste defects, biogenic amine (BA)

FOOD SCIENCE & TECHNOLOGY

Review

The influence of cheese manufacture parameters on cheese microstructure, microbial localisation and their interactions during ripening: A review

C.D. Hickey[a,b], M.A.E. Auty[a,*], M.G. Wilkinson[b,1] and J.J. Sheehan[a,1]



frontiers in MICROBIOLOGY

REVIEW ARTICLE
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Growth and location of bacterial colonies within dairy foods using microscopy techniques: a review

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The growth, location, and distribution of bacterial colonies in dairy products are important factors for the ripening and flavor development of cheeses, yogurts, and soured creams. Starter, non-starter, spoilage, and pathogenic bacteria all become entrapped in the developing casein matrix of dairy foods. In order to visualize these bacterial colonies and the environments surrounding them, microscopy techniques are used. The use of various microscopy methods allow for the rapid detection, enumeration, and distribution of starter, non-starter and pathogenic bacteria in dairy foods. Confocal laser scanning microscopy is extensively utilized to identify bacteria location via the use of fluorescent dyes. Further study is needed in relation to the development of micro- gradients and localized ripening parameters in dairy products due to the location of bacteria at the protein–fat interface. Development in the area of bacterial discrimination using microscopy techniques and fluorescent dyes/tags is needed as the benefits of rapidly identifying spoilage/pathogenic bacteria early in product manufacture would be of huge benefit in relation to both safety and financial concerns.

Keywords: lactic acid bacteria, milk fermentation, bacterial location, cheese, microscopy, fat-protein interface

BACTERIA WITHIN DAIRY PRODUCTS

Bacteria are naturally present and are used extensively across all areas of dairy and food fermentation, either as natural microflora, or as starter cultures added under controlled conditions (Yang et al., 2012). Their fermentative ability, especially that of lactic acid bacteria (LAB) is based on the creation of an acidic environment through the breakdown of carbohydrates such as lactose, maltose, lactulose and sucrose thereby ensuring preservation of food stuffs. Fermented dairy products are often not manufactured under sterile conditions or with sterile milk (unpasteurized) and this can allow non-starter LAB as well as spoilage or pathogenic bacteria access to the fermenting food system (Montville and Matthews, 2005). LAB's commonly found in dairy products include strains of *Streptococcus*, *Lactococcus*, *Lactobacilli*, *Bifidobacteria*, *Enterococcus*, and *Pediococci*. Within these species there are numerous strain types which can be used in fermentation processes to give specific acidification and flavor profiles to the final product.

Bacteria associated with dairy fermentations can grow over a wide temperature range from 4 to 50°C. Mesophilic bacteria have an optimum growth range of 25–35°C, while thermophilic species have an optimum range of 37–45°C (Johnson and Steele, 2013). The growth of bacterial cells within dairy foods is heavily influenced by parameters such as pH, water activity and salt-in-moisture levels as well as temperature.

The use of starter bacteria is needed in order to acidify the cheese milk before and during dairy food production. These starter bacteria are inoculated into the milk at their optimum growth temperature (described above) and then stored post manufacture at temperatures ranging from 4 to 12°C (depending on the type of product) in order to slow the growth and acidification of these bacteria. Adjunct cultures such as *Propionibacterium* become active via exposure to warmer temperature ranging from 20 to 25°C for a set period of time and are directly involved in the metabolism of lactate to propionic and acetic acid, water, and CO_2 (Choisy et al., 2000; Hayaloglu and McSweeney, 2014).

LACTIC ACID BACTERIA

Lactic acid bacteria are the most common and important starter cultures used in fermented dairy products and may originate from the microflora of raw milks (e.g., bovine, ovine, caprine) but more frequently are inoculated intentionally during product manufacture. The initial role of LAB is to control pH of ripening milk and subsequent dairy products via the conversion of naturally occurring lactose found in milk to lactic acid (glycolysis). The rapid reduction (4–8 h) of pH to below 5.3 in cheese or 4.6 in fermented milk products allows for the control of non-starter microflora as only acid-tolerant bacteria can survive in those conditions (Johnson and Steele, 2013). The secondary function of LAB's in dairy fermentations is flavor development. Intracellular enzymes released by starter and non-starter bacteria during manufacture and ripening are the main contributors to flavor development via the three main biochemical pathways (glycolysis, lipolysis, and proteolysis). The breakdown of caseins is the most important pathway for flavor development in hard and semi- hard type cheese, which LAB contribute heavily to with the formation of small peptides and free amino acids which can then be further converted to form various alcohols, aldehydes, acids, and esters (Smit et al., 2005). Examples of dairy foods produced through LAB fermentations include cheeses, yogurts and sour creams such

Agriculture and Food Development Authority

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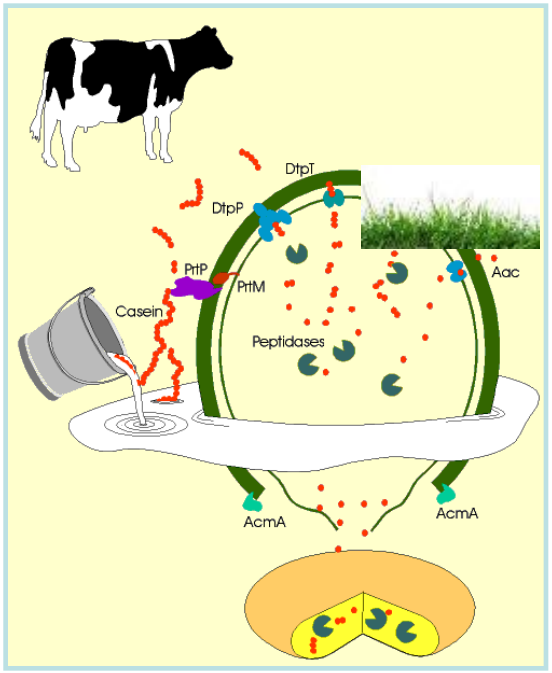
Cheese

- Global cheese sales expected >$100bn (€92bn) by 2019 (Transparency Market Research)
  - Relationship between manufacture parameters and ripening, quality and consistency
  - Explore relationship between cheese matrix/ microstructure and bacteria entrapped within
- A protein network/matrix made up of micelles which fuse together forming chains becoming more tightly bound to form a dense matrix in which
  - fat globules, free fat, soluble and casein bound minerals such as calcium, water and sodium chloride fractions are all interspersed

Cheese ripening

Cheese ripening is a slow process

- Enzymatic & Metabolic Reactions of Microbes (Starter & Non Starter Lactic Acid Bacteria)

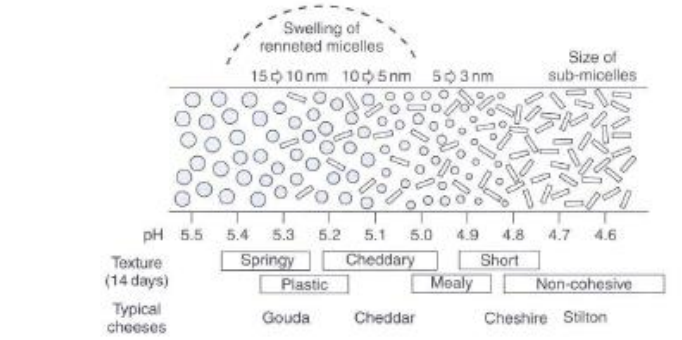


- Cheese is a sub-optimal environment for microbes
- Competition for nutrients
  - Cell Growth – metabolic & enzymatic reactions
  - Cell Death – lysis - enzymatic reactions
  - Dormant State – metabolic & enzymatic reactions!!

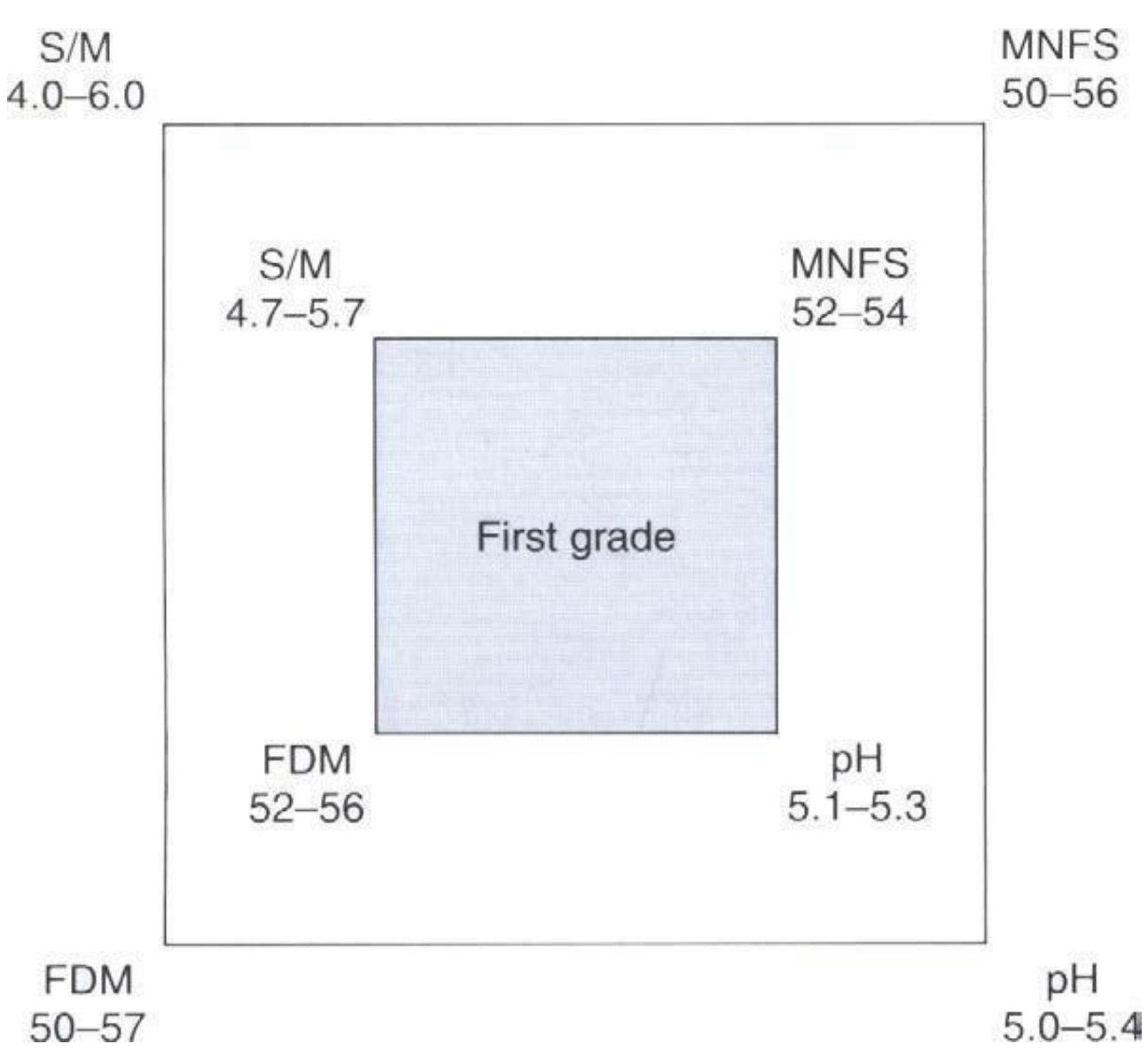
Cheese Ripening

Matrix: What influences these metabolic & enzymatic reactions?

- Cheese composition (Key Quality Parameters)
  - Salt in moisture
  - Moisture in non fat substances
  - Fat in dry matter
  - pH
- Water activity
  - Free water
- Ripening regimes
  - Temperature & time



Diagrammatic representation of the effect of the pH on the microstructure and texture of cheese.


S/M
4.0–6.0
MNFS
50–56
S/M
4.7–5.7
MNFS
52–54
First grade
FDM
52–56
pH
5.1–5.3
FDM
50–57
pH
5.0–5.4

Changes in milk- external to cheese plant

- More cross breeding- Influence on cheese manufacture ?
- Breed, feed cause variation in casein micelle size (Lodes *et al.,* 1996; Glantz *et al.,* 2010; Bijl *et al.,* 2014a).
- Milk with smaller casein micelles (diameter 147 -183 nm) vs larger (200- 266 nm)
  - Coagulates faster
  - Gives a firmer coagulum, (Walsh *et al.,* 1998; Auldist *et al.,* 2002; Glantz *et al.,* 2010; Gustavsson *et al.,* 2014b; Logan *et al.,* 2014; Bland *et al.,* 2015).

- Interactive effect of fat globule size and casein micelle size

- Smaller casein micelles & larger fat globules
  - faster coagulation and gave a firmer curd than milk with large casein micelles and larger fat globules.

- Larger fat globules are tightly packed in the pores generated by smaller casein micelles, giving a structural rigidity and hence firmer curds (Logan et al., 2014, 2015).

Recent research to inform cheese manufacture proces

Parameter 1	Parameter 2	Impact 1	Impact 2	Ref
Calcium Chloride		Microstructure	Fat loss	Ong *et al., 2013*
Calcium Chloride	Lower drain pH	Microstructure	Fat retention	Ong *et al., 2015*
Calcium Chloride	Lower drain pH	Manufacture process	Quality	Soodam et al., 2015
Milk pH at renneting		Texture	Increase yield	Ong *et al., 2012*
Rennet		Microstructure	Composition	Soodam *et* al., 2015
Coagulation temp		Microstructure	Composition	Ong *et al.,* 2011

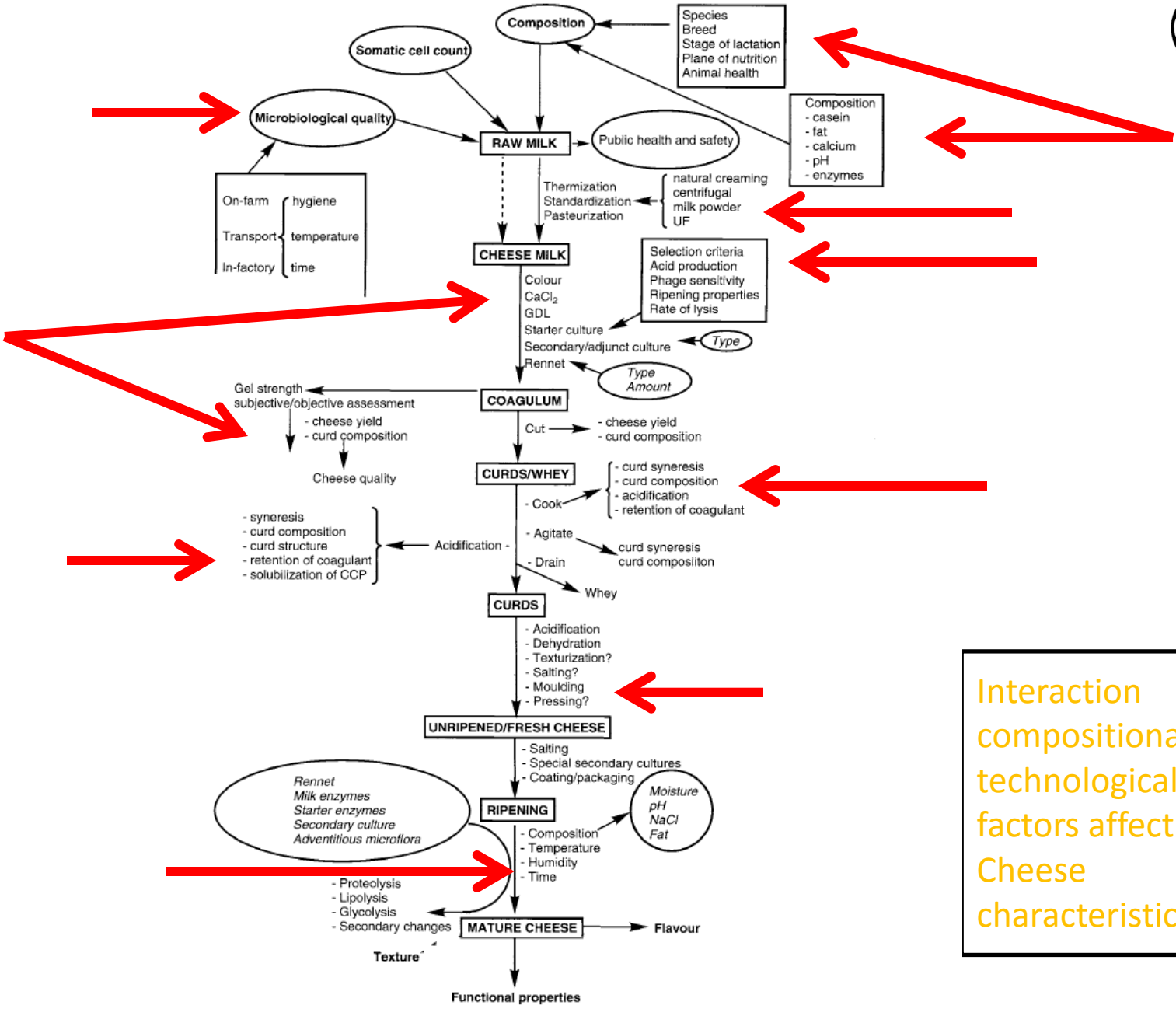


Figure 2 Interaction of compositional and technological factors that affect the quality of cheese.

Interaction of compositional & technological factors affecting Cheese characteristics

Ref: Factors that affect cheese Quality, Fox, P.F. and Cogan, T.M., 2004

Microbes and cheese: Diversity and location

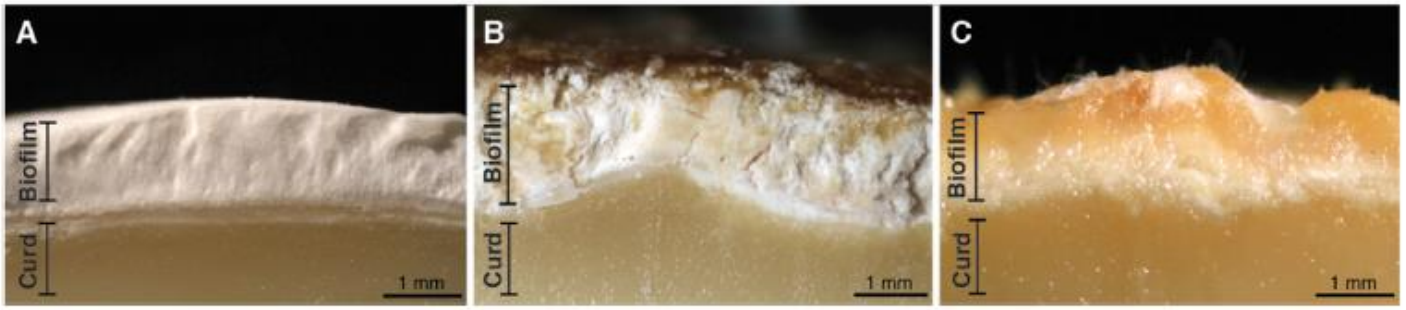


Figure 1. Microbial Communities Form on the Surfaces of Naturally Aged Cheeses

Cross-sections through naturally aged cheeses show rind biofilms growing on the surface of the cheese curd. (A–C) (A) A bloomy rind biofilm, (B) a natural rind biofilm, and (C) a washed rind biofilm.

Wolfe *et al.,* 2014. Cell 158, 422-433,

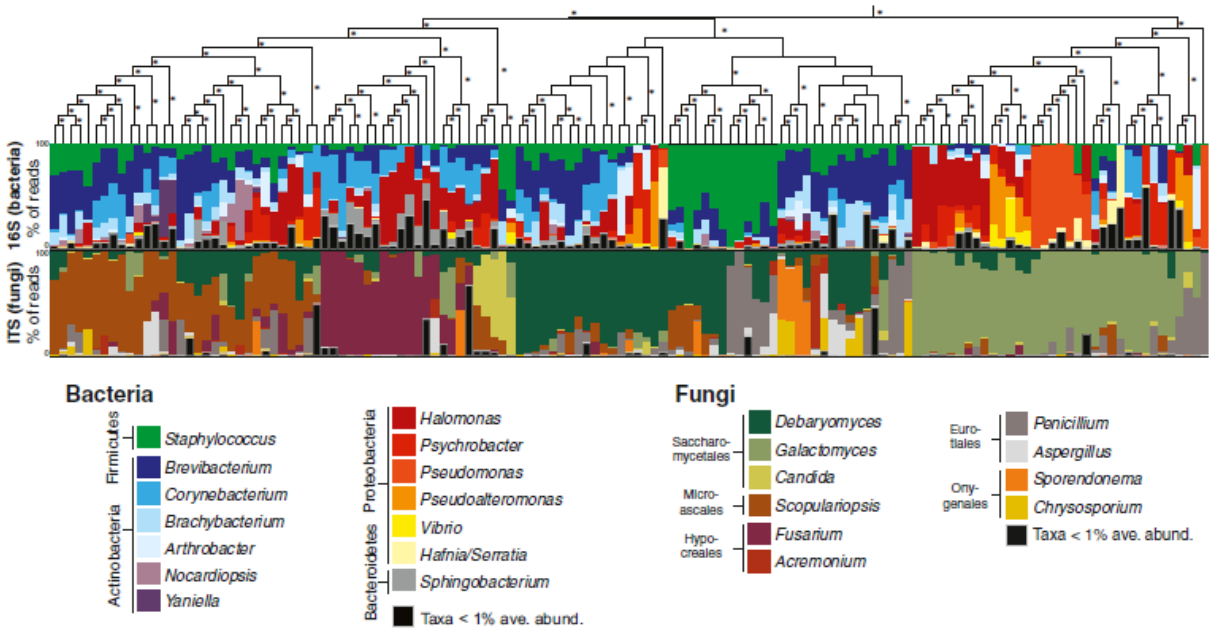


Figure 2. Distribution of Abundant Genera across Cheese Rind Communities

Microbes and cheese: Diversity and location

Ripening time

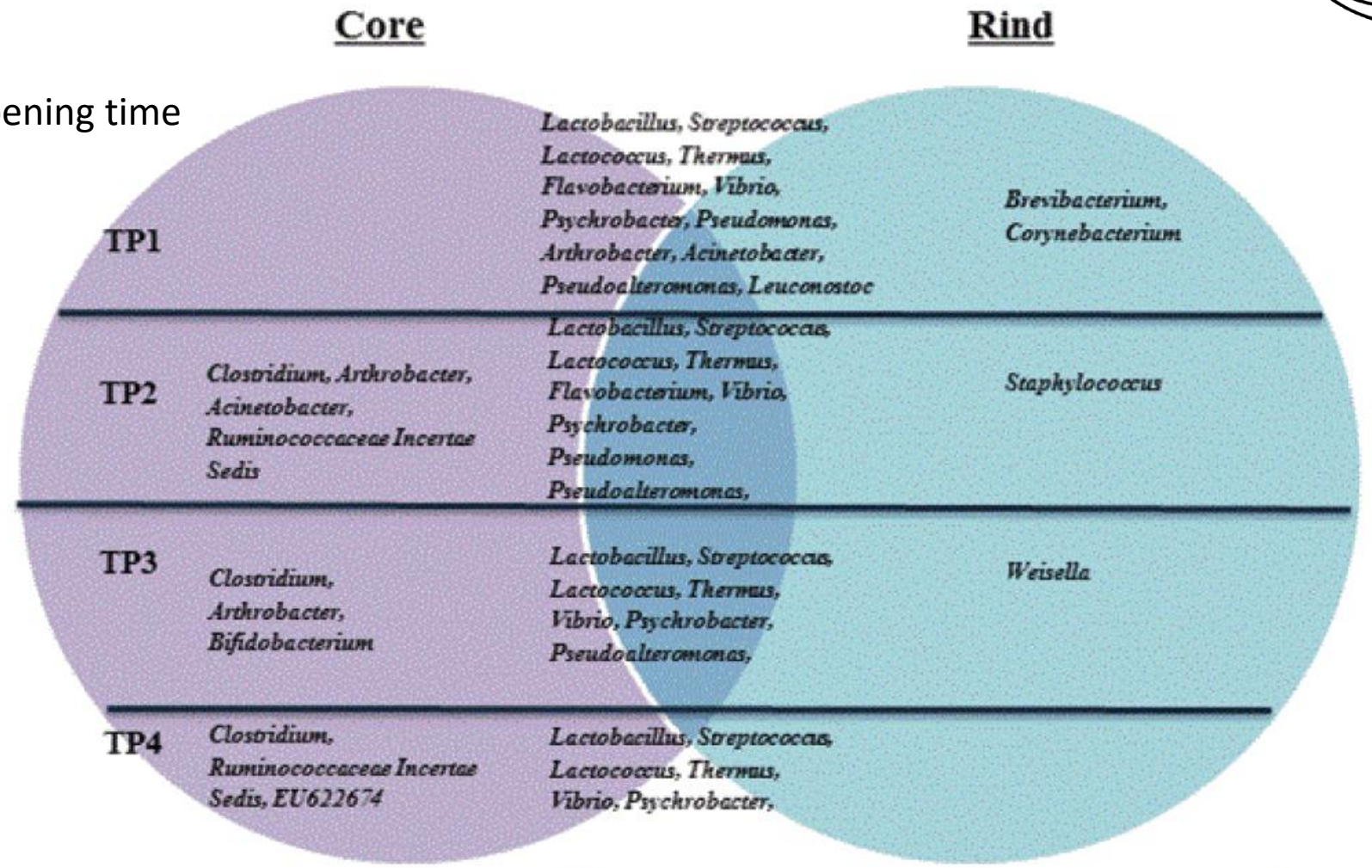
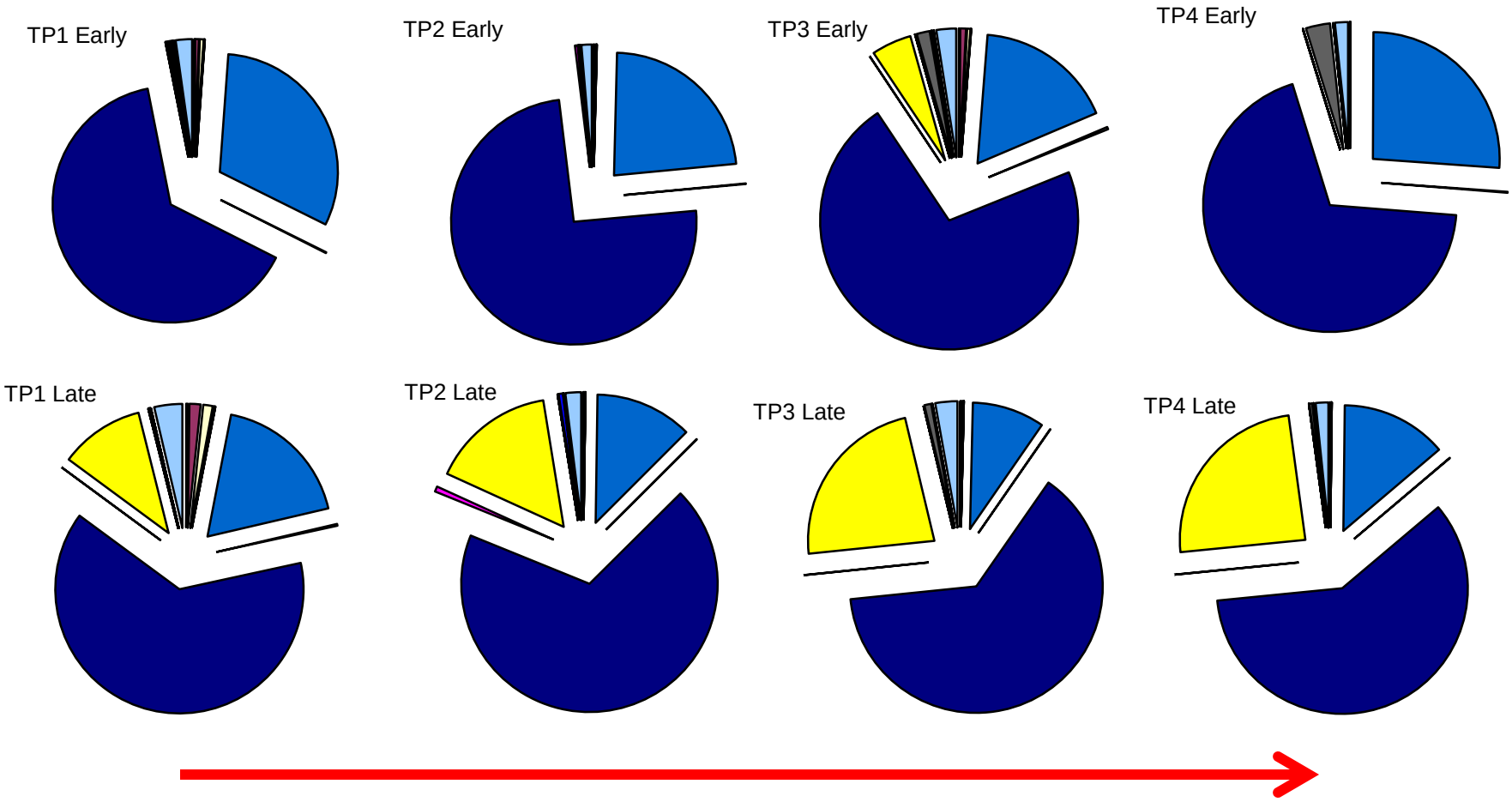


FIG 4 Venn diagram depicting spatial differences in the microbial composition at each time point. Genera located in the intersecting region were detected in both the core and the rind, while those on the periphery were detected exclusively in the core or the rind.

O'Sullivan *et al., 2015. Applied Environmental Microbiology,81 (7), 2525-2533.*

Microbes and cheese: Diversity with manufacture time



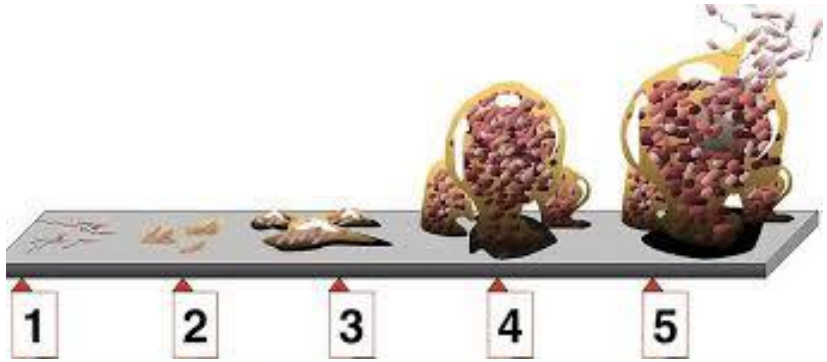
Modified from : O'Sullivan *et al., 2015. Applied Environmental Microbiology,81 (7), 2525-2533.*

Microbes: Points of entry to cheese curd

- Starter Inoculation /adjunct

- Survival of pasteurisation ? (NSLAB)
  - Some thermo resistance reported : assays involving milk (Jordan and Cogan, 1999).
  - Strains of *Lactobacillus brevis* did not survive pasteurisation
  - Strains of *Lb. buchneri* and *Lb. curvatus* were partially resistant (reduction on treatment of ~ 2 logs) (Sanchez-Llana, Fernanadez & Alvarez, 2011)

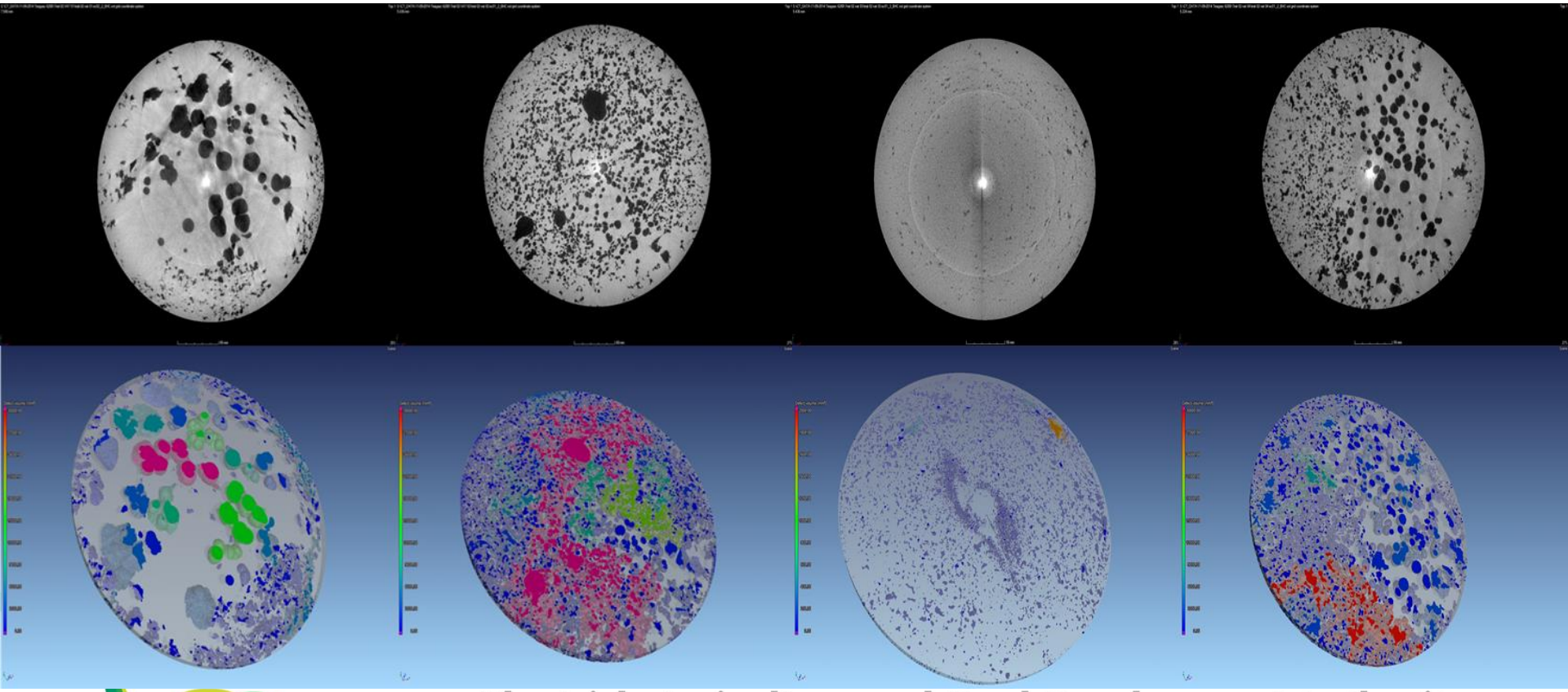
- Biofilms
  - Growth in plate heat exchangers
    - Streptococci (Sheehan, 2011)
    - *T. thermophilus* (Langeveld et al., 1995)



Compromised *Lb. helveticus* starter activity in the presence of facultative heterofermentative *Lb. casei* DPC6987 results in atypical eye formation in Swiss-type cheese

O'Sullivan *et al.,* 2016. Journal of Dairy Science

CTL SPC SLC SLPC



Microbes – where are they in the cheese matrix ?

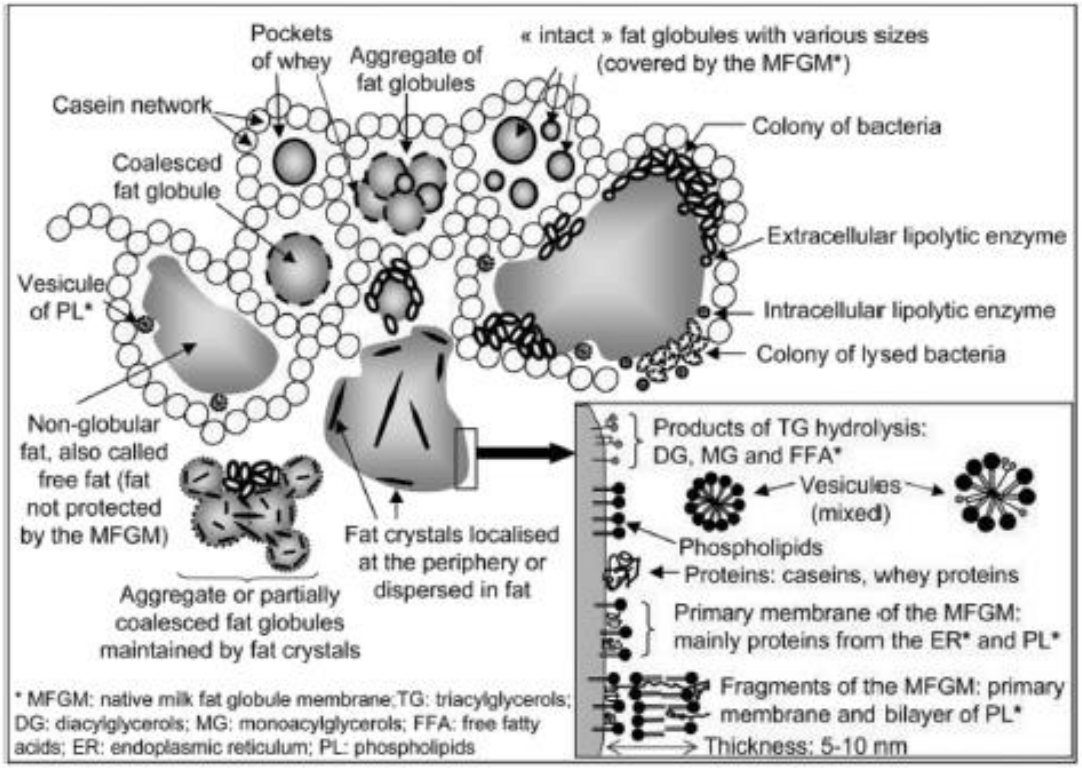
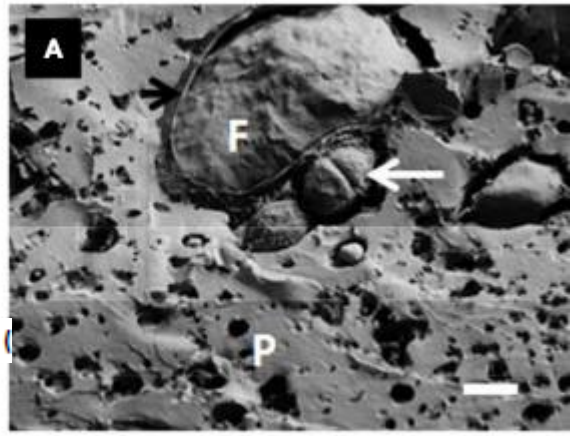


Fig. 2. Schematic representation of the supramolecular structure of milk fat in dairy products. Reprinted with permission from Lopez *et al.* (2006). Copyright (2006) American Chemical Society.

Microbes – where are they in the cheese matrix ?

- Bacterial distribution is not homogenous in cheese- random distribution of bacterial colonies (Fitzsimons et al., 2001).
- Each bacterial cell is believed form a colony and potentially undergo immobilisation within the matrix (Jeanson et al., 2011).
- Bacteria have been shown to preferentially locate at the fat–protein interface and sometimes within whey pockets in dairy products. (Hannon et al, 2006; Lopez et al., 2006, 2007; Pitino et al., 2012; Ong et al., 2013).
- Close proximity or in direct contact with milk fat globules and their membranes (Laloy et al., 1996).

Hickey et al., 2015



scale bar = 1 μm. (

FIGURE 4 | (A) Cryo-SEM image of a Cheddar-type cheese showing the location of the starter bacteria (*S. thermophilus*) (white arrow) and fat globule (F), including fractured MFGM (black arrow), within the protein

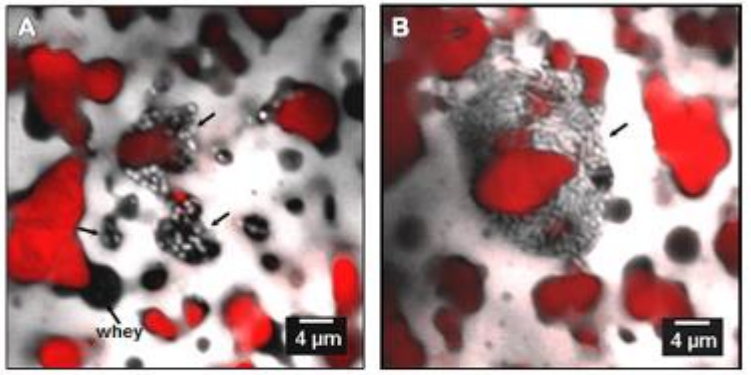


FIGURE 6 | Confocal laser scanning microscopy images of bacteria in Emmenal cheese after 1 day of ripening, showing location of bacterial colonies (light color) in whey pockets (A, black areas) and at the interface (B) between fat (red) and protein (gray). Adapted from Lopez et al. (2006) with permission from the authors.

Microbes – where are they in the cheese matrix ?

- Bacterial populations are directly related to the fat content of the cheese

Fat free	50 % reduced	full fat cheese
Bacterial pop.s	30-100 %	4- 10 fold higher

As ripening progressed (> 1-2 months) bacteria become embedded in MFGM
(Laloy et al., 1996)

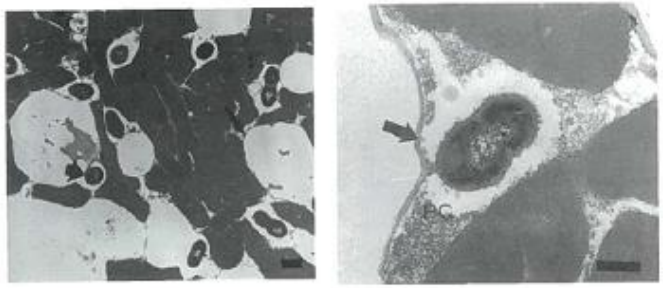


FIGURE 1 | Transmission electron microscopy (TEM) images (ultrathin sections) of starter bacteria (*Lactococcus lactis* subsp. *cremoris* KB) location in full-fat Cheddar cheese in image 1 (left). Image 2 (right) shows the starter strain KB locating in close proximity to the MFGM (arrow) together with proteolysed casein protein (PC). The Bar represents 400 nm. Reprinted with permission from Laloy et al. (1996) and Elsevier. Copyright (1996) Elsevier.

S. aureus – on surface rather than core
Aerated core- large colonies (Fleurot at al., 2014)

Microbes- Interaction with the matrix

- Colonies consist of bacterial cells in various physiological states of growth
- Bacterial cells which are in the exponential phase of growth are located on colony exterior touching the matrix-

Suggests that larger the interfacial area- the greater the bacterial activity on the matrix, in turn influencing ripening. (McKay et al., 1997).

- Increased inoculum levels (10^7 CFU/g) vs (10^4 CFU/g) (Jeanson et al., 2011)

  - smaller colonies and further away
  - 7 fold increase in interfacial area of exchange with cheese matrix

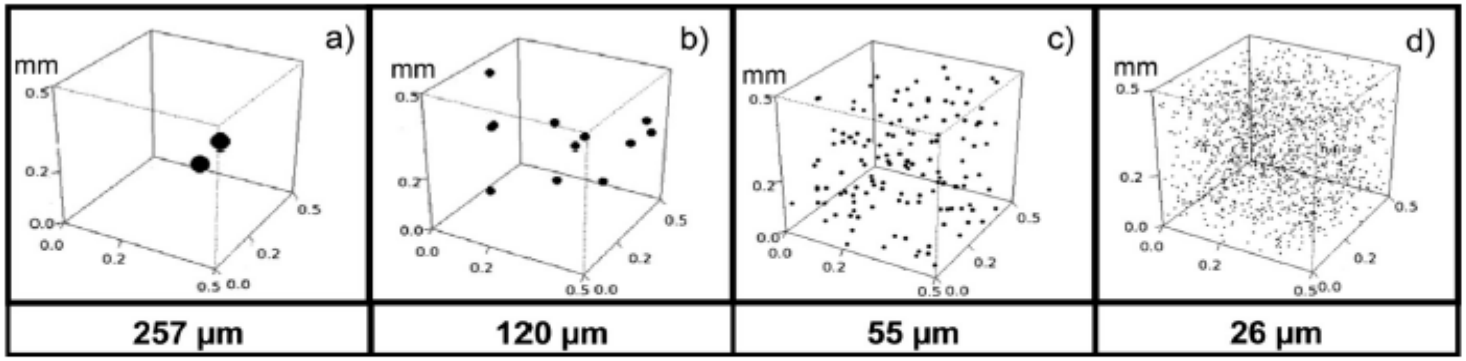


FIG. 2. Theoretical distribution of bacterial colonies in a volume (0.5 by 0.5 by 0.5 mm), such as a piece of cheese, assuming that they are evenly distributed at 10^4 CFU/cm^3 (a), 10^5 CFU/cm^3 (b), 10^6 CFU/cm^3 (c), and 10^7 CFU/cm^3 (d), and associated mean 3D theoretical distances to the nearest neighbor colony.

Jeanson, S., Chadæuf, J., Madec, M., Aly, S., Floury, J., Brocklehurst, T., et al. (2011). Spatial distribution of bacterial colonies in a model cheese. *Appl. Environ. Microbiol.* 77, 1493–1500. doi: 10.1128/AEM.02233-10

Microbes- Interaction of colonies with the matrix

- Question: Growth and physiology of bacterial cells in colonies influence ?
  - The microenvironment around a colony, or alternatively
  - The cells within a colony may modify the microenvironment (e.g., pH, redox potential) due to their metabolic activity

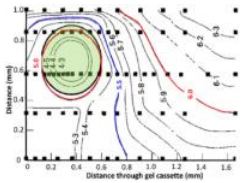
- pH micro-gradients did not occur around microbial colonies
  - unripened non-fat UF model cheese system
  - lactococci rather than thermophillic species.

(Jeanson et al., 2013 Applied and Environmental Microbiology, 6516–6518)

- Micro colonies (radius<100–200µm) - no pH micro-gradients
- Macro-colonies (radius>200µm): pH micro-gradients observed in and around colonies

  Jeanson et al., 2015 (Frontiers in Microbiology)

  Skandamis and Jeanson, 2015 (Frontiers in Microbiology)



Potential impact of varying pH levels on cheese ripening

- Factors determining pH of curd
  - the extent of acidification during manufacture
  - the availability of substrate for fermentation (principally lactose)
  - buffering capacity of the cheese curd
  - Salt in moisture levels
  - Bacterial colonies?

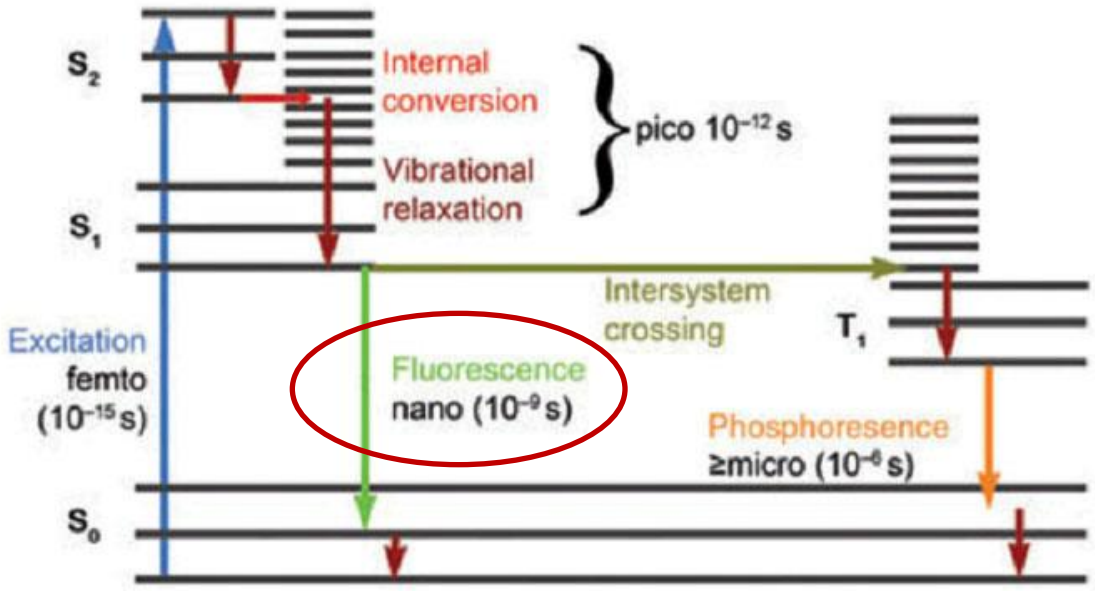
- Cheese pH affects the degree of casein hydration (Euston et al., 2002; Kilcast and Angus, 2007).

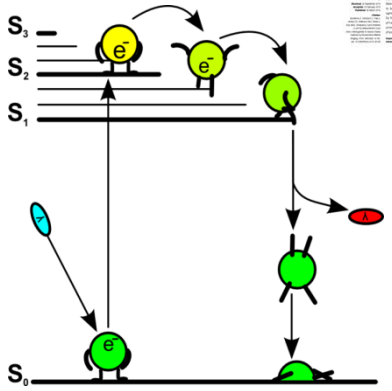
- pH influences activity of enzymes
  - plasmin (Grufferty and Fox, 1988)
  - coagulant, both retention of and the activity of the enzyme

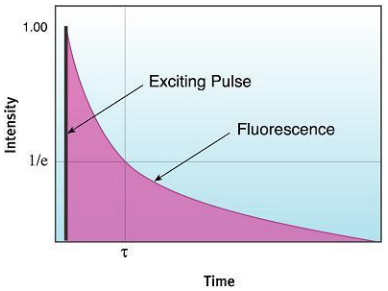
  (Holmes et al., 1977; Stadhouders et al., 1977; Visser, 1977; Creamer et al., 1985; Garnot et al., 1987)

- pH influences the metabolic activity of lactic acid bacteria (Meldrum et al., 2003; Kajfasz and Quivey, 2011; Jeanson et al., 2013). E.g., amino acid decarboxylase activity.

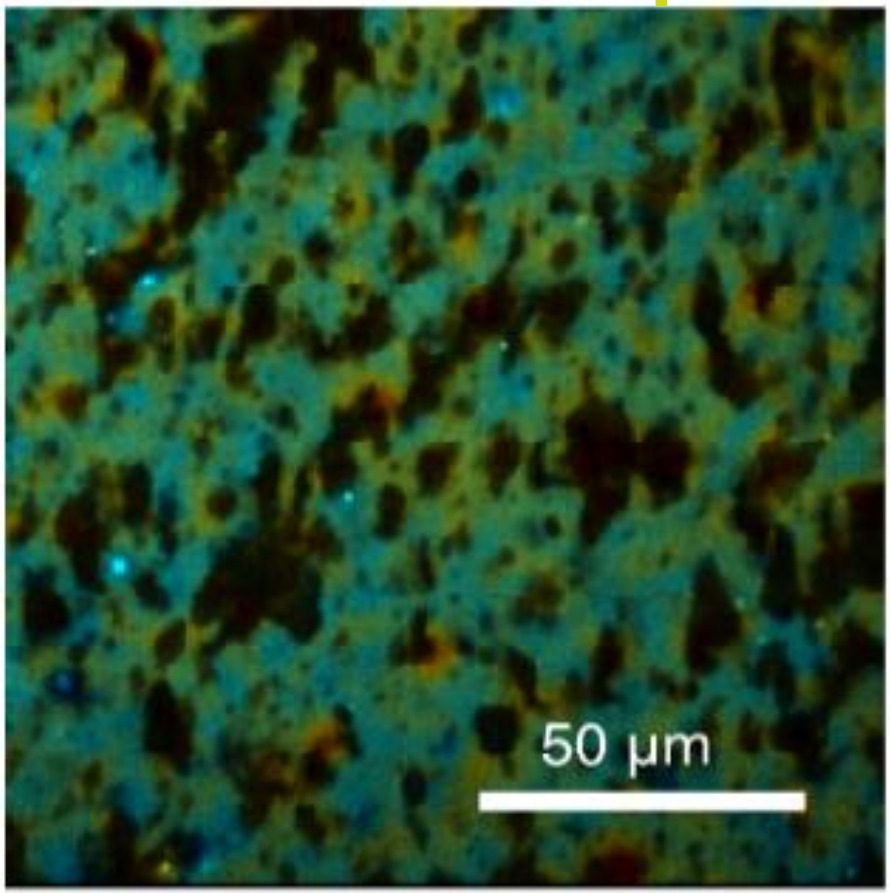
Microstructure and microbes: Fluorescence lifetime imaging Microscopy (FLIM)

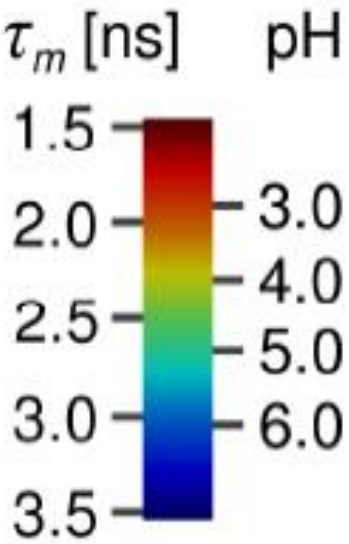


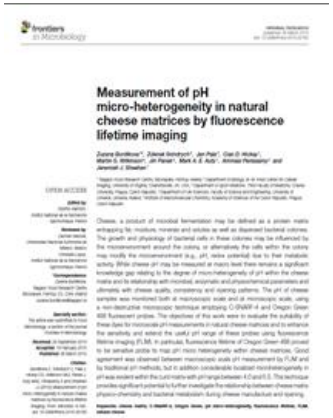




Localised pH in cheese






Measurement of pH micro-heterogeneity in natural cheese matrices by fluorescence lifetime imaging

- Dark areas most likely represent fat within the matrix
- Localised variation in pH is evident

Significance of results

- Suggests the pH of a cheese matrix is not homogenous at micro-scale but contains localized variation.

- This may be due to
  - localized differences in the aqueous phase or
  - concentrations of constituents of the aqueous phase including lactose, lactate, minerals or salt.
  - It may also be influenced by variations in buffering capacity of the surrounding cheese matrix.
  - Colony size and location ?

- Currently investigating
  - Patterns of micro heterogeneity in different cheese types
  - Influence of varying manufacture processes on pH at local level
  - Relationship with bacterial colonies

Starter and NSLAB during ripening

- LAB undergo lysis during ripening (?) (Sheehan et al., 2009)
- NSLAB and minute quantities of starter bacteria remain active and intact
- NSLAB evolution over ripening
- NSLAB survive for an extended period of time on the monosaccharides galactose and glucose found in bovine MFGM.
- This ability is due to the many types of glycolytic enzymes possessed by NSLAB. (Moe, Porcellato, and Skeie, 2013)

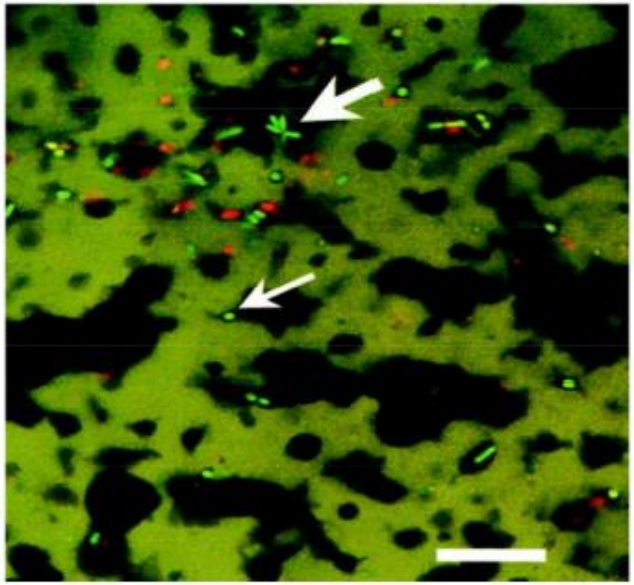


FIGURE 5 | Confocal laser scanning microscopy image of probiotic cheddar cheese showing star shaped clusters of live (bright green), presumptive Bifidobacteria, (large arrow), and dead (red) bacterial cells at fat (black)/protein (green) interface and presumptive NSLAB bacteria (small arrow). Scale bar = 25 μm. Reprinted with permission from Auty et al. (2001) and American Society for Microbiology (ASM).

Auty et al., (2001) Appl. Environ Microbiol. 67:420–425.

- Live v Dead v (Injured/ non culturable/ metabolically active/ enzyme release)

Salt- influence on ripening

- Salt distribution
  - Macro level- Brine salt diffusion
  - Micro or localised level

- Chromaphores for salt
- Application of CSLM

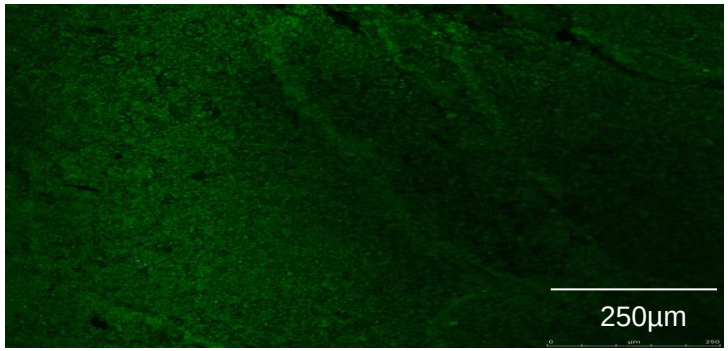
- Salt penetration → CoroNa^{+} Green

↓

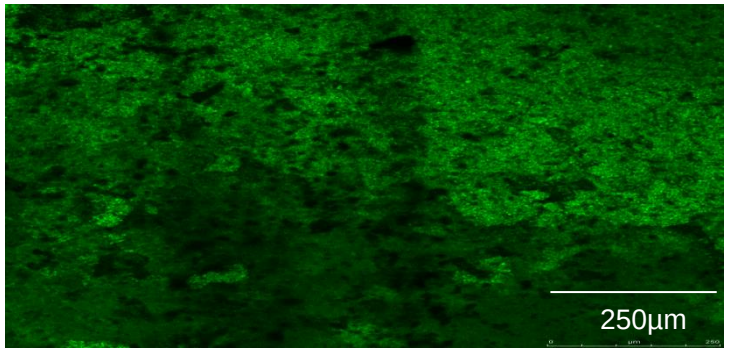
Excitation/emission = 488/510-530nm

- Analytical tool for further research

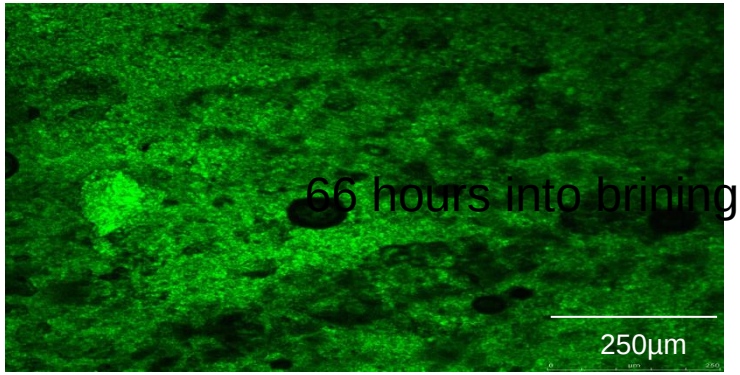
Salt penetration into cheese matrix



2.5cm from surface

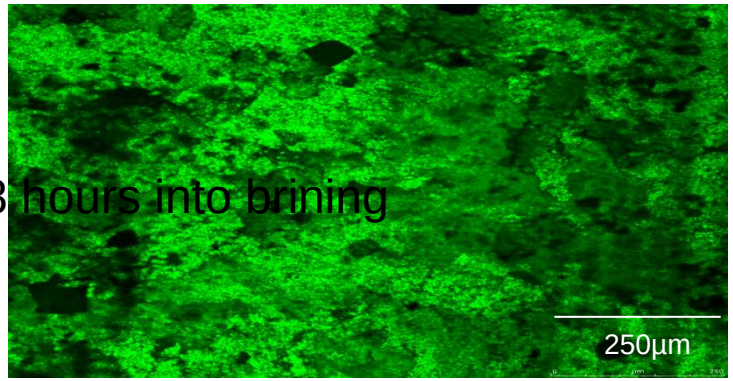


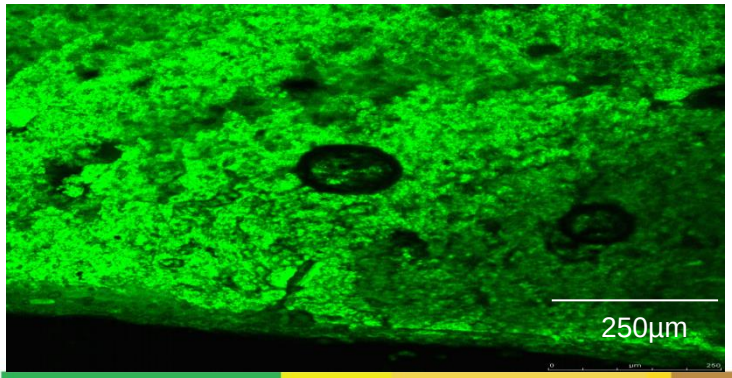
66 hours into brining



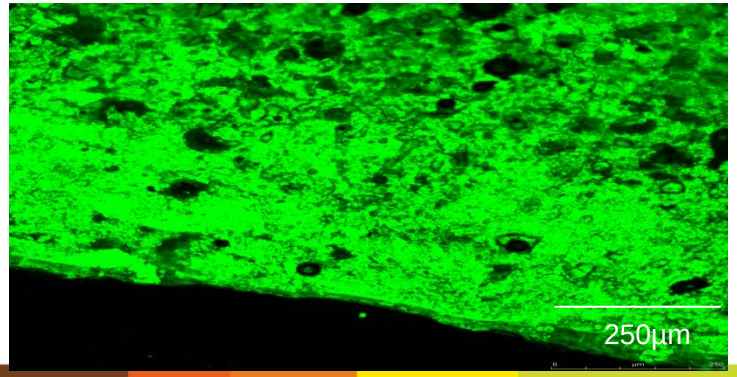
~1cm from surface

18 hours into brining



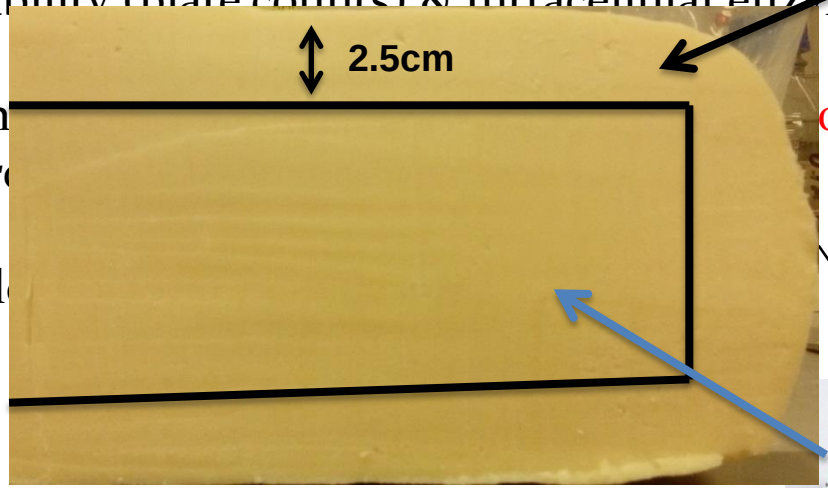


Cheese surface



Experimental design

- 2 sampling locations used in order to determine effect of salt :
  - Chemical composition (Moisture, Fat, Protein, pH)
  - Microbial viability (plate counts) & Intracellular enzyme activity (LDH, PEP X & N)
  - Membrane in
    (damaged/br
  - Cell morphol

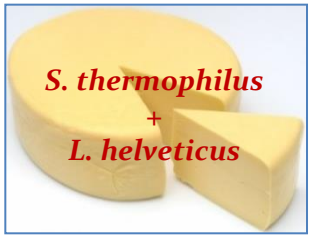


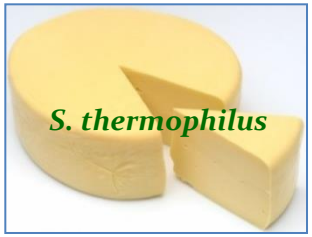
Outside (high salt)

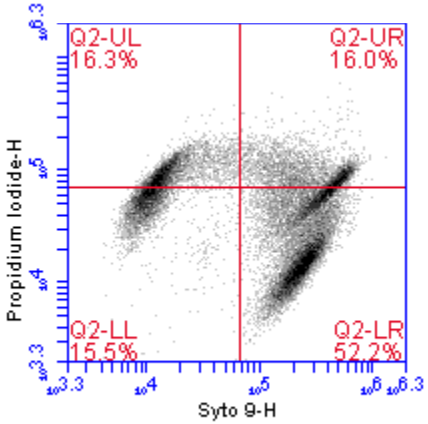
opidium Iodide

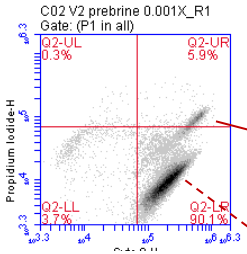
Inside (low salt)

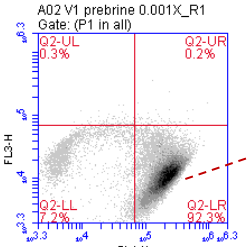
Experimental design

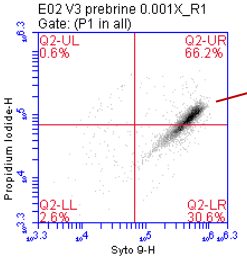


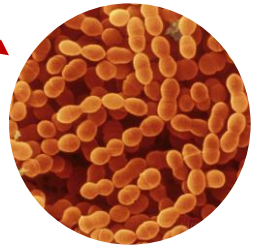


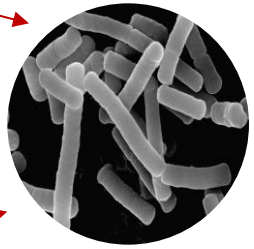




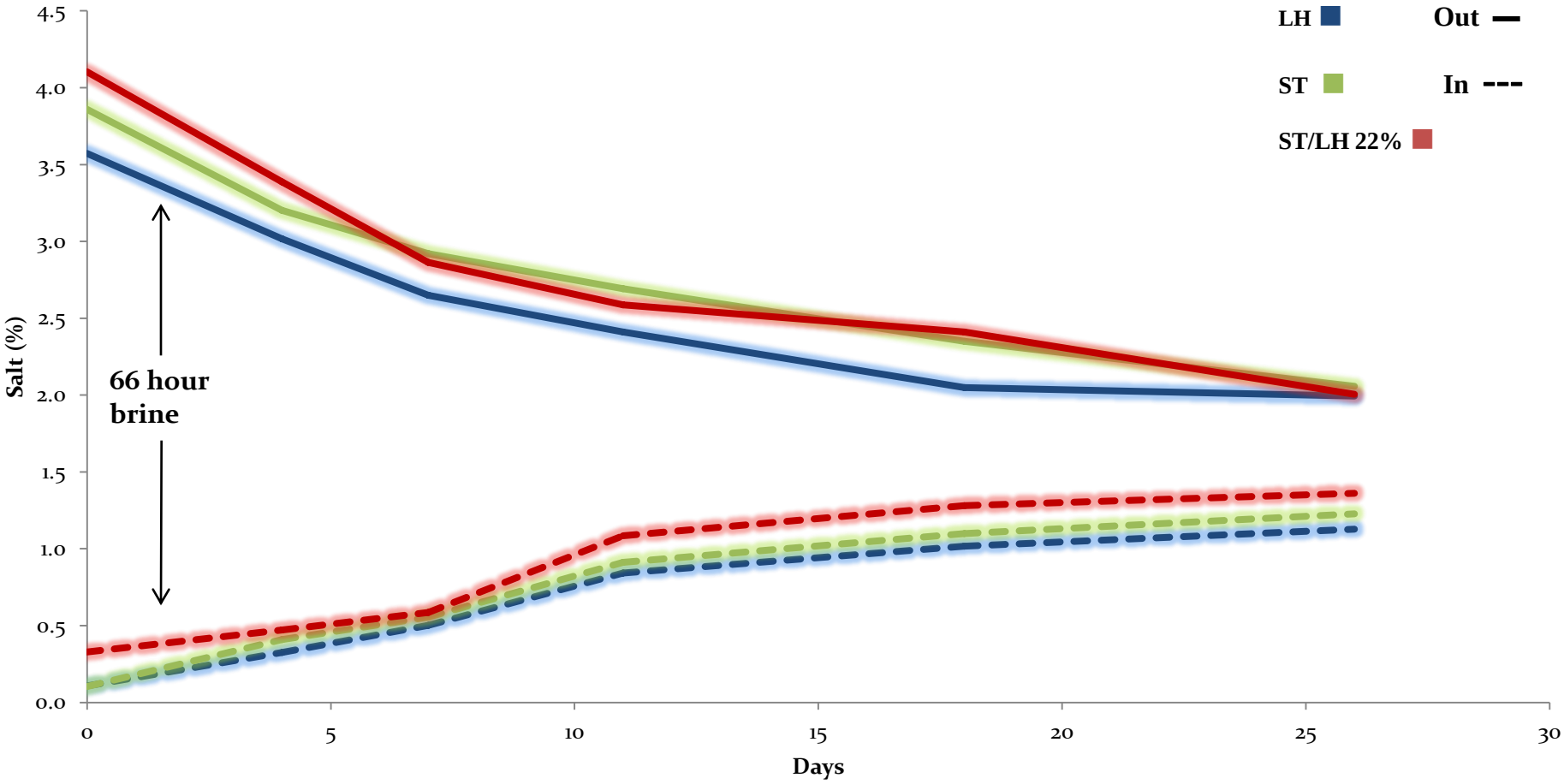




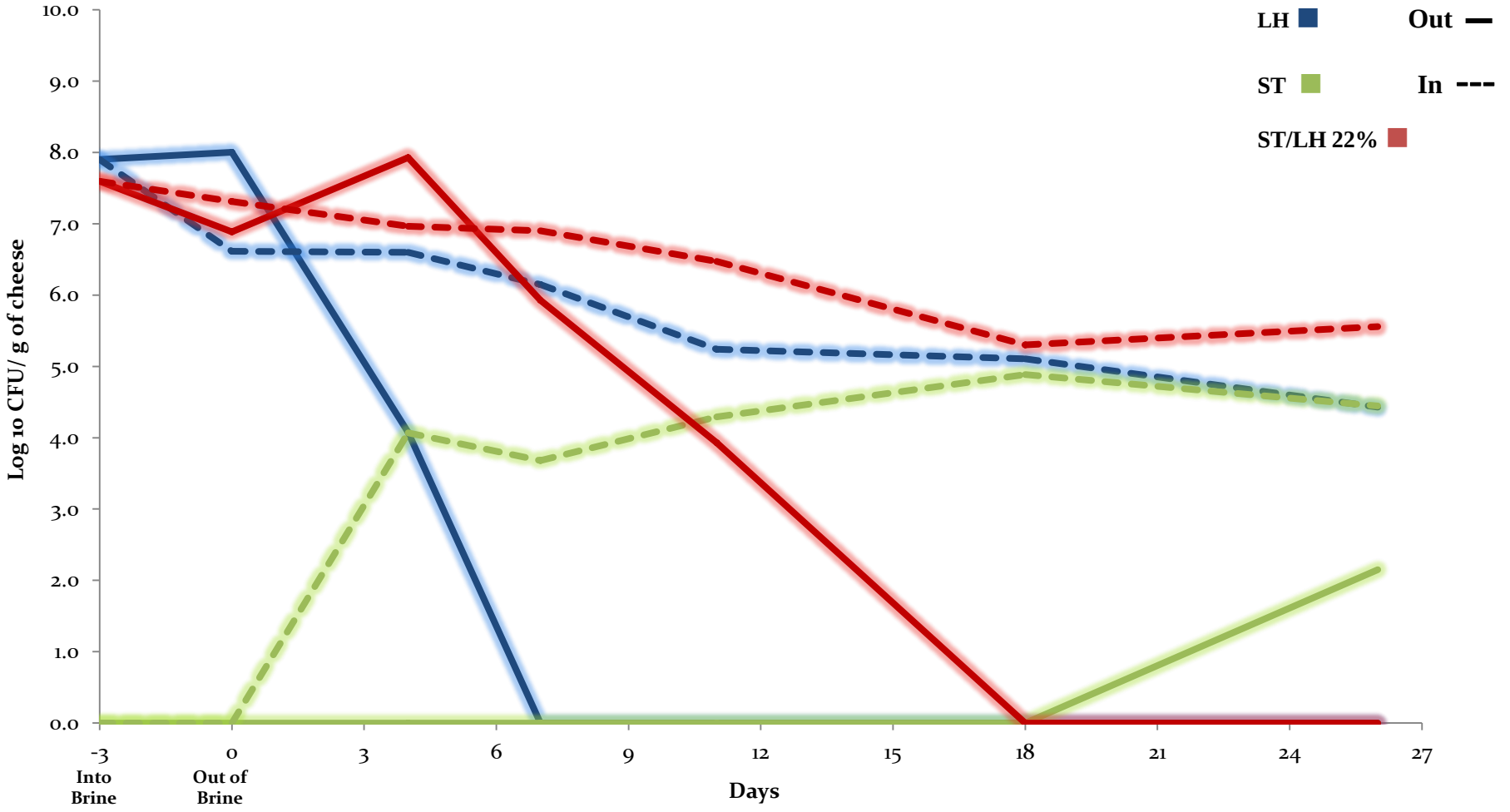




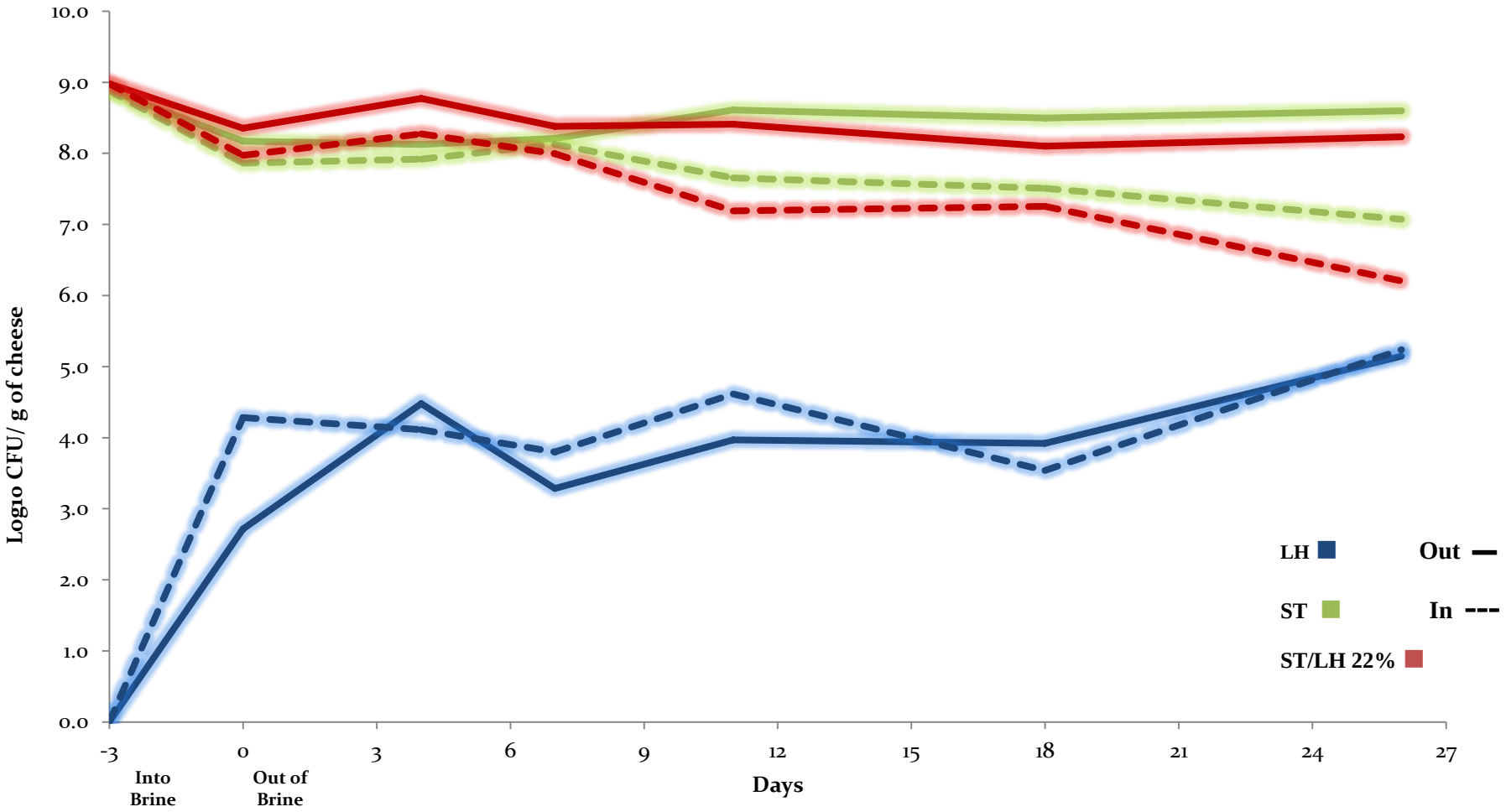
Results: Salt conc. post brining



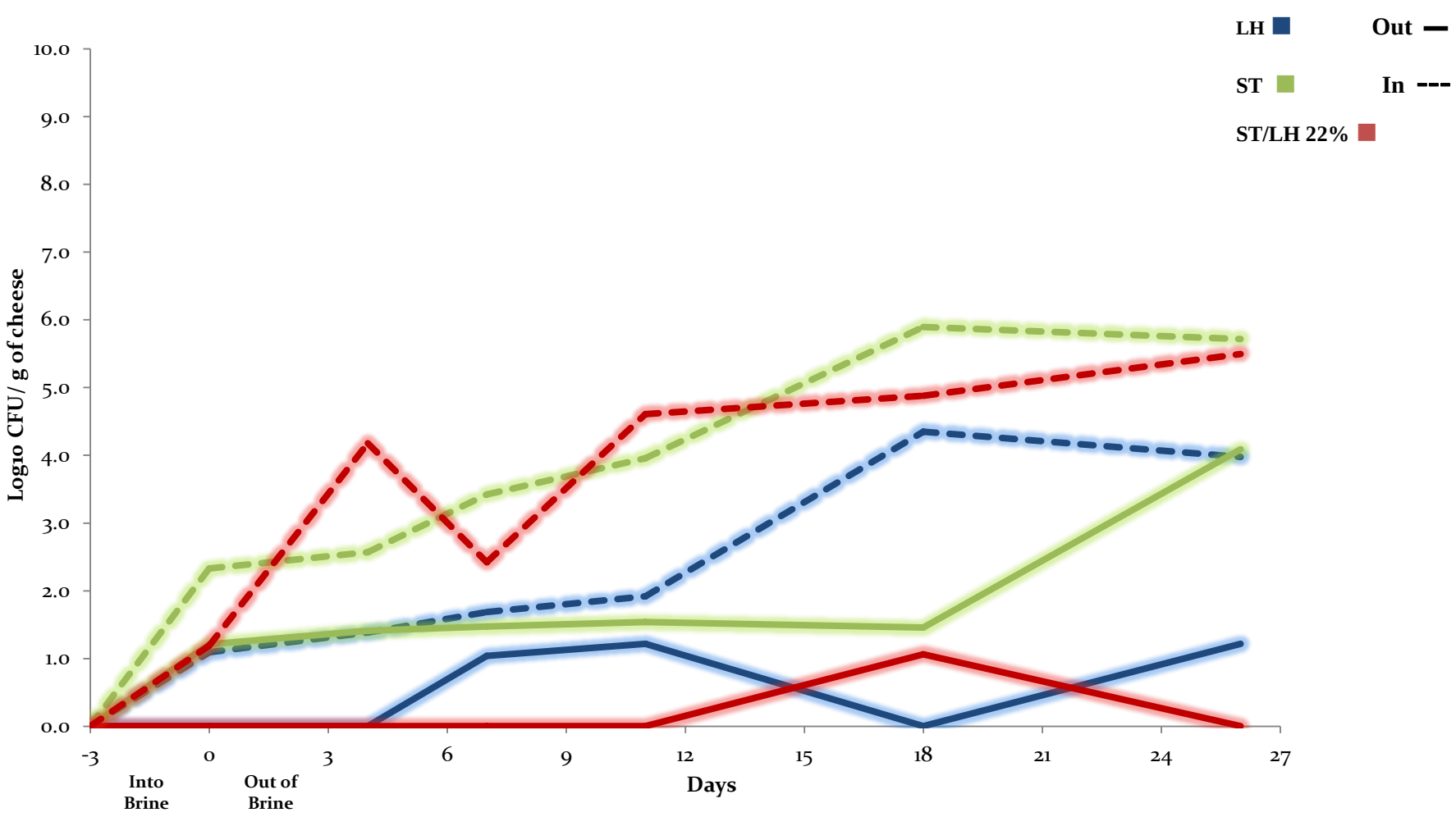
L. helveticus viability (plate count)



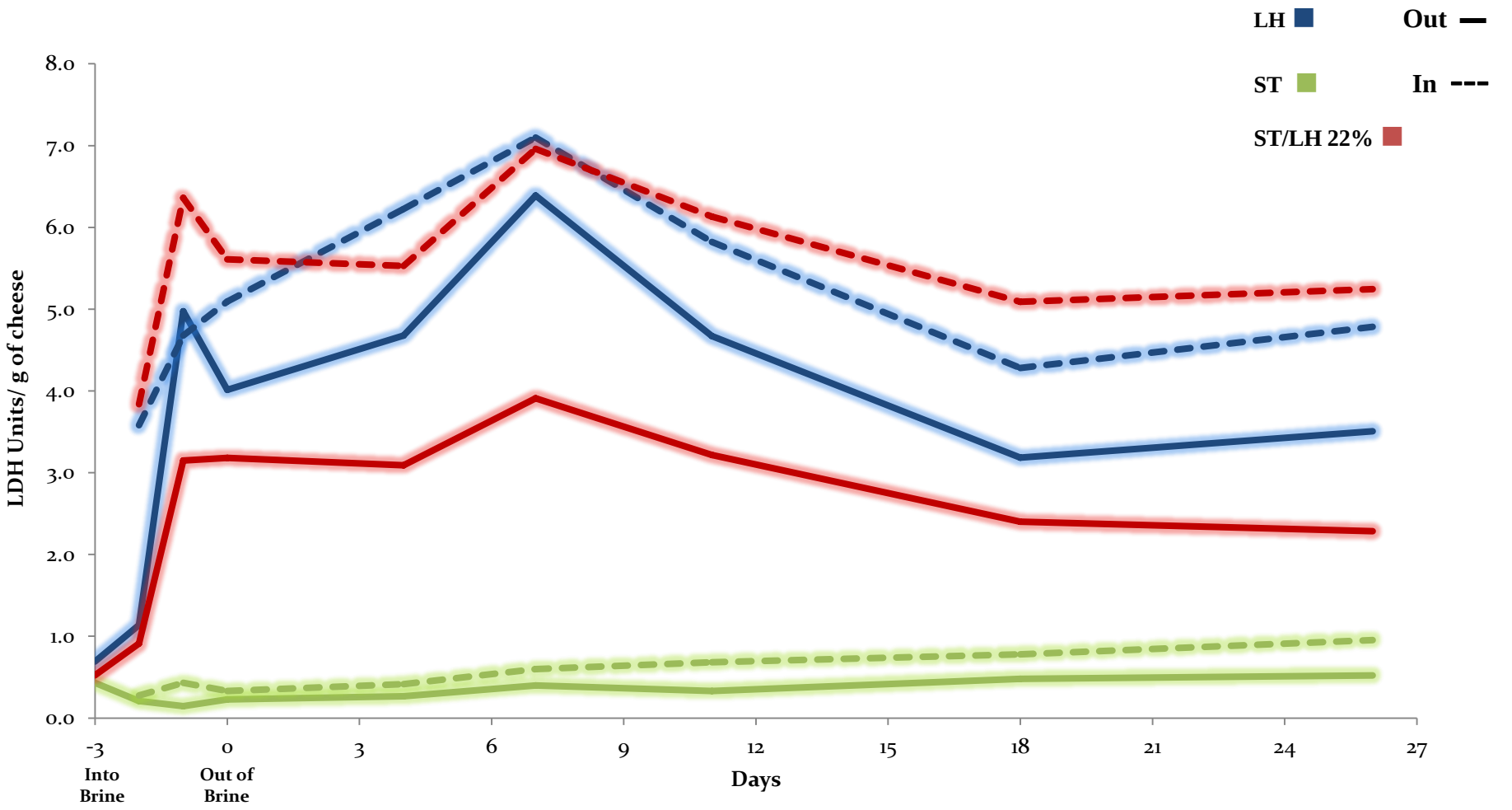
S. thermophilus viability (plate count)



NSLAB viability (plate count)

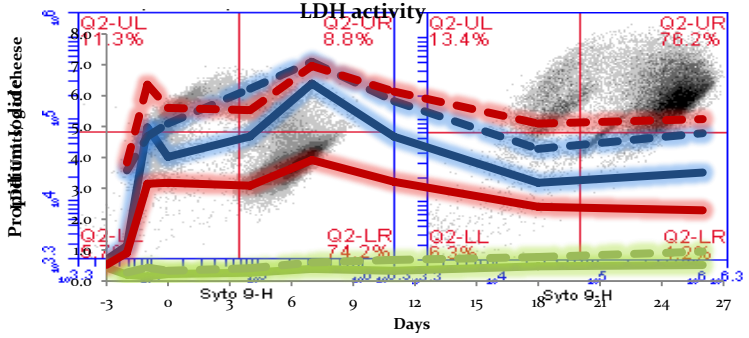


LDH enzyme activity

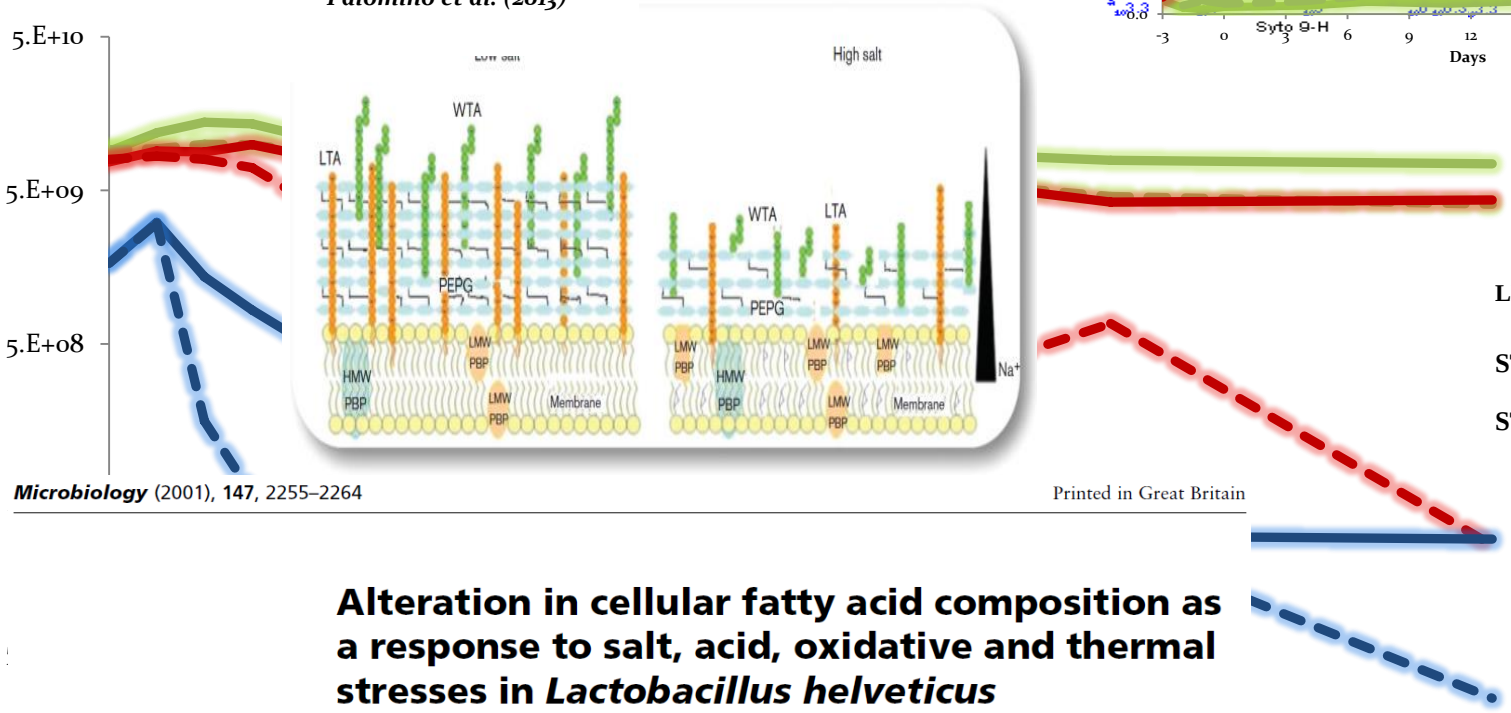


Membrane integrity (FC)

(Total live cells)



Palomino et al. (2013)

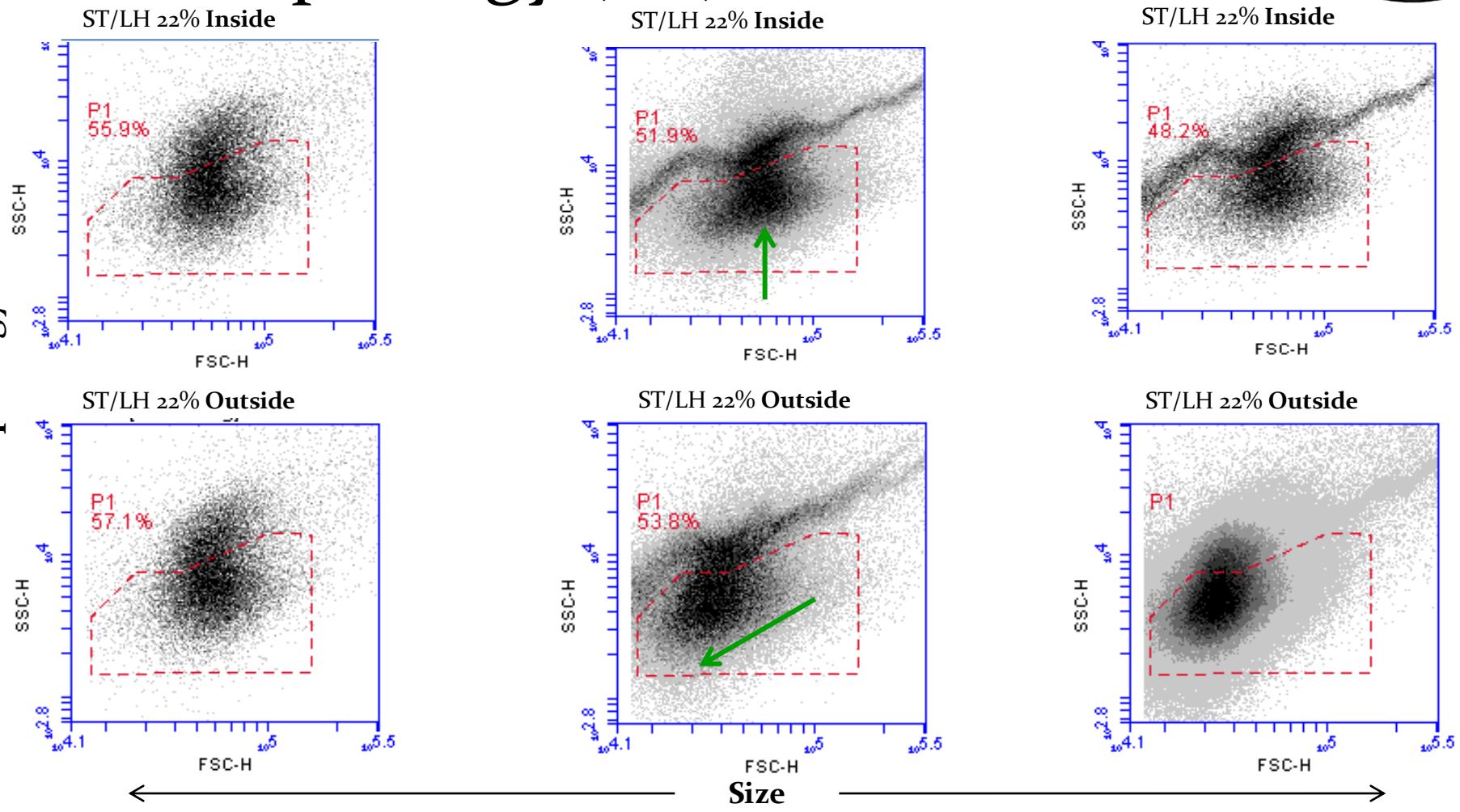


Microbiology (2001), **147**, 2255–2264 Printed in Great Britain

Alteration in cellular fatty acid composition as a response to salt, acid, oxidative and thermal stresses in *Lactobacillus helveticus*

M. Elisabetta Guerzoni,[1] Rosalba Lanciotti[1] and P. Sandro Cocconcelli[2]

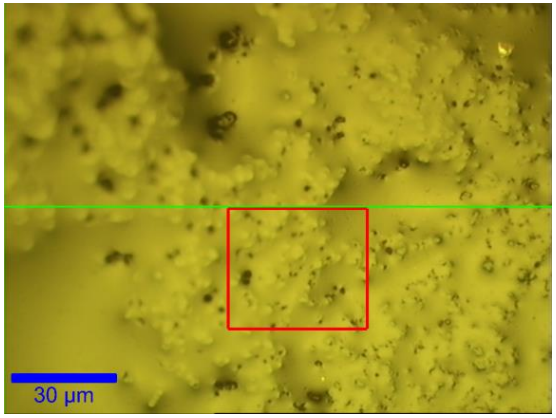
Cell morphology (FC)

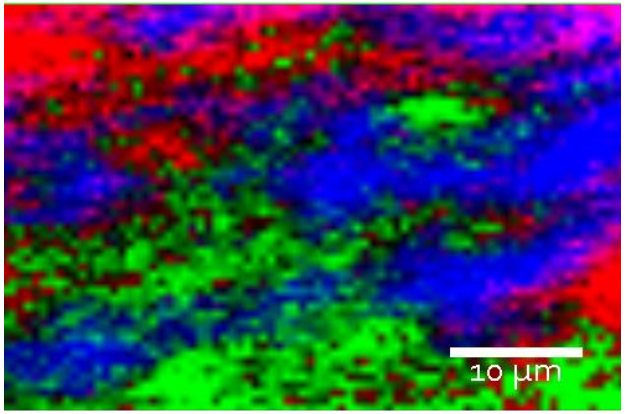


Conclusions

- High salt = no significant effect on *S. thermophilus* viability
- High salt = ↓ *L. helveticus* viability
- High salt = ↓ enzymatic activity
- Possibly due to osmotic stress → bacterial cell membrane alteration
- identified inactive starter population which does not contribute effectively to cheese ripening, most likely due to bacterial membrane alteration,
- Potential for heterogeneity of ripening (cold or hotspots) in developing cheeses
- Impact on Probiotics ?

Distribution of other components in the matrix





Cheese components:

- Fat (in red)
- Protein (in green)
- Water (in blue)

Burdikova et al, (2015). *Dairy Science and Technology, 95, 687 – 700.*

Relationship between bacteria and matrix components

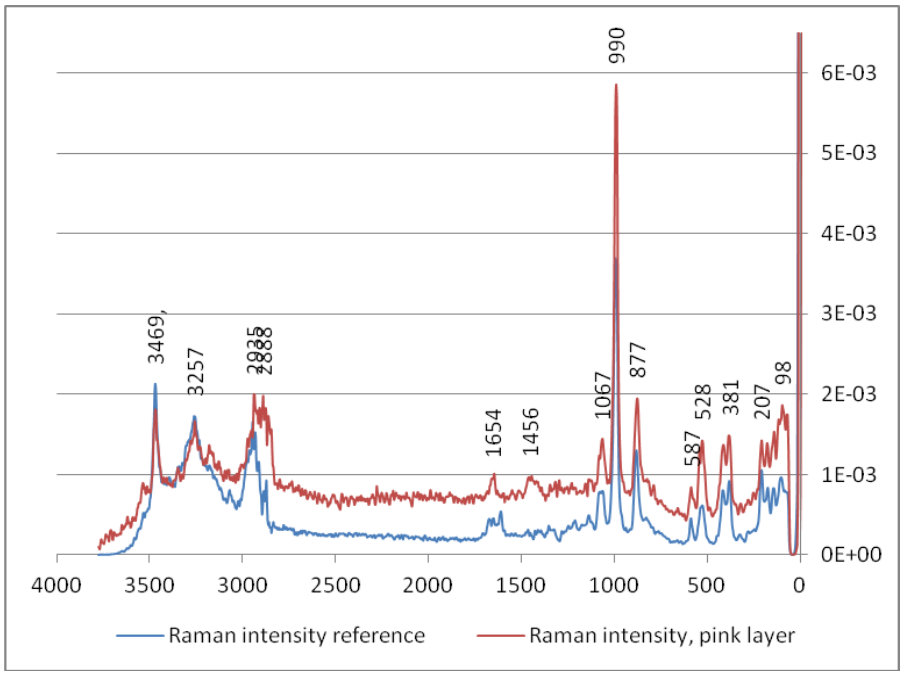


Fig. Vibrational characteristics of biomolecules in natural cheese in the pink area (red line) and outside the pink area (blue) line,

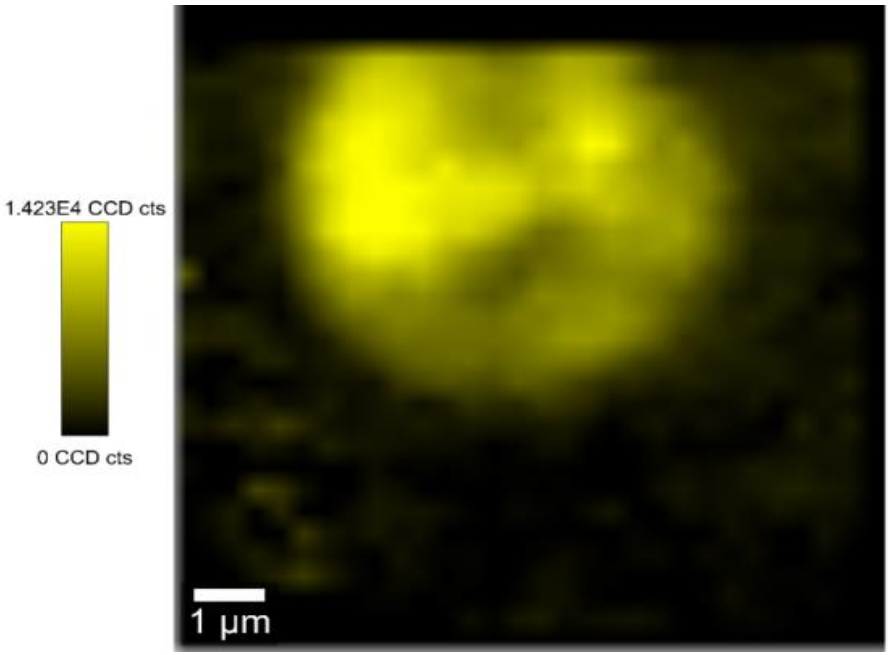


Fig. Localised intensity distribution of Raman signal from the pink layer and the surrounding cheese matrix (Confocal Raman microscopy

Relationship between bacteria and matrix components

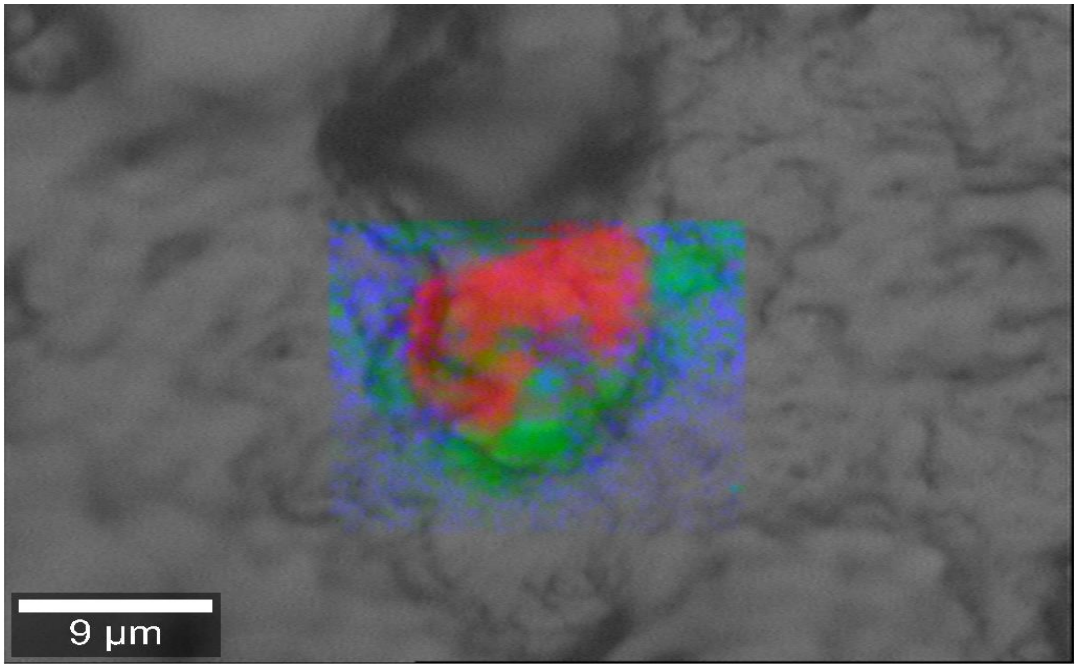


Fig. Overlay of intensity image of the studied cheese matrix (grey) and the maps of the chemical composition obtained from local Raman spectra: red – carotenoids, mainly present in pink layer; blue - proteins; green - lipids.

Thermus and the pink discolouration defect in cheese
Quigley et al., Accepted for publication pending minor revisions

Probiotics

- Probiotic bacteria such as *Bifidobacterium longum,Bif. lactis*, *Lactobacillus acidophilus, L. casei* and *L. paracasei* are usually added to yoghurt and other fermented milks (Heller 2001), and to cheese (Gardiner et al. 1998) as delivery vehicles for human consumption.

- However, probiotic bacteria must survive in foods to reach the human gastrointestinal system and further modify gut microbiota (Kramer et al. 2009; Yu et al. 2009).

Cheese – Environment for starters, adjuncts and NSLAB

- *Lactococcus lactis* is used as a starter culture

- Probiotic bacteria are usually added to cheese milk and thus sequentially undergo
  - physico-chemical stresses such as heat, acid, salt and cold during initial manufacture
  - changes in redox potential over storage and distribution (Rallu et al. 1996; van de Guchte et al. 2002), as do other adventitious or added lactic acid bacteria (LAB).

- NSLAB survive in cheese and grow over ageing, of which lactobacilli are the dominant species,
  - probiotic lactobacilli species may also remain viable in Cheddar cheese during ageing until consumption to provide health benefits.

Enumeration of bacteria in cheese

- Estimates of bacterial viability in different foods and environments vary based on the enumeration techniques used.
  - Growth media-based enumeration discounts possible alternate physiological states of bacteria, such as nonculturability (*Fenelon et al. 2000; Ganesan et al. 2007*).

- Such growth-based observations led to a previous hypothesis that starter bacteria die and lyse to subsequently provide substrates that accelerate NSLAB growth (*Branen and Keenan 1969; Crow et al. 1995; Buist et al. 1997, 1998*).

- However, lactococci, NSLAB become nonculturable in carbohydrate-depleted media while remaining metabolically active (Ganesan et al. 2004, 2007)

- The declining lactococcal counts in cheese may represent a subpopulation of replicating cells, while a nonculturable population of cells that is unable to divide and is hence not enumerated on growth media (Kilcawley et al. 2011) coexists.

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ORIGINAL ARTICLE

Probiotic bacteria survive in Cheddar cheese and modify populations of other lactic acid bacteria

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Keywords
Cheddar cheese, low fat, nonculturability, probiotic, survival.

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Abstract

Aims: Starter lactic acid bacteria in Cheddar cheese face physico-chemical stresses during manufacture and ageing that alter their abilities to survive and to interact with other bacterial populations. Nonstarter bacteria are derived from milk handling, cheese equipment and human contact during manufacture. Probiotic bacteria are added to foods for human health benefits that also encounter physiological stresses and microbial competition that may mitigate their survival during ageing. We added probiotic *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus paracasei* and *Bifidobacterium animalis* subsp. *lactis* to full-fat, reduced-fat and low-fat Cheddar cheeses, aiming to study their survival over 270 days of ageing and to determine the role of the cheese matrix in their survival.

Methods and Results: Probiotic and other lactic acid bacterial populations were enumerated by quantitative PCR using primers specifically targeting the different bacterial genera or species of interest. Bifidobacteria were initially added at 10^6 CFU g^{-1} cheese and survived variably in the different cheeses over the 270-day ageing process. Probiotic lactobacilli that were added at 10^7 CFU g^{-1} cheese and incident nonstarter lactobacilli (initially at 10^6 CFU g^{-1} cheese) increased by 10- to 100-fold over 270 days. Viable bacterial populations were differentiated using propidium monoazide followed by species-specific qPCR assays, which demonstrated that the starter and probiotic microbes survived over ageing, independent of cheese type. Addition of probiotic bacteria, at levels 100-fold below that of starter bacteria, modified starter and nonstarter bacterial levels.

Conclusions: We demonstrated that starter lactococci, nonstarter lactobacilli and probiotic bacteria are capable of surviving throughout the cheesemaking and ageing process, indicating that delivery via hard cheeses is possible. Probiotic addition at lower levels may also alter starter and nonstarter bacterial survival.

Significance and Impact of the Study: We applied qPCR to study multispecies survival and viability and distinctly enumerated bacterial species in commercial-scale Cheddar cheese manufacture.

Introduction

Probiotic bacteria are defined as 'live micro-organisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO 2002; Morelli and Capurso 2012). The consumption of probiotic bacteria is reported to confer many health benefits such as preventing gut inflammation, immunomodulation, preventing

Summary of studies with Probiotic addition

Table 1 Previous studies of probiotic addition to Cheddar cheese

Study	Probiotic bacteria used	Scale of cheese manufacture	Targeted level of probiotics (CFU g^{-1})	Detection method in cheese	Viability assessment in cheese	Detected levels of probiotics (CFU g^{-1})	Cheese ageing period (months)
Dinakar and Mistry (1994)	*Bifidobacterium bifidum* (immobilized; added during salting)	100 kg	10^6	Microbial plating on selective media	Microbial plating on selective media	10^6 to 10^7	6
Gardiner *et al.* (1998)	*Lactobacillus salivarius*, *Lact. paracasei*	25, 450 l (2 reps × 2 strains only)	NS	RAPD-PCR/gel electrophoresis on DNA extracted from grown colonies	Microbial plating on selective media	10^8	8
Daigle *et al.* (1999)	*Bifidobacterium infantis*	250 l	5×10^7	Microbial plating on selective media	Microbial plating on selective media	10^7	3
Gardiner *et al.* (1999a)	*Enterococcus faecium*	450 l	4×10^8	Antibiotic resistance mutant	Microbial plating on selective media	4×10^8	15
Gardiner *et al.* (1999b)	*Ent. faecium*	450 l	10^8	Antibiotic resistance mutant	Microbial plating on selective media	3×10^8	9
Mc Brearty *et al.* (2001)	*Bif.* sp.	450 l	10^8	RAPD-PCR/gel electrophoresis on DNA extracted from grown colonies	Microbial plating on selective media	10^5 to 10^8	6
Auty *et al.* (2001)	*Lact. paracasei*, *Bif.* sp. BB-12	450 l	10^8	Microbial plating on selective media, staining and confocal microscopy	Microbial plating on selective media, staining and confocal microscopy		NS
Phillips *et al.* (2006)	*Lactobacillus acidophilus*, *Bifidobacterium* sp., *Lact. casei*, *Lact. paracasei* and *Lact. rhamnosus*	10 l	10^8	Microbial plating on selective media	Microbial plating on selective media	10^3 to 10^8	9
Sharp *et al.* (2008)	*Lact. casei*	136 kg	10^7	Antibiotic resistance mutant	Microbial plating on selective media	10^7	3
Ong and Shah (2008)	*Lact. acidophilus* and *Lact. helveticus*	20 l	NS	Microbial plating on selective media	Microbial plating on selective media	10^8	6
Achilleos and Berthier (2013)	*Lact. paracasei*	Not disclosed	10^8	Microbial plating on selective media and qPCR	Microbial plating on selective media and qPCR	10^8	0

NS, not specified.

Enumeration of bacteria in cheese

- According to these studies, even the same strains or species survive variably,
  - one group showing survival throughout ageing, but
  - another demonstrating loss of viability of the same in 6– 8 weeks.
  - Some studies were conducted in smaller scale (10–20 l of milk)
  - none of these studies enumerated survival of probiotic bacteria at the species level or

Probiotic cultures used

Supplier	Organism	Name
Chr. Hansen, Milwaukee, WI, USA	*Lactococcus lactis*	DVS850
Cargill Inc., Waukeshaw, WI, USA	*Bifidobacterium lactis*	Bif-6
Chr. Hansen	*Bif. lactis*	BB-12
Chr. Hansen	*Lactobacillus acidophilus*	LA-5
DSM Food Specialties, Logan, UT, USA	*Lact. acidophilus*	L10
DSM Food Specialties	*Lactobacillus casei*	L26
Chr. Hansen	*Lact. casei*	CRL-431
Chr. Hansen	*Lactobacillus paracasei* subsp. *paracasei*	F19

To achieve: 10^6–10^7 CFU/g

Approach used

- Extraction of genomic DNA from cheese
- Addition of DNAase to destroy DNA from lysed cells
- qPCR
- Live-Dead / Viable bacterial qPCR assay using propidium monoazide

Results

- Even at this high initial NSLAB level, still distinguish added probiotic from NSLAB,
- Added probiotic lactobacilli survived in cheese over 270 days of ageing, even growing 10 to 1000-fold
    - Other studies that compared survival of different lactobacilli in cheese have confirmed the presence of *LB . paracasei* up to 300 days of cheese age (Gardiner *et al*. 1998; Fitzsimons *et al*. 2001).
- At any time, probiotic lactobacilli levels were only 1–10% of that of total lactobacilli

Fold change of different probiotic bacterial populations in cheeses over 270 days of ageing

Probiotic organism	Fold change in populations (CFU g^{-1} ratio of 270 days/0 days)		
	Full fat	**Reduced fat**	**Low fat**
Lactobacillus ***acidophilus*** LA-5	−1·9 ± 2·3	13 ± 0·9	−16 ± 0·4
Lact. ***acidophilus*** L10	2·3 ± 1·0	230 ± 0·9	−37 ± 1·4
Lactobacillus casei CRL-431	4·1 ± 2·0	9·3 ± 0·9	71 ± 0·6
Lact. casei L26	16 ± 0·4	7·4 ± 0·2	3·5 ± 0·3
Lactobacillus paracasei F19	1·5 ± 0·5	2·2 ± 1·0	5·2 ± 0·8
Bifidobacterium *lactis* Bif-6	−12 ± 10	2·8 ± 1·1	−6900 ± 10
Bif. *lactis* BB-12	−3200 ± 100	1500 ± 10	−2·7 ± 1·0

Significant factors influencing NSLAB and Probiotic levels

Probiotic	Statistically significant (p≤0.05) effects and interactions	
	NSLAB levels	**Probiotic levels**
LA-5 acidophilus	-	-
L-10 acidophilus	Fat	-
F 19 paracasei	Fat	Fat
L-26 casei	Fat	-
CRL-431 casei	-	Time
BB-12	-	Fat
Bif-6	-	-
Control – probiotic not added	-	Not applicable

Composition of cheeses

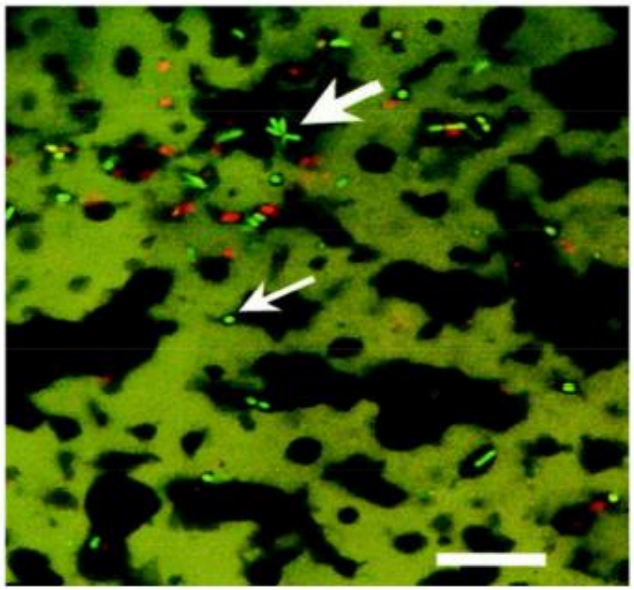
Cheese type	Moisture (%)	Fat (%)	Salt (%)	% Salt-in-moisture	% Fat in dry matter	% Moisture in Non Fat substance
Full fat	38.8	31.5	1.2	3.0	51.5	56.2
Reduced fat	45.8	17.1	1.9	4.1	31.5	55.3
Low fat	50.5	7.5	2.0	3.9	15.2	54.6

Cheeses ripened at 3 C and sampled at 5 days, 1, 2, 3, 4, 6 and 9 month of age

Bifidobacteria survival

- Bifidobacteria survival in cheese has been previously demonstrated (Mc Brearty *et al*. 2001)

- The two *Bif. lactis* strains added to cheese showed differing survival patterns in cheeses,
  - Suggestion that alteration of fat level effectively changes physico-chemical conditions inside the cheese matrices and thus alters the survival of members of the same genus.



Lactobacilli and Lactococci

- `Suggested that some probiotic lactobacilli influenced the levels of total lactobacilli in Cheddar cheese,
  - Interesting, considering that probiotics were added at 10- to 1000-fold lower levels than total lactobacilli

- Using selectively permeating PMA that binds intracellular nucleic acids in dead or membrane-compromised cells,
  - found that lactococci, other NSLAB and probiotic lactobacilli all remained viable in Cheddar cheese over 270 days of ageing

- Potentially, with casein-derived amino acids being abundant in cheese, lactococci may survive in the nonculturable state in cheese and acquire metabolic energy
  - via Arg and branched chain amino acid degradation

Finding of individual study

- Starter LAB and some probiotic bacteria survived throughout ageing, indicating the suitability of semi-hard, aged cheeses as suitable vehicles for probiotic delivery.
- Probiotic lactobacilli survived differently at species level.
- Some added probiotics also altered the levels of total lactobacilli and starter bacteria, even when present at levels 10- to 1000-fold lower.
- Starter, NSLAB and probiotic bacteria remained viable with an intact cell membrane throughout the period of cheese ageing.

Overall Conclusions

- Cheese – a complex system even with a long standing knowledge base
- Need for further understanding of factors influencing
  - Matrix Microstructure
  - Bacteria entrapped within
    - Types
    - Metabolic acitivity
- Interactions between factors influencing the matrix and entrapped bacteria

- Cheese provides a good matrix for probiotic growth and delivery
  - “Cheese matrix effect”- different impact of LDL vs other saturated fat products
- Cheese matrix relationship with microflora within cheese
- This could be expected to impact of Probiotics as well as other cheese microflora

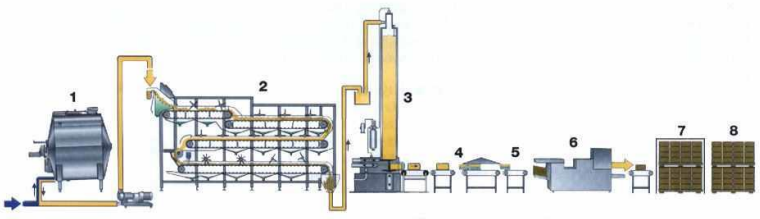


Fig. 14.35 Flowchart for mechanized production of Cheddar cheese.

1 Cheese vat
2 Cheddaring machine
3 Block former and bagger
4 Vacuum sealing
5 Weighing
6 Carton packer
7 Palletizer
8 Ripening store

Milk
Curd/cheese

The Cheese matrix-physicochemical and microbial considerations for probiotic delivery

Diarmuid (JJ) Sheehan

Teagasc Food Research Centre Moorepark, Ireland

Chemical composition

	Sample location	Moisture	MNFS	Fat	FDM (% w/w)[2]	Salt	S/M	pH
S. thermophilus	Outside	35.82^{C}	$51.54^{B,C}$	29.39^{A}	45.80^{C}	2.92^{A}	**7.92^{A}**	**5.52^{A}**
	Inside	$37.74^{A,B}$	$53.26^{A,B}$	29.61^{A}	47.39^{A}	0.55^{B}	1.33^{B}	**5.59^{A}**
L. helveticus	Outside	34.43^{C}	49.34^{C}	30.23^{A}	45.83^{C}	2.65^{A}	**8.12^{A}**	**5.25^{B}**
	Inside	38.53^{A}	54.72^{A}	29.14^{A}	47.02^{A}	0.50^{B}	1.14^{B}	**5.29^{B}**
ST/LH 22% brine	Outside	34.81^{C}	49.89^{C}	29.83^{A}	45.81^{C}	2.71^{A}	**8.21^{A}**	**5.27^{B}**
	Inside	38.02^{A}	$53.25^{A,B}$	29.19^{A}	$46.93^{A,B}$	0.59^{B}	1.55^{B}	**5.21^{B}**

Enumeration of bacteria in cheese

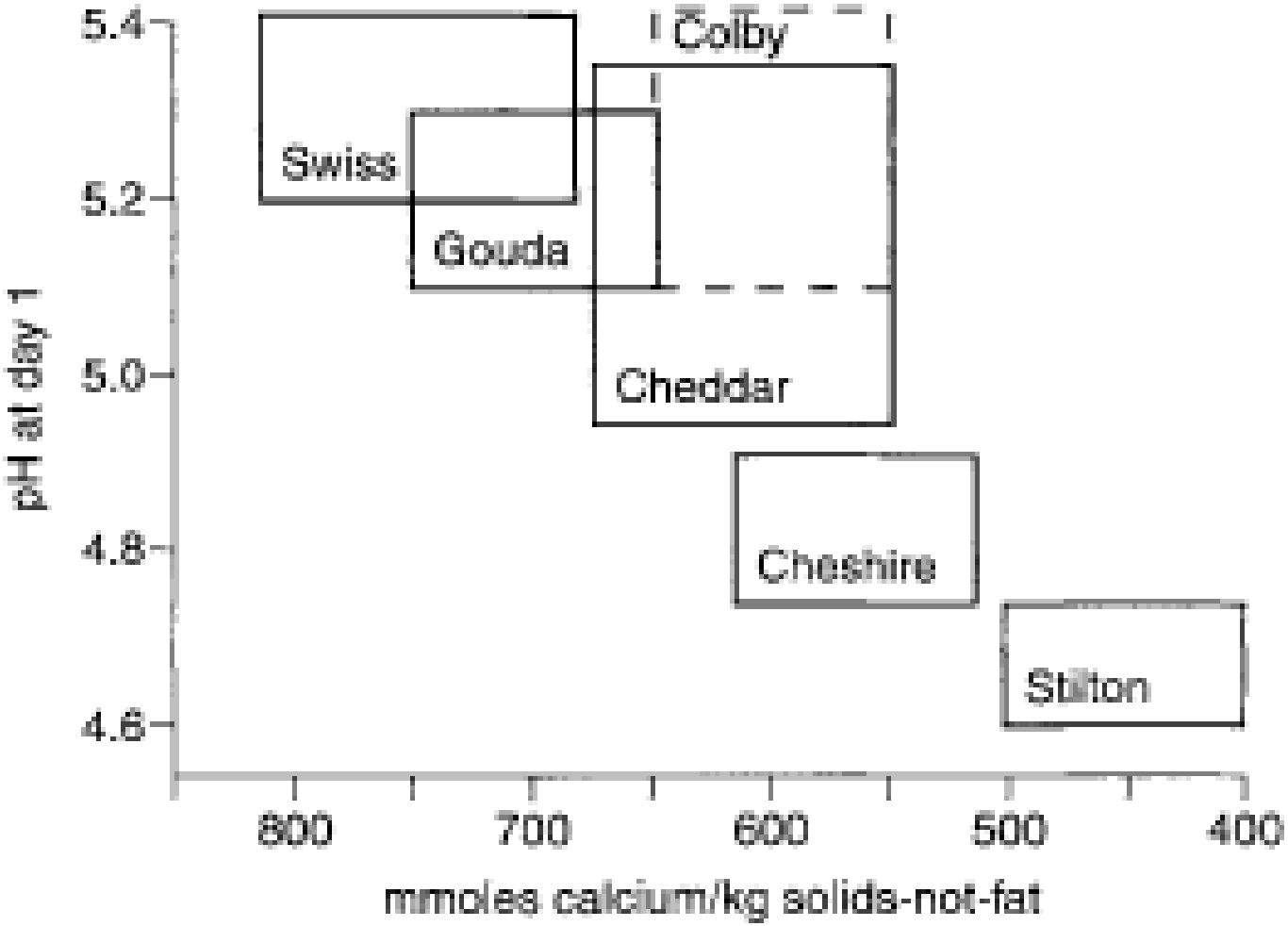
- Hypothesized that the addition of specific probiotic bacteria to cheese during manufacture modifies starter and NSLAB lactobacilli survival in Cheddar cheese at different fat levels
- Assessed whether addition of probiotics at levels below that of starter bacteria altered starter or NSLAB levels.
- The viability of the three groups of bacteria was determined using propidium iodide-based qPCR assays

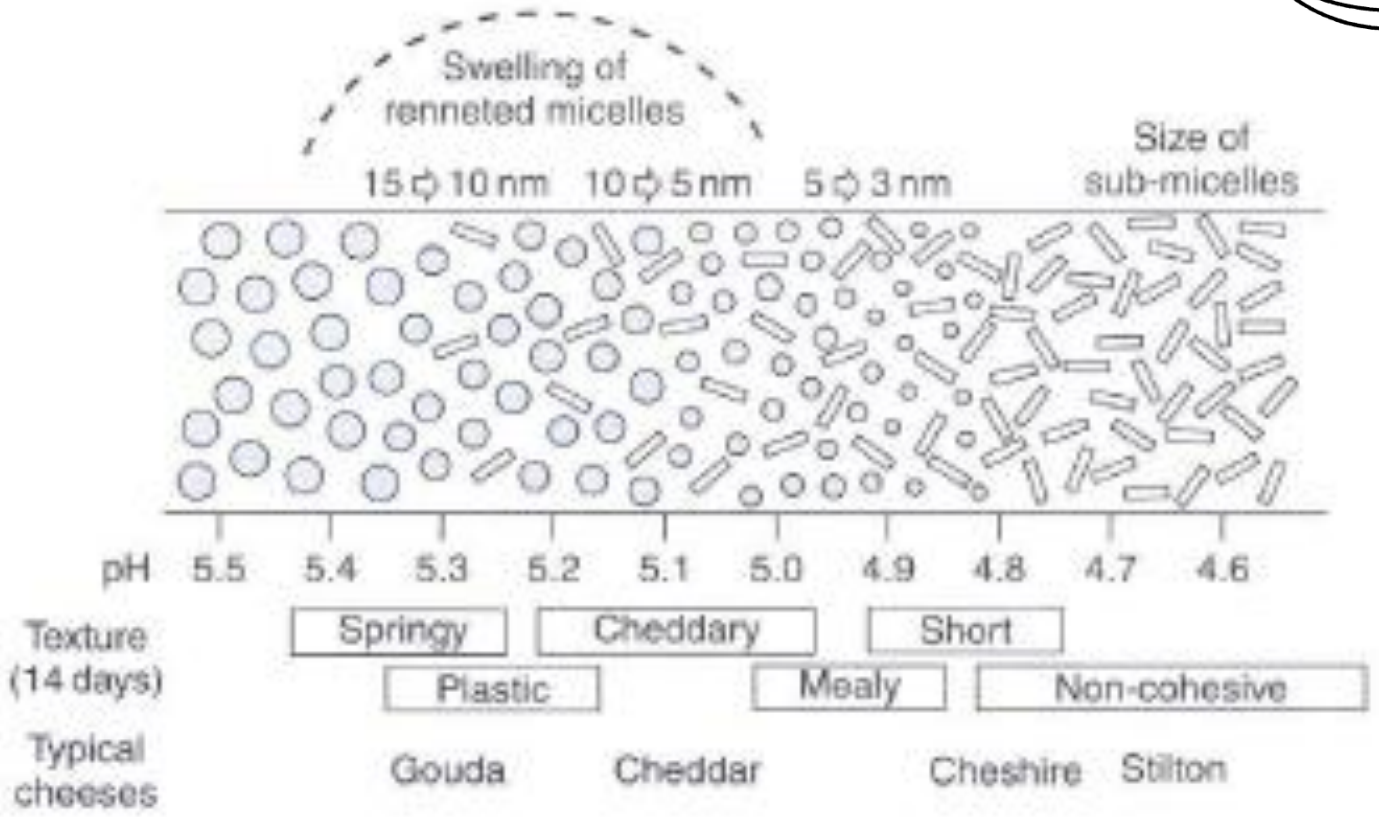
Possible explanations to variable growth w.r.t fat content

- One explanation is lower **salt-in-moisture** (3%) in full-fat cheese compared to reduced- or low-fat cheeses (4%) that may allow **starter bacteria** to metabolize **lactose faster, leading to sugar starvation and further, an earlier shift into the nonculturable state** (Ganesan *et al*. 2007).

- The **nondividing lactococci may hence be a lesser challenge to the added lactobacilli**, whereas the later the lactose reduction, starter nonculturability is delayed and so is growth of lactobacilli. However, this explanation only fits the increase in levels of strains CRL-431 and F19, and not L26, which survived better in full-fat cheese.

- **Additional genes** in the genomes of lactobacilli outside the common core set (Makarova *et al*. 2006) may be involved in the ability of probiotic lactobacilli to survive differently in cheese.


5.4
5.2
5.0
4.8
4.6
pH at day 1
Colby
Swiss
Gouda
Cheddar
Cheshire
Stilton
800
700
600
500
400
mmoles calcium/kg solids-not-fat



Diagrammatic representation of the effect of the pH on the microstructure and texture of cheese.