Doctoral Thesis

Impact of different packaging films on smear microflora composition, metabolic activity and quality of red-smear cheeses during cold storage

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IMPACT OF DIFFERENT PACKAGING FILMS ON SMEAR MICROFLORA COMPOSITION, METABOLIC ACTIVITY AND QUALITY OF RED-SMEAR CHEESES DURING COLD STORAGE

A thesis to attain the degree of

DOCTOR OF SCIENCES of ETH ZURICH

(Dr. sc. ETH Zurich)

presented by

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2014
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Abbreviations

AA  Amino Acids
AP  Active Packaging
bp  Base pair
CaCl$_2$  Calcium Chloride
cDNA  Complementary DNA
cfu  Colony forming units
cn  Copy numbers
CO$_2$  Carbon dioxide
DNA  Deoxyribonucleic Acid
EVOH  Ethylene Vinyl alcohol
FAHA  Facultative Anaerobic Halophilic and Alkaliphilic
H$_2$O  Water
LAB  Lactic Acid Bacteria
LDPE  Low-density Polyethylene
LLDPE  Linear LDPE
MAP  Modified Atmosphere Packaging
mgo  malate quinone oxidoreductase gene
mRNA  Messenger Ribonucleic Acid
NH$_3$  Ammonia
NSLAB  Non Starter Lactic Acid Bacteria
NPN  Non Protein Nitrogen
PCR  Polymerase Chain Reaction
PA  Polyamide
PE  Polyethylene
PLA  Polylactide, Poly Lactic Acid
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<tr>
<td>PMA</td>
<td>Propidium Monoazide</td>
</tr>
<tr>
<td>PP</td>
<td>Polypropylene</td>
</tr>
<tr>
<td>PVDC</td>
<td>Polyvinylidene chloride</td>
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<td>qPCR</td>
<td>Quantitative PCR</td>
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<tr>
<td>RH</td>
<td>Relative Humidity</td>
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<tr>
<td>rRNA</td>
<td>Ribosomal Ribonucleic Acid</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse Transcription PCR</td>
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<tr>
<td>SLAB</td>
<td>Starter Lactic Acid Bacteria</td>
</tr>
<tr>
<td>TN</td>
<td>Total Nitrogen</td>
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<tr>
<td>TTGE</td>
<td>Temporal Temperature Gradient gel Electrophoresis</td>
</tr>
<tr>
<td>VBNB</td>
<td>Viable But Not Culturable</td>
</tr>
<tr>
<td>v-qPCR</td>
<td>Viability quantitative PCR</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per volume</td>
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<tr>
<td>WSN</td>
<td>Water Soluble Nitrogen</td>
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<tr>
<td>w/w</td>
<td>Weight per weight</td>
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Summary

Red-smear cheeses such as Appenzeller, Gruyère, Tilsit and Tête de Moine are extensively produced and of great economic importance in Switzerland. The packaging of fully ripened red-smear cheese portions for transportation and sale in self-service shelves is a practice more and more done due to retailers logistic and consumer’s convenience demand. The so far unsolved problem of a defective, wet, smudgy and off-odorous smear is known since red-smear cheeses are film-prepacked. Therefore, the purpose of this thesis was to investigate the cheese surface defect and to maintain the high quality of film-prepacked red-smear cheese portions by development of new strategies to control the smear microflora.

Two hypotheses were formulated for cheese surface defect development. The first claimed a single microorganism or a shift in smear-microbiota composition due to changing conditions through film-packaging and subsequent storage to be responsible for cheese surface defect. The second hypothesis claimed a changed activity and/ or metabolism of the microbiota due to the switch to anaerobiosis caused by film packaging.

To investigate the problem of smear defect in film-prepacked cheese portion the microbial composition of 47 cheese surfaces of two Swiss varieties M and T were analyzed in a polyphasic approach using culture dependent and culture independent methods (Chapter 2). The cheese smear of variety M was characterized by 27, variety T by 29 microbial species of which 11 and 12 represented typical species, belonging to Arthrobacter, Brachybacterium, Brevibacterium, Corynebacterium, Microbacterium and Staphylococcus in a range of $10^7$-$10^8$ cfu cm$^{-2}$. Further cheese smear isolates were members of Bacillus ($10^7$-$10^8$ cfu cm$^{-2}$), Citrobacter (>10$^5$), Enterococcus ($10^3$-$10^6$ cfu cm$^{-2}$), Facklamia ($10^7$-$10^8$ cfu cm$^{-2}$), Morganella ($10^3$-$10^5$ cfu cm$^{-2}$), Proteus ($10^3$-$10^5$ cfu cm$^{-2}$) and Vagococcus ($10^7$-$10^8$ cfu cm$^{-2}$). An unexpected stable microbial composition was revealed for all surface smear samples independent from the cheese production plant, packaging technology and time point of storage for both variety M and T. A hypothesis of a rather complex etiology of the observed
smear defect was stated, where more than a single factor seems to contribute to the development of the defect during storage. According to this study a metabolic shift of the smear microflora due to the change of environmental factors within a vacuum package was rather assumed than a dramatic change in microbial composition. Therefore 21 cheese surface of each variety were analyzed in a storage test either wrapped in standard film or in one of the four alternative packaging films differing in gas and water vapor permeability (Chapter 3). Environmental factors such as redox potential, water activity and pH development were measured and showed defective smear to be associated with anaerobic conditions, an increase of water activity (0.005-0.01) and a pH drop (0.5-1.3) over 8 weeks at 8°C. Microbial growth and activity behavior were measured using qPCR and RT-qPCR. A decrease of 0.7-1.6 log total bacteria, 0.6-1.2 log C. casei and 0.6-1.6 log Bacillus spp. 16S rRNA copy numbers measured by RT-qPCR monitored for both varieties under packaging conditions suggested a drop of metabolic activity in these microbial groups. Cheese portions wrapped in three out of the four films developed a defect giving evidence that packaging cheese in plastic film is generating the problem of a wet, smudgy and off-odorous smear. These experiments demonstrated that the film properties influence the physicochemical characteristic of the cheese smear and consequently the cheese smear microflora. A better quality smear combined with aerobic conditions and a high pH but with the drawback of mold growth was achieved in alternative film D. A combination of film D with mold growth inhibition, oxygen control or oxygen tight film was suggested as a promising approach. These goals were approached experimentally by mold growth prevention by application of an antifungal component and biopreservative measures by addition of a protective culture containing Lactobacillus paracaseii and Propionibacterium jensenii in combination with previously promising film D (Chapter 4). Further, approaches of cheese surface defect prevention as drying the cheese loaves before cutting and packaging, dry salt addition on the cheese surface and a further packaging film E in combination with and without an antifungal component were tested. A satisfying dry, sparse, nice colored and non-off-odorous smear
could only be achieved by the low-gas and high-water vapor permeable film E. This promising film was shown to prevent cheese smear defect in a challenge test for variety T and in a repetition and an industrial scale up trial for both varieties T and M (Chapter 5). A high impact of packaging on metabolic activity of the cheese surface microflora was observed by the significant decrease of the protein coding *mqo* gene transcripts of *C. casei* in standard film additionally to 16S RNA. A significant drop of living bacterial cells of 1.2 log for *C. casei* and 0.9 log for *Bacillus* measured by viability qPCR (v-qPCR) combined with enhanced proteolysis over storage in defective smear suggested a high influence of dying cells by cell lysis and release of intracellular enzymes on cheese defect development. The alternative film created a better environment for cheese smear microflora compared to standard film exhibited in micro-aerobic conditions instead of anaerobic conditions and a diminished pH drop resulting in a smaller decrease of living bacterial cells shown for *C. casei* and *Bacillus* spp. combined with a slower proteolysis. The contribution of more than one single factor to cheese smear defect development could be demonstrated by this study. The influence of intrinsic factors as pH or redox potential on the cheese smear microflora and the impact of the cheese smear microbiota on defect development could be proofed. The cheese smear wrapped in alternative film E was significantly better evaluated by potential consumer with respect to cheese smear humidity, smearyness and appearance and allowed a convenient handling of the cheese. Film E represents a possible prevention solution for red-smear cheese surface defect ready for industrial application ideally for whole and half cheese loaves.
Zusammenfassung

Rotschmierkäse wie Appenzeller, Gruyère, Tilsiter und Tête de Moine sind in der Schweiz ein bedeutender wirtschaftlicher Faktor und werden in grossen Mengen produziert. Für den Transport oder um als vorverpackte Käseportionen in das Selbstbedienungsgestell im Supermarkt zu gelangen, werden immer mehr komplett gereifte Rotschmierkäse portioniert und in Folien verpackt. Dabei entsteht eine defekte Schmiere, die sich als nasse, schmierige und geruchsintensive Oberfläche äussert. Dieses Problem ist bekannt, seit Rotschmierkäse in Folien verpackt werden.

Das Ziel dieser Doktorarbeit war die Untersuchung des Käseoberflächendefekts und die Erhaltung der hohen Qualität von in Folien verpackten Rotschmierkäseportionen durch neue Strategien um die Mikroflora der Schmiere zu kontrollieren. Zur Entwicklung des Käseoberflächendefekts wurden zu Beginn zwei Hypothesen aufgestellt. Gemäss der ersten Hypothese ist ein Mikroorganismus oder eine Verschiebung in der Zusammensetzung der Schmieremikroflora als Folge der veränderten Bedingungen durch das Verpacken in Folie und der anschliessenden Lagerung für die Entwicklung des Defekts verantwortlich. Der zweiten Hypothese nach entsteht der Defekt durch eine veränderte Aktivität und/oder Metabolismus der Mikroorganismen, ausgelöst durch den Wechsel zu anaeroben Bedingungen durch das Verpacken in Plastikfolie.

Um den Defekt der Käseoberfläche zu untersuchen, wurde die Zusammensetzung der Mikroorganismen von 47 Käseoberflächen der beiden Schweizer Käsesorten M und T in einem polyphasischen Ansatz mit Kultur abhängigen und Kultur unabhängigen Methoden analysiert (Kapitel 2). Die Käseschmiere der Sorte M wurde durch 27, die Sorte T durch 25 Arten charakterisiert, von welchen 11, respektive 12, typischen Arten entsprechen. Zu den typischen Käseschmierearten wurden Arthrobacter, Brachybacterium, Brevibacterium, Corynebacterium, Microbacterium und Staphylococcus gezählt, die in $10^7$-$10^8$ KBE cm$^{-2}$ vorkamen. Weitere Käseschmiere-Isolate gehörten zu Bacillus ($10^7$-$10^8$ KBE cm$^{-2}$),
Zusammenfassung

Citrobacter (>10^2 KBE cm^2), Enterococcus (10^3-10^6 KBE cm^2), Facklamia (10^7-10^8 KBE cm^2), Morganella (10^3-10^5 KBE cm^2), Proteus (10^3-10^5 KBE cm^2) und Vagococcus (10^7-10^8 KBE cm^2). Für beide untersuchten Sorten M und T konnte eine unerwartet stabile Zusammensetzung der Mikroorganismen unabhängig von der Produktionsstätte, Verpackungstechnologie und Zeitpunkt der Lagerung aufgezeigt werden. Darauf wurde die Hypothese aufgestellt, nach der eher eine komplexe Zusammensetzung von verschiedenen Faktoren als einem Einzelnen zum Schmieredefekt während der Lagerung beitragen. In dieser Studie wurde eher eine Verschiebung der metabolischen Aktivität durch die anaeroben Bedingungen in der Verpackung als Grund für die Entstehung der defekten Schmieremikroflora gesehen als eine Verschiebung in der Zusammensetzung der Mikroorganismen.

Deshalb wurden 21 Käseportionen von jeder Sorte in Standardfolie oder in eine der vier alternativen Folien verpackt und in einem Lagerungsversuch getestet. Die vier alternativen Folien unterschieden sich in der Gas- und Wasserdampfdurchlässigkeit (Kapitel 3). Umweltfaktoren, wie das Redox Potential, die Wasseraktivität und die pH-Entwicklung wurden gemessen. Die Defektentwicklung konnte während der achtwöchigen Lagerung bei 8°C eindeutig mit anaeroben Bedingungen, einer Zunahme der Wasseraktivität um 0.005-0.01 und einer pH-Abnahme um 0.5-1.3, in Verbindung gebracht werden. Das Verhalten des Wachstums und der Aktivität der Mikroorganismen wurde mit quantitativer PCR (qPCR) und Reverstranskriptions-qPCR (RT-qPCR) gemessen. Die Abnahme von 0.7-1.6 log der gesamten Bakterien, 0.6-1.2 log von C. casei und 0.6-1.6 log der Bacillus spp. 16S rRNA Kopien Zahl, gemessen mit RT-qPCR - für in Folien verpackte Portionen beider Sorten - deutet auf einen starken Abfall der metabolischen Aktivität dieser Gruppen der Schmieremikroflora hin. Ein Defekt entwickelte sich in drei der vier getesteten alternativen Folien und deutet darauf hin, dass das Verpacken in Folie an sich problematisch ist. Mit dieser Studie konnte aufgezeigt werden, dass die Eigenschaften der Folien die physikalischen und chemischen Parameter der Käseschmieremikroflora beeinflussen. Eine bessere Qualität der Oberfläche verbunden mit aeroben Bedingungen und einem pH Wert um 8 wurde in der alternativen Folie D
Zusammenfassung


auf die Defektentwicklung konnte bestätigt werden. Potentielle Konsumenten beurteilten die in Folie E verpackten Käseoberflächen in Bezug auf die Feuchtigkeit, Schmierigkeit und allgemeine Erscheinung signifikant besser als die in Standardfolie verpackten. Der Umgang mit der in Folie E verpackten Käseportionen wurde als unproblematischer empfunden. Mit der Folie E steht eine Lösung für die industrielle Anwendung zur Bekämpfung für das Problem des Schmieredefekts für ganze und halbe Käselaibe bereit.
Chapter 1

General introduction
1. Cheese

1.1. Background

Cheese is the generic name for a group of fermented milk-based food products produced worldwide in around 1000 varieties differing in aroma, texture and form (Jany & Barbier, 2008). Cheese manufacture is essentially a method for preserving the nutritive value of milk through fermentation, removal of moisture and addition of salt (Beresford, Fitzsimons, Brennan, & Cogan, 2001) by using the four basic ingredients: milk, rennet, salt and microorganisms for most cheeses (Beresford, et al., 2001; Jany & Barbier, 2008). It is the most diverse group of dairy products, academically interesting and scientifically challenging because unlike many other foods, which are relatively biologically and physically stable, cheeses are dynamic and therefore unstable (McSweeney, 2007a). Cheese making dates back to some 8000 years ago originating from the Middle East where the first fermented milk-based foods were made. Long before 20th century smear cheeses have already been produced without knowledge of the bacterial nature of the surface flora (Bockelmann & Hoppe-Seyler, 2001). In Switzerland cheese is an important comestible, almost half of the Swiss milk is processed into cheese of which more than 450 varieties are produced. Cheese consumption per capita achieved a new maximum with 7.2 kg fresh, 2 kg soft, 6.1 kg semi-hard and 4.6 kg hard cheeses in 2010 (Schweizer Bauernverband, 2013). Furthermore, the export of Swiss Cheese is playing a more and more important role since the last years (Bundesamt für Statistik (BFS), 2013).

1.2. Cheese production process

The milk for cheese making comes from cows, sheep, goats and buffaloes. As the main ingredient, its quality and preparation are of vital importance. Hygienic milk harvesting, refrigeration and gentle handling are essential features of milk harvesting and transport to the factory. An important issue affecting the final product is the control of the microbiology of the cheese milk, and there is an ongoing vociferous debate on the merits of raw milk cheese
versus pasteurized milk cheeses (Bennett & Johnston, 2004). If raw milk is to be stored for a long period before use, thermisation (typically below 66°C/variable time) is performed to prevent the growth of psychrotropic organisms and their associated production of lipases and proteinases. The milk enters the cheese vat, typically at 32°C and is mixed by stirring (Fig. 1). Starter cultures, typically lactic acid bacteria, are added. The pH reduction during manufacture due to production of lactic acid from lactose, and the biochemical and physical changes during the curing or ripening phase after manufacture of the initial cheese curd are the primary roles of the microorganisms (Settanni & Moschetti, 2010). Starter adjunct are organisms with the primary role of which is post-initial manufacture. Starter and adjunct cultures are commonly mixed into the milk in the cheese vat, although for some varieties, such as smear-ripened cheese, the formed cheese may be inoculated with the culture (Fig. 1). The most fundamental step in cheese making process occurs in the cheese vat and involves the conversion of the liquid milk into a semi-solid gel. Subsequent syneresis, or shrinkage and loss of whey from this gel, results in the formation of cheese curd. Coagulation involves the aggregation of the casein and is normally achieved by addition of a coagulant to the milk in the vat stage of manufacture, although it can also be accomplished by pH reduction through acidification for some varieties. Rennet, derived from the abomasum of young milk-fed calves, in which the principal active ingredient is chymosin, has been traditionally the coagulant of choice (Bennett & Johnston, 2004). Nowadays, chymosin is also microbially produced with the aid of genetically modified organisms (GMO) like Aspergillus niger or Kluyveromyces lactis and marketed as Chy-Max (Chr Hansen, Horsholm, Denmark) and Maxiren (DSM, Delft, Netherlands) or as GMO-free product in fermentation based production Chy-Max-M (Chr Hansen). Calcium chloride (0-0.5 g L\(^{-1}\) CaCl\(_2\)) is added to the cheese milk for coagulation assistance, cheese making process improvement and yield increase (Ong, Dagastine, Kentish, & Gras, 2013). Once a satisfactory coagulum has been formed, usually after 30-40 min, the gel is cut into cubes to encourage moisture expulsion (syneresis). In most cheese making processes, the curd/whey mixtures are heated “cooked” to a higher temperature while lactose is fermented by the starter bacteria and acid is
produced. The cooking process has an important role in controlling syneresis by influencing curd shrinkage and acid development. After cooking the curd/whey mixture is stirred until the drain pH is reached and curd/whey separation is initiated by pumping out the vat’s contents of curds and whey into molds. The cheese mold is a specialized container designed to hold and form the curd into the desired shape, permitting the further loss of whey and the application of pressure. After the cheese loaf is formed in the mold brining is performed. Brine salting basically involves the immersion of the cheese loaf in a brine bath, a solution containing 19-21 % w/w NaCl and appropriate level of CaCl$_2$ (e.g. 0.2%, w/w) for semi-hard and hard cheeses. Its pH should be close to the cheese pH (typically 5.2-5.3) and its temperature should be 10-18°C for many cheeses. The salt concentration of the brine must be maintained as salt moves into the cheese and water/whey moves out, causing dilutions (Bennett & Johnston, 2004; Bockelmann, 2007). Once the cheese has been brined for the required period, it is stored for ripening under defined conditions and in case of red-smear cheeses is brushes with saline and bacterial culture mixture regularly. The most widely accepted approach for classification of cheeses is based on moisture content, with further division depending on milk type and the role of microorganisms in cheese ripening (Little, et al., 2008). According to this classification cheeses are soft, semi-hard or hard cheeses made with raw or pasteurized milk and fresh, mold, mold-surface or bacterial surface-ripened.
1.3. Cheese ripening and red-smear formation

Bacterial surface ripened cheeses develop their characteristic flavor and red, glistening appearance during ripening of the cheese. After brining, smear cheeses are either deliberately inoculated with commercial preparations containing different combinations of *Brevibacterium linens*, *Arthrobacter arilaitensis*, *Corynebacterium variabilis*, *Debaryomyces hansenii* and/or *Geotrichum candidum* (Bockelmann & Heller, 2010; Winkler, Jakob, & Amrein, 2008) or are old-young smeared. The traditional old-young smearing, young cheeses are smeared or washed with smears from older cheeses, ensures that microorganisms required for surface ripening are transferred to young cheeses (Brennan, 2004). The main disadvantage of this approach is the contamination of all young cheeses with undesired contaminants, such as *Listeria* (Goerges, et al., 2011; Roth, Schwenninger,
Hasler, Eugster-Meier, & Lacroix, 2010; Schoder, Skandamis, & Wagner, 2013), Staphylococcus aureus or Gram-negatives, if they are present on the surface of the old cheese (Bockelmann, 2002, 2007; Bockelmann & Heller, 2010; Rademaker, Peinhopf, Rijnen, Bockelmann, & Noordman, 2005). Limited biodiversity is rather the disadvantage of controlled inoculation (Bockelmann, Hoppe-Seyler, Krusch, Hoffmann, & Heller, 1997; Grattepanche, Miescher-Schwenninger, Meile, & Lacroix, 2008), but the use of cultures could be shown to assist in fast smear development without old-young smearing (Bockelmann & Heller, 2010). Further, it could be shown that the use of cultures did not provoke an imbalance of the adventitious house flora and the “house flora microorganisms” were found on the cheese smear along the species from the commercial cultures (Bockelmann & Heller, 2010). Typical source of the adventitious smear microorganisms are cheese milk, cheese brines, the air of ripening rooms, ripening shelves and the human skin (Bockelmann, 2007). Before ripening, the pH of the cheese surface is about 5.0 and cheeses are ripened at high relative humidity (RH >90%) at temperatures of 12-14°C. These conditions result in rapid development of the smear, which is also supported by spreading the microcolonies which develop on the surface with a brush dipped in brine or automated brushing machine (Bockelmann, 2007). Distribution of the smear is vital, as rapid spreading of the microorganisms of the cheese surface ensures uniform ripening and reduces the risk of undesired contaminants, colonizing the cheese surface. As a result a uniform bacterial smear develops giving red-smear cheeses their typical red, glistening appearance.

Red-smear cheeses are made using lactic acid bacteria (LAB) as starters (SLAB); their flavor is determined primarily by the growth of the surface microflora. The biochemical activity of the surface flora results in the development of a cabbagy, garlicky or putrid flavor during ripening, mainly due to the production of volatile aromatic sulphur compounds originating from cysteine and methionine, particularly methanthiol, α-ketobutyrate and ammonia (Bockelmann, 2007). B. linens might be the most well-known producer of aroma compounds, but also other smear bacteria such as Corynebacterium glutamicum, Arthrobacter nicotianae,
Staphylococcus equorum and Micrococcus luteus generate aromatic sulphur compounds to a lower but still significant extent (Bockelmann, 2007; Brennan, 2004). The microbial composition of the smear of these cheeses is dominated by halotolerant yeasts mainly Debaryomyces hansenii, Debaryomyces candidum and Geotrichum candidum, begin to grow on the cheese surface and cause a pH increase by metabolizing lactate into CO$_2$ and H$_2$O (Bockelmann & Hoppe-Seyler, 2001; Brennan, 2004; Mounier, Monnet, Jacques, Antoinette, & Irlinger, 2009). This deacidification favors the growth of a complex Gram-positive bacterial flora comprising various coryneform bacteria including Micrococceae, Brevibacteriaceae and Corynebacteriaceae (Brennan, 2004; Roth, Schwenninger, Hasler, Eugster-Meier, & Lacroix, 2010). Also marine bacteria like Facklamia spp. and Marinilactibacillus spp. known as facultative anaerobic halophilic and alkaliphilic bacteria (FAHA) have been detected on cheese surface (Feurer, Irlinger, Spinnler, Glaser, & Vallaeya, 2004; Ishikawa, et al., 2007; Roth, Schwenninger, Hasler, Eugster-Meier, & Lacroix, 2010; Roth, Schwenninger, Eugster-Meier, & Lacroix, 2011). Gram-positive bacteria belonging to the genus Arthrobacter, Brachybacterium, Brevibacterium, Corynebacterium, Microbacterium, Micrococcus and coagulase-negative Staphylococcus represent desired smear microorganisms, whereas enterococci that are frequently found in cheese smear are assumed to represent hygiene indicators. Gram-negative Halomonas spp. and Vibrio spp. in cheese were reported and advanced the hypothesis that these bacteria might be indicative of hygienic problems (Maoz, Mayr, & Scherer, 2003), but since this study Gram-negative bacteria have been isolated from other traditional European cheeses, including farmhouse and industrially-produced cheeses (Feurer, et al., 2004; Ishikawa, et al., 2007; Mounier, et al., 2005). Therefore, they may be considered as component of the cheese surface microbiota originating from the adventitious microflora of the cheese-making environment. Some of the cheese smear microorganisms are pigmented, which leads to the characteristic red-orange color of smear cheeses. B. linens contribute to color and aroma formation, proteolysis and lipolysis (Ghosh, Bockelmann, & Heller, 2009; Valdés-Stauber, Scherer, & Seiler, 1997). B. linens is widely used in smear inocula for red-smear cheeses but recent
research has suggested that this organism is usually a minor component of the smear flora (McSweeney, 2007b). In German Tilsit cheese the development of red color could be promoted by mixed-cultures of yellow Arthrobacter strains and B. linens (Bockelmann, et al., 1997). A study on three different French red-smear cheeses (Epoisses, Mont d’Or and Maroilles) demonstrated in pigment fingerprints that the yellow-red-brown coloration came from three groups of pigments; group I produced by yellow bacteria like Micrococcus gubbenense and Arthrobacter spp., group II aromatic carotenoids (isorenieratene, 3-hydroxy-isorenieratene, 3,3-di-hydroxy-isorenieratene) produced by B. linens and related species, and group III β-carotene derived from milk (Galaup, et al., 2007). Most pigments observed were in common, but the relative composition of these pigments allowed obtaining a different and specific fingerprint. The pigments of B. linens were only found in one of the three cheeses indicating that B. linens are not solely responsible for the color of the smear (Galaup, et al., 2007). The deacidification by the yeast increasing the surface pH from ≤5 to >6.5 not only enables the desirable bacteria to grow but also enhances the action of enzymes (Gripon, 1997) and further modifies the rheological properties of the cheese. The pH of the surface also increases during ripening due to the catabolism of lactate by yeast and the production of NH₃ through deamination of amino acids by the surface microorganisms (Brennan, 2004; Mounier, et al., 2009). Cheese ripening involves a very complex set of biological, biochemical and chemical reactions which can be classified into four groups: glycolysis and the catabolism of lactic and citric acids, lipolysis and the catabolism and modification of fatty acids, proteolysis and catabolism of amino acids and the interaction between the products of the previous reactions (Fig. 2). These reactions are catalyzed by living microorganisms or enzymes derived from milk, coagulant, primary starter, adjunct cultures, non-starter lactic acid bacteria (NSLAB) and adventitious microflora (Fox & Cogan, 2004; Settanni & Moschetti, 2010). The main contribution to cheese ripening is played by microbial enzymes released by SLAB, NSLAB and other microorganisms naturally present in milk or added by the cheese maker as the milk enzymes, mainly plasmin and acid protease acting on β- and αS1-casein, find unfavorable pH and rennet enzymes are
inactivated during curd cooking and are inhibited by salt (Settanni & Moschetti, 2010).
Proteolysis is of main importance for the final texture and flavor of the cheese. LAB possess
a complex proteolytic enzymatic system and play the main role in degradation of casein and
peptides, leading to free amino acids (FAA). The fundamental features that influence the
dynamics of the cheese ecosystem are physical parameters such as moisture, salt
concentration, pH and redox potential which change during manufacture and ripening and
are themselves influenced by the microflora and biological features such as those resulting
from the interactions between microorganisms (Beresford, et al., 2001; Jany & Barbier, 2008;
Ndoye, Rasolofo, LaPointe, & Roy, 2011). Bacterial counts during ripening can exceed $10^9$
cm$^{-2}$ while those of yeasts are generally $\sim 10^7$ cm$^{-2}$ (Bockelmann, 2007).
Fig. 2. Lactose catabolism and glycolysis (A), lipolysis (B) and casein degradation and proteolysis (C) during cheese ripening (adapted from Bennet & Johnston, 2004). Rennet is added in the beginning of cheese making and plasmin derives from the milk, starter lactic acid bacteria (SLAB), non-starter lactic acid bacteria (NSLAB) and the surface smear microflora are contributing to cheese ripening of red-smear cheeses.
1.4. Factors influencing cheese quality

The typical traits of rennet-coagulated cheeses develop mainly during ripening and frequently depend on the growth of a secondary microflora, which are not always reproducible. During ripening, a complex array of microbiological, biochemical and chemical reactions occur and therefore also many opportunities for problems to develop (Fox & Cogan, 2004). There are several aspects to quality of the cheese like safety from a public health viewpoint, nutritional, flavor, texture and appearance. Cheese must conform to the expected characteristics of the variety although a certain degree of variability is tolerated and acceptable. Consumers expect that a product will be reproducible and consistent between batches and over time with respect to flavor, texture, appearance and functionality, especially for the principal industrial produced varieties, some variability is tolerated, perhaps even expected in artisanal cheeses (Fox & Cogan, 2004).

Various quality aspects are introduced starting from the raw material to the end product of fully ripened cheese. The quality of the milk and its possible contamination with spoilage microorganisms and pathogens is a crucial aspect of all cheeses (Bennett & Johnston, 2004; Little, et al., 2008), although high moisture, raw-milk cheeses are of more concern but most of these have a low pH (4.6) after ripening and appear to be safe. It is probably significant that raw-milk cheeses are made on a small/very small scale from very fresh milk from healthy cows (Fox & Cogan, 2004). Pasteurized milk brings the advantage of killing spoilage microorganisms like coliforms, pseudomonads and yeasts preventing off-flavor and spoilage. Some of the adventitious microorganisms in raw milk, especially the NSLAB, probably contribute positively to cheese flavor (Little, et al., 2008; Settanni & Moschetti, 2010). It is generally accepted that the flavor of raw-milk cheese is more intense, although more variable, than that of pasteurized milk cheese (Fox & Cogan, 2004).

Salting, one of the classical methods for food preservation, operates by reducing the water activity ($a_w$) of the product. Probably all cheeses are salted by one of the four methods either by mixing dry salt with milled or chipped curd (e.g. Cheddar-type cheese), brine salting of the molded/pressed cheese through what NaCl diffuses into the cheese in response to the
difference in osmotic pressure between the brine and the aqueous phase of the cheese (e.g. Swiss red-smear cheeses), surface application of dry salt to the surface of pressed cheese (e.g. Blue cheeses) or salting of cheese milk performed for few varieties (e.g. Domiati) (Fox & Cogan, 2004). The principal effects of salt in cheese are an inhibitory and selective effect on the microflora, a significant effect on the activity of many enzymes and through this two an indirect effect on the ripening, flavor and quality of cheese, further a direct effect on flavor (Fox & Cogan, 2004; Guinee & Fox, 2004; Guinee, 2007).

During ripening the characteristic flavor, texture, appearance and functionality develop along lines predetermined by the microbiology and composition of the curd established during manufacturing. However, the cheesemaker can influence the rate and, to some extent, the pattern of ripening by controlling the temperature and humidity of the environment. Many cheeses develop a characteristic microflora on the surface consisting of yeast, mold and/or bacteria during ripening and this microflora has a major effect on the sensory qualities of the cheese. Traditionally this secondary microflora was adventitious, acquired from the milk and/or environment, and the growth of certain, desirable, contaminating microorganisms was promoted by selecting certain environmental conditions such as pH, temperature, humidity, oxygen concentration, salt concentration and moisture level. The adventitious was likely to be variable leading to inconsistencies of the cheese quality and is therefore replaced by selected secondary cultures in modern cheese technology, although the adventitious microorganisms may still grow (Limburger), and even dominate in some cases (French soft cheese and German Tilsit cheese) (Bockelmann & Heller, 2010; Fox & Cogan, 2004; Rademaker, et al., 2005).

The appearance of the cheese is a very important quality criterion and includes features such as depth and uniformity of color, presence or absence of mechanical opening or eyes due to gas formation and presence or absence of mold. Usually, appearance of cheese is the only attribute by which the purchaser can assess the quality of cheese and hence is of the utmost importance (Fox & Cogan, 2004). Flavor is probably the most important quality attribute for cheese consumed as table cheese although flavor and texture are strongly interactive. The
flavor of cheese is in subtle balance between several hundred compounds. Commercially, cheese quality is usually assessed by subjective sensory evaluation (Fox & Cogan, 2004). The quality of cheese is influenced by its composition especially moisture content, NaCl concentration, pH, moisture-in-non-fat substances and percentage fat-in-dry matter. Moisture content, percentage salt in moisture and pH are the key determinants of cheese quality (Fox & Cogan, 2004).

1.5. Cheese packaging

“The microbial safety and stability as well as the sensory and nutritional quality of most foods are based on an application of combined preservative factors called hurdles. The most important hurdles used in food preservation are temperature (high or low), water activity (a_w), acidity (pH), redox potential (Eh), preservatives (e.g. nitrite, sorbate, sulfite), and competitive microorganisms (e.g. lactic acid bacteria)” (Leistner, 2000). The packaging is part of the hurdle technology and the first packages used were made from contemporary natural materials available, for instance wooden boxes, potteries and baskets of reeds. Overtime, processed materials as glass and later aluminum and plastics were introduced, in future biobased packaging materials will be used as an alternative for traditional film materials but more research is being needed for evaluation of the commercial use (Peelman, et al., 2013). A packaging has to accomplish several purpose such as physical and barrier protection, transmission, information, communication, convenience and marketing. For certain cheese varieties, particularly those with an active surface microflora like bacterial surface ripened red-smear cheeses the packaging plays a critical role in controlling the ripening and post-ripening of the cheese through its moisture and gas permeability characteristics. There are several stages of packaging of many cheese varieties. Many hard cheese type made on a large industrial scale are bulk-packaged immediately after manufacture before ripening (Kelly, 2007) and ripened within a plastic film. But also packaging of fully ripened portions for distribution and sale in self-service shelves is increasingly important because consumer often purchase cheese directly in the supermarkets (Picque, et al., 2011). Increasing attention is
today being paid to packaging of cheese, and what was once regarded as an inert and passive protectant for cheese is being acknowledged as a potentially significant means of controlling ripening, quality and safety (Kelly, 2007). Red-smear cheese portions are often associated with a cheese smear defect when cut in portions and wrapped in plastic film for distribution and sale.
2. Smear microbiota analysis of red-smear cheese

2.1. Culture dependent versus culture independent methods

In cheese manufacture the composition and activity of the cheese microflora is the least controllable of all parameters and cheese microbial populations still remain difficult to control due to their complex dynamics and to their interactions (Beresford, et al., 2001; Ndoye, et al., 2011). Therefore, more knowledge of the structure and dynamics of the whole microbial community of cheese were requested for better understanding of how cheese characteristics vary with respect to microbial growth and metabolism (Jany & Barbier, 2008). Such microbial communities can be analyzed in vitro by both culture dependent and culture independent methods. Culture dependent methods consist of isolating and culturing microorganisms prior to their identification according to either morphological, biochemical or genotypic characteristics (Ercolini, Moschetti, Blaiotta, & Coppola, 2001). Cultivation is time consuming, due to labor-intensive techniques followed by long culture periods and their major bias is that the selectivity of the chosen medium directly influences the observed diversity. Numerically more abundant microbial species often out-compete species occurring in low numbers and some species may be even unable to grow under these artificial conditions. Biodiversity may consequently be underestimated or misinterpreted (Jany & Barbier, 2008). The main advantage of cultivation methods are the collection of the microorganisms, various species and strains that might be important for further scientific characterization or possible industrial application in starter-, adjunct- or protective cultures. Culture independent methods based on analysis of DNA or RNA directly extracted from the food matrix are more and more used for community-level studies. Coupled with a global analysis they are fast and potentially more exhaustive and well suited for analyzing microbial communities over time and may provide the possibility of exploring cheese microflora dynamics (Quigley, et al., 2011). Global approaches used for bacterial cheese communities are PCR- denaturing gradient gel electrophoresis (PCR-DGGE), PCR- denaturing temporal temperature gradient gel electrophoresis (PCR-TTGE), single-strand conformation polymorphism-PCR (SSCP-PCR),
terminal restriction fragment length polymorphism (T-RFLP), denaturing high-performance liquid chromatography (DHPLC) and DNA microarrays. SSCP-PCR was used for evaluation of the structure and dynamics of *Staphylococcus* population during cheese making and bacterial communities in artisanal St. Nectaire cheese (Delbès & Montel, 2005; Delbès, Ali-Mandjee, & Montel, 2007). SSCP-PCR was applied for comparison of bacterial diversity between traditional and industrial cheese (Feurer, et al., 2004) and SSCP-PCR combined with SSCP-RT-PCR for evaluation of the relationships between diversity, dynamics and activity of the bacterial community in raw-milk Salers cheese (Duthoit, Callon, Tessier, & Montel, 2005; Duthoit, Tessier, & Montel, 2005). Diversity and dynamics in Tilsit cheese was analyzed by T-RLFP (Rademaker, et al., 2005), in Sicilian cheese by PCR-DGGE and RT-DGGE (Randazzo, Torriani, Akkermans, de Vos, & Vaughan, 2002) and in Fontina PDO cheese (Dolci, et al., 2013). An optimized protocol was used to apply PCR-DGGE for *Clostridium* spp. detection associated with late blowing defects (Cocolin, et al., 2007). TGGE was used to evaluate the diversity of milk, experimental and commercial cheese (Ogier, Son, Gruss, Tailliez, & Delacroix-Buchet, 2002; Ogier, et al., 2004) as well as the diversity of *Clostridium* populations in commercial and experimental cheese (Le Bourhis, et al., 2005). Nowadays, high throughput sequencing methods are more and more important and are also applied in food microbiology for investigation of complex ecosystem as in Irish artisanal cheeses (Quigley, et al., 2012).

### 2.2. Temporal temperature gradient gel electrophoresis (TTGE)

The application of the molecular approach of temporal temperature gradient gel electrophoresis (TTGE) allows a more accurate characterization of smear cheese surface microbial diversity than only cultivation method (Mounier, et al., 2009; Ndoye, et al., 2011; Ogier, et al., 2004). A polyphasic approach in which cultivation is completed with TTGE analysis is our choice to investigate the differences between defective and non-defective red-smear cheese surface ecosystem (Fig. 3). TTGE is based on the separation of PCR amplicons of the same length but with different base pair sequences. The DNA fragments
are separated on the basis of a decreased electrophoretic mobility and partially melted double-strand DNA molecules (Le Bourhis, et al., 2005; Ogier, et al., 2004). Separation is performed in polyacrylamide gels by varying the temperature over time. Consequently, reaching the specific domain and therefore branched molecules are created and DNA mobility in the gel reduced. A GC clamp (30-40 bp) prevents the double-strand DNA from complete dissociation during TTGE analysis. As a result, amplicons of the same size can be separated based on differences in melting behavior. The banding patterns reflect the complex microflora of the smear. The bands are identified either by comparison to a previous established molecular ladder used in each gel or by direct extraction of the DNA from the acrylamide gel and sequencing. TTGE and DGGE are the most extensively used methods for the study of microbial communities in food microbiology including dairy products and more specifically to monitor the structure and even dynamics of microbial communities in cheese (Cocolin, Alessandria, Dolci, Gorra, & Rantsiou, 2013).

2.3. Quantitative real-time PCR (qPCR)

For monitoring specific microbial community members two distinct applications are quantitative PCR (qPCR) and reverse transcription quantitative PCR (RT-qPCR). The qPCR technique is used for DNA amplification, whereas the RT-qPCR is devoted to study expressed genes (RNA) which will be discussed in 2.4. The qPCR technology is based on the detection and quantification of a fluorescent marker corresponding to amplified target DNA molecules, being either a specific probe or a DNA-intercalating agent such as SYBR Green for effective detection, identification, and quantification of bacteria in different types of samples or products (Fierer, Jackson, Vilgalys, & Jackson, 2005; Monnet, Correia, Sarthou, & Irlinger, 2006). On the basis of sensitivity and precision, qPCR can solidly support the accuracy of the data obtained by methods such as T/DGGE or T-RFLP (Rasolofo, St-Gelais, LaPointe, & Roy, 2010). Additional advantages of qPCR are speed, reproducibility and small quantities of samples. The quantification limit has been estimated at $10^3$-$10^4$ cfu g$^{-1}$ in cheese (Rantsiou, Alessandria, Urso, Dolci, & Cocolin, 2008). The specificity depends entirely on the
PCR primers (Monnet, et al., 2006). The main disadvantage of qPCR is the poor distinction between viable and non-viable bacteria. *Corynebacterium casei* as model cheese microbe in cheese smear was quantified by q-PCR and proposed to use this quantification method for understanding the ecology and the functional properties of the bacteria in smear-ripened cheese (Monnet, et al., 2006).

2.4. **Reverse transcription (RT)-qPCR**

The microbial ecology and dynamics of cheese is characterized by the presence of metabolically active population that is not suitably detected by culture dependent methods. Bacteria may remain metabolically active, but not necessarily be able to divide and thus are not detected as cultivable microorganisms in growth media, also referred as viable but not culturable (VBNC) organisms (Ndoye, et al., 2011). In the last years the usefulness of reverse transcription PCR (RT-qPCR) to target RNA molecules in order to characterize microorganisms and metabolic activities that are expected during the most important phase of cheese making are understood. Reverse transcription has been used in cheese analysis coupled to other molecular tools such as RT-DGGE (Dolci, Alessandria, Rantsiou, Bertolino, & Cocolin, 2010; Dolci, et al., 2013) or RT-PCR-SSCP (Duthoit, Callon, et al., 2005) for several occasions to monitor microbial activity. The use of quantitative PCR systems coupled with the appropriate procedure of RNA extraction from the cheese matrix was recommended to better understand which of the important activities are being carried out in certain steps of cheese production (Coppola, Blaiotta, & Ercolini, 2008; Desfossés-Foucault, LaPointe, & Roy, 2013). The detection of cDNA from RNA transcripts is a nice tool to detect metabolically active organisms in cheese and therefore a measure for cell viability (Monnet, Ulve, Sarthou, & Irlinger, 2008). To control for mRNA losses during RNA extraction and inefficiencies in reverse transcription, a technique that utilizes the exogenous internal reference luciferase mRNA along with mRNA absolute standard curves was developed (Johnson, Lee, Holmes, & Alvarez-Cohen, 2005). In general for gene expression analysis a gene has to be proven to be stably expressed under given conditions and expression of other genes are normalized against this gene (Carraro, et al., 2011; Falentin, et al., 2010; Falentin, et al., 2012; Monnet,
et al., 2013). In a complex ecosystem like cheese it is not possible to proof for a stable gene expression against which normalization can be done. Therefore Monnet et al. (2013) proposed to normalize the gene expression of interest against the whole amount of RNA or against the weight of cheese. The targeting of 16S rRNA is popular although also in discussion due to its rather long stability compared to mRNA of protein coding genes with general shorter half-life time (Cenciarini, Courtois, Raoult, & La Scola, 2008). For the generation of a global picture it is still a valuable molecule to address as its multiple operons generate high abundance and therefore no problem with detection limits are expected (Desfossés-Foucault, et al., 2013; Dolci, et al., 2013). Especially if 16S rRNA transcripts of bacteria in food are detected over an extended timespan of two months they can be considered as newly synthesized (Falentin, et al., 2012). Alternatively, the metabolic activity of microorganisms can be indicated by quantification of more labile mRNA transcripts of protein encoding genes that are probably better viability markers as for their instability after the death of bacteria (Cenciarini, et al., 2008; Sheridan, Masters, Shallcross, & Mackey, 1998). The malate quinone oxidoreductase (MQO), which is also called malate dehydrogenase, is a membrane-bound enzyme that takes part in the citric acid cycle. It transfers an electron to a quinone and oxidizes malate to oxaloacetate. The reduced quinones are oxidized by the electron transfer chain. Flavin-adenine dinucleotide (FAD) acts as cofactor of MQOs (Kabashima, Sone, Kusumoto, & Sakamoto, 2013; Molenaar, Van der Rest, & Petrovic, 1998). As for Corynebacterium glutamicum the MQO was shown to be essential for the malate oxidation to oxaloacetate in the citric acid cycle (Bott & Niebisch, 2003). In many cases, this enzyme is reported to be essential for growth and especially important for the adaption to changing growth environments (Kabashima, et al., 2013). For this reason the expression of mqo gene encoding a protein seem to be a good indicator for the metabolic activity and therefore also a good indicator for the vitality of the microflora. A good correlation between malate quinone oxidoreductase transcripts detection and colony morphotype assignment of Corynebacterium casei in retail cheese, as well as a good
agreement between the abundance of mRNA and that of rRNA was demonstrated (Monnet, et al., 2013).

2.5. Propidium monazide (PMA)-qPCR

For quantifying the relative abundance of viable bacteria, the use of a DNA intercalating dye, ethidium monoazide (EMA) or propidium monoazide (PMA) is a promising strategy (Nocker, Cheung, & Camper, 2006). These dyes penetrate only into the non-viable cells with compromised membrane integrity and covalently bind to the DNA by photo-activation. In comparison to EMA, PMA proved to be more selective in penetrating only dead microbial cells. Originally it was described that the crosslinkage of PMA to DNA renders the DNA insoluble and therefore not available for further analysis step through wash out by DNA extraction (Nocker, et al., 2006). More recent studies described that the covalent bound between PMA and DNA prevents the amplification by PCR (Elizaquível, Sánchez, & Aznar, 2012; Kim & Ko, 2012). Therefore only viable bacteria cells are quantified by PMA-qPCR, also referred as viable qPCR (v-qPCR) (Fig. 3). The suitability of a PMA-qPCR assay has been confirmed by several studies using different bacterial groups and samples. Nocker et al. (2006) used Gram-negative and Gram-positive species including *Escherichia coli* for his comparative study on EMA and PMA. More recent studies applied PMA-qPCR to focus on *Bacillus subtilis* (Kim & Ko, 2012) and on foodborne pathogenic organisms like *E. coli* O157:H7, *Listeria monocytogenes* and *Salmonella* in spinach and mixed salad (Elizaquível, et al., 2012). Desfosses-Foucault et al. (2012) used PMA-qPCR to monitor probiotic viability in Cheddar cheese. They successfully demonstrated that the use of PMA-qPCR is better suited to quantify cellular viability in cheese than with cultivation and that DNA from dead cells was efficiently blocked by the PMA molecules.
Fig. 3. Polyphasic approach including cultivation and culture independent methods such as TTGE and qPCR for investigation of defective and non-defective red-smear cheese surface. RT-qPCR is used to monitor metabolic activity of cheese smear bacteria and PMA-qPCR to detect living bacterial cells over storage of film-prepacked cheese portions.
3. Cheese surface defect prevention strategies

3.1. Salt as an ancient additive

The use of salt (NaCl) as a food preservative dates from ancient times and is one of the classical methods of food preservation together with fermentation and dehydration (air/sun) (Guinee & Fox, 2004). Salt in cheese serves two major roles, it acts as preservative and contributes directly to flavor and quality. The preservative action of NaCl is due to its depressing effect on the water activity ($a_w$) of the cheese. The osmotic pressure of the aqueous phase of foods is increased by the salt, causing dehydration of bacterial cells, killing them or at least preventing their growth (Guinee, 2004).

3.2. Biopreservation and the cheese surface

In spite of modern advances in technology, the preservation of foods is still a debate issue. Amelioration of economic losses due to food spoilage, lowering the food processing costs and avoiding transmission of microbial pathogens through the food chain while satisfying the growing consumers demands for foods that are ready to eat, fresh-tasting, nutrient and vitamin rich, and minimally-processed and preserved are major challenges for the current food industry (Galvez, Abriouel, Lopez, & Ben Omar, 2007; Picque, et al., 2011).

Biopreservation can be defined as the addition of antagonistic cultures or of their metabolic products (organic acids, hydrogen peroxide, enzymes and bacteriocins) to food to inhibit pathogenic and spoilage microorganisms and/or extend shelf life while changing the sensory properties of the product as little as possible (Holzapfel, Geisen, & Schillinger, 1995; Schwenninger & Meile, 2004). Protective cultures should in the first instance be considered as additional safety factor improving the microbiological safety of food (Holzapfel, et al., 1995). Biopreservation is achieved by either application of antimicrobial metabolites without the producing strain (e.g. bacteriocin), application of an adjunct culture producing antimicrobial metabolites in situ or ex situ that does not influence food quality, or application of a technological flora harboring protective effects (Grattepanche, et al., 2008). Nisin and
Bacteriocins like pediocin and nisin are well-known bacteriocins with strong antimicrobial activity, including against bacteria outside the genus of the producer and killing a broad spectrum of Gram-positive bacteria including important pathogens (Nes, Yoon, & Diep, 2007). Nisin is used for processed cheese, salad dressings, canned foods, and meats to extend shelf life (Muppalla, Sonavale, Chawla, & Sharma, 2012). Pediocin is mainly known through its high anti-Listeria activity (Mathys, et al., 2007), but it is also proposed for inhibition of food spoilage organisms and food contaminating molds (Narayanan, Neera, Mallesha, & Ramana, 2013). A further naturally produced antimicrobial is natamycin, a fungicide with a broad spectrum against most yeasts and fungi (Vierikova, Hrnčiarikova, & Lehotay, 2013) approved by the European Food Safety Authority (EFSA) for surface treatment of semi-hard and semi-soft cheese and cured sausages (1mg dm$^{-2}$) (EFSA, 2009). The bacteriocin nisin (E234) is allowed to use in Switzerland for Mascarpone (10 mg kg$^{-1}$), for ripened cheese (12.5 mg kg$^{-1}$) and for processed cheese products only for cheese spread (12.5 mg kg$^{-1}$), natamycin (E325) is allowed for cheese surfaces treatment of extra-hard, hard and semi-hard cheeses (1mg dm$^{-1}$) and is not allowed on the surface of cut cheese (Zusatzstoffverordnung, 2007) but Swiss cheese makers of ripened cheese (extra-hard, hard-, semi-hard and soft cheese) have a trade codex not to use neither nisin nor natamycin in cheeses manufacture (Switzerland Cheese Marketing, 2007). Application of live cultures producing antimicrobial metabolites is an advantageous alternative biopreservatives for food producers in demand of natural preservation techniques, as the application of a food-grade microorganism does not need to be indicated on the product label.

### 3.3. Packaging materials for food and cheese

The preservation of cheese as it passes through the distribution networks requires an extended shelf life to remain saleable. The function of packaging material is not always as simply to act as an impermeable membrane isolating the contents from its surrounding. The requirements for cheese are particularly varying with cheese type (Barlow & Morgan, 2013). Different polymers have different permeabilities, so that packaging barrier properties can be
tailored to the product by choice of the material and film thickness. EVOH (Ethylene vinyl alcohol), PVDC (Polyvinylidene chloride) and PA (Polyamide) are the most common barrier polymer, PE (Polyethylene) and PP (Polypropylene) are used for mechanical strength, PE for attractiveness and LDPE (Low-density Polyethylene) for packaging closure by heat sealing, LLDPE (linear LDPE) is a shrink wrap, flexible film that fits closely around the product. Depending on the combination of strength, durability and barrier function food packaging films vary in thickness between about 10 µm and 250 µm. Cheese packaging is in average between 100 µm and 200 µm of which 20-30% compromise the barrier layer (Barlow & Morgan, 2013). Different materials optimized for each of these functions are combined into a single multi-layer film commonly consisting of three, five seven or even more layers.

Modified atmosphere packaging (MAP) alters the natural gas surrounding of the product in the package to delay deteriorative changes. MAP is a preservative-free method to extend shelf life and to keep food products fresh for a longer time (Khoshgozaran, Azizi, & Bagheripoor-Fallah, 2012). Passive or active methods can modify the atmosphere inside a package. In passive MAP, the rate of change and the final gas composition in the package depend on both the packed product and the permeability of the packaging material. The main disadvantage of this technique is the long time required for optimal gas composition which could be important for products with short shelf life. Active MAP is usually accomplished by first creating a vacuum and then injecting the desired gas mixture in the package. The desired atmosphere is directly achieved at the beginning of storage time and remains unchanged. The disadvantages of active MAP over passive MAP are the higher costs in equipment and gases (Khoshgozaran, et al., 2012; Rodríguez-Aguilera & Oliveira, 2009). Active MAP packaging has to be designed of handpicking the film type and size for each product (Farber et al. 2003) and is currently applied for fresh cheeses like cottage (Maniar et al. 1994, Mannheim and Soffer, 1996), cheeses in brine like Mozzarella (Alves et al. 1996, Eliot et al. 1998) and Greek whey cheese (Papaioannou et al. 2007) and surface mold-ripened cheese (Rodríguez-Aguilera, Oliveira, Montanez, & Mahajan, 2011a, 2011b). Garabal et al. 2010 (Garabal, Rodríguez-Alonso, Franco, & Centeno, 2010) showed that
vacuum packaging is the best method for retaining the sensory quality of San Simón da Costa smoked semi-hard cheeses. Because permeability to $O_2$, $CO_2$ and water vapor transmission rates for packaging films are among the most essential factors in determining the package atmosphere composition, which may influence the product’s deterioration rate, the final decision on packaging film should be made based on vast assessments (Khoshgozaran, et al., 2012).

Another concept to extend shelf-life and improve sensory properties is active packaging in which the packaging material interacts actively with the product. Antimicrobial films, moisture absorbers and biodegradable coatings have been successfully applied in the past (Rodríguez-Aguilera & Oliveira, 2009). The application of lysozyme/EDTA in combination with MAP has been shown to prolong shelf life of Burrata cheese (Conte, Brescia, & Del Nobile, 2011) and recently an antimicrobial polylactic acid (PLA) embedded copper nanoparticles was successfully applied for Fiordilatte cheese (Conte, et al., 2013). Silver has attracted particular attention because of its broad efficacy against bacteria and other organisms and its relatively low toxicity to humans. Silver is incorporated into polymer matrices by reduction as a silver complex trough on site precipitation or as a particulate additive (Loher, Schneider, Maienfisch, Bokorny, & Stark, 2008).

4. Aim of the thesis

4.1. Background and aim of this thesis

The development of cheese smear is characterized by a succession of microbial populations and influenced by environmental factors such as pH, water activity, ripening temperature and salt content of the cheese. A balanced smear is a major quality criterion and plays an important role during cheese ripening by preventing spoilage due to growth of undesired microorganisms by competitive mechanisms. For sale a dry, non-odorous, sparse and nicely colored smear is required. Our modern lifestyle demands a guaranteed quality and safety of the product over an extended timespan. In addition, the increasing importance of Swiss cheeses as export goods requires long lasting high quality products. As consequence of
consumer habit and retailers logistic more and more fully-ripened red-smear cheeses portions are wrapped in plastic film, transported to retailers and sold as film-prepacked cheese from self-service shelves. This wrapping procedure is usually followed by a period of cold storage at the retailers and in the households of consumers that is often associated with a marked deterioration in the smear quality, characterized by an unpleasant off-odorous and a wet, sticky and smudgy cheese smear upon opening of the packed cheese. The problem of sticky smear formation is known since cheese portions are wrapped in plastic films and described a decade ago (Bachmann, et al., 2003; Winkler & Amrein, 2001). The problem arises more often since film-prepacking of small cheese portions is increasingly applied (Bachmann, et al., 2003; Eliskases-Lechner, Ginzinger, Rohm, & Tschager, 1999). Numerous trials to solve the problem by pre-treating cheeses prior to packaging such as drying the rind, removing the rind, retarded smear ripening, different packaging materials and applying water-binding components to the cheese rind have been performed (Winkler, et al., 2008) in co-operation with Swiss cheese retailers. However, none of the treatments provided the desired solution to solve the problem sufficiently. Hitherto, a thorough scientific investigation of the described problem of development of defective smear due to film pre-packing of red-smear cheese has not been conducted. It is assumed that the exclusion of oxygen through film-packaging might favor the growth of facultative and strictly anaerobic bacteria and/or a change of the metabolic activity of certain smear microorganisms. This would suggest a microbiological cause for the development of defect smear upon film-packaging.

The aim of this project was to elucidate semi-hard red-smear cheese surface defect mechanism of fully ripened semi-hard cheeses and design a defect prevention solution according to the cause. Therefore defective and non-defective red-smear cheese surfaces of two Swiss varieties were analyzed and compared for their microbial composition. Culture dependent method including cultivation, isolation and identification of pure cultures by 16S rRNA gene sequencing as well as culture independent techniques such as temporal temperature gradient gel electrophoresis (TTGE) and quantitative real-time PCR (qPCR).
were applied (Chapter 2, Fig. 4). A list of atypical organisms harboring a potential for defect contribution was postulated. Microbial composition revealed to be very similar in defective and non-defective smear of both varieties (Amato, et al., 2012). The focus was therefore set on microbial transcriptional activity, environmental factors such as pH, water activity value and redox potential in combination with latest alternative packaging materials. Three films differing in gas- and water vapor permeability’s as well as an antimicrobial film containing nanosilver particles were tested in challenge tests over 8 week of storage at 8°C. The redox potential, water activity and pH development as well as transcriptional activity of the cheese smear microflora were monitored (Chapter 3, Fig. 4). Various corrective measurements especially to improve the promising performance of a high gas- and water vapor permeable film in combination with a protective culture as well as an antifungal component and a new low gas and high water vapor permeable film in combination with an antifungal component was applied (Chapter 4, Fig. 4). Malate quinone oxidoreductase gene transcripts of Corynebacterium casei were followed as an alternative to 16S rRNA. Metabolic activity measurements gave evidence for the importance to distinguish between dead and living bacterial cells development over storage. Therefore a combined approach of propidium monoazide (PMA) staining and qPCR (PMA-qPCR or v-qPCR) was evaluated for its application in cheese smear and used to follow living bacteria cells during storage in film-prepacked portions. The combination of culture dependent and culture independent methods used in this project could enlighten various aspects of the complex etiology of cheese surface defect development. Finally an alternative packaging solution could be found presenting a dry, sparse, non-odorous and nice colored cheese surface until the end of storage time (Chapter 5, Fig. 4).
4.2. Hypotheses

The following hypotheses were formulated for this thesis:

- The cheese smear can get wet, smudgy and off-odorous because of growth of single or a group of microorganisms or a shift in smear-microbiota composition due to the changing conditions through film-packaging and subsequent storage
- The cheese smear can develop a defect because of changed activity and/or metabolism of the microflora due to the switch to anaerobiosis caused through film packaging
- The cheese smear defect is rather caused by multiple factors than by only a single one
- A balanced cheese smear microflora can be achieved by controlling physicochemical conditions

4.3. General objectives

The general objectives of this thesis are to acquire knowledge of the diversity of the microbiota of regular and defective film-prepacked red-smear cheeses, to explain microbial dynamics and the mechanism of cheese surface defect formation in film-prepacked portions and to gain increased acceptance of prepacked cheese by retailers and consumers through defect prevention measurement.
4.4. Specific objectives

- Investigation and identification of the composition of semi-hard red-smear cheese microflora of regular and defective film-prepacked cheeses by culture dependent and culture independent methods

- Development and evaluation of molecular culture-independent technologies for further defect characterization

- Identification of red-smear cheese defect causing factors by monitoring physicochemical parameters and metabolic activity of the cheese surface microflora

- Monitoring microbial dynamics in cheese smear over storage by living bacteria cells detection

- Laboratory and industrial scale test of defect prevention measures
Investigation of the smear microflora | Physicochemical parameters and metabolic activity of the cheese smear during defect development | Various approaches for defect prevention | Explanation and prevention of red-smear cheese defect

<table>
<thead>
<tr>
<th>Non-defective - defective smear</th>
<th>pH – a_w – redox potential</th>
<th>Storage tests Pretreatments: salting – drying biopreservation – alternative film</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultivation and identification</td>
<td>qPCR and RT-qPCR</td>
<td>Defect prevention solution</td>
</tr>
<tr>
<td>TTGE</td>
<td>DNA extraction</td>
<td>PMA-qPCR</td>
</tr>
</tbody>
</table>

**Fig. 4.** Research strategy used for investigation and prevention of defect development in film-prepacked red-smear cheese portions. Investigation of the cheese surface microflora composition (Chapter 2), physicochemical parameters, impact of metabolic activity of the smear microflora and influence of packaging material (Chapter 3). Various approaches tested for defect prevention (Chapter 4), an explanation and a solution strategy of red-smear cheese defect (Chapter 5).
Chapter 2

Microbial composition of defect smear - a problem evolving during foil*-prepacked storage of red-smear cheeses

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#the two first authors contributed equally to the project
*the term foil in this chapter is used equally as film in the following chapters

Abstract

To elucidate the problem of smear defect in foil-prepacked fully-ripened red-smear cheese portions, the microbial composition of 47 smear samples from 2 cheese varieties was analyzed. Cheese variety M was characterized by a diversity of 27 different bacterial species of which 11 represented typical smear microorganisms belonging to Arthrobacter, Brachybacterium, Brevibacterium, Corynebacterium, Microbacterium and Staphylococcus. For variety T 29 different species were identified with 12 isolates representing typical smear microorganisms. Further pure isolates represented members of Bacillus, Citrobacter, Enterococcus, Facklamia, Morganella, Proteus and Vagococcus. Culture-dependent analysis revealed a stable microbial composition which was confirmed by culture-independent techniques such as TTGE analysis or qPCR. However, several bacteria with potential to contribute to smear defects by off-flavor or exopolysaccharide production were identified. Hence, our results provide evidence that the cause of the defect might not be assigned to a single microbial group but rather due to the contribution of various microorganisms and to their metabolic activity within the foil.
1. Introduction

Red-smear cheeses are characterized by the development of microorganisms on their surface that contribute to the red, glistening appearance. In contrast to mold-ripened cheeses, the major part of microorganisms involved in surface ripening of red-smear cheeses are bacteria. Accordingly, this type of cheese is also known as bacterial surface-ripened cheese (Brennan, 2004; Mounier, et al., 2005). Well known varieties like Appenzeller, Gruyère, Tilsit, Tête de Moine, Livarot, Gubbeen and Reblochon, are extensively produced and are of great economic importance in European countries like Austria, Belgium, Germany, France and Switzerland (Brennan, 2004; Larpin-Laborde, et al., 2011; Mounier, et al., 2005; Mounier, et al., 2009). The cheese smear is a complex microbial ecosystem evolved through a dynamic process started by de-acidification of the cheese surface through yeast growth and metabolism, followed by the colonization with bacterial populations, mainly coagulase-negative staphylococci and coryneform bacteria including Micrococcaceae, Brevibacteriaceae and Corynebacteriaceae (Brennan, 2004; Roth, Schwenninger, Hasler, Eugster-Meier, & Lacroix, 2010). Also marine bacteria like Facklamia spp. and Marinilactibacillus spp. known as facultative anaerobic halophilic and alkaliphilic bacteria (FAHA) have been detected on cheese surface (Feurer, et al., 2004; Ishikawa, et al., 2007; Roth, Schwenninger, Hasler, Eugster-Meier, & Lacroix, 2010; Roth, Schwenninger, Eugster-Meier, & Lacroix, 2011). Gram-positive bacteria belonging to the genus Arthrobacter, Brachybacterium, Brevibacterium, Corynebacterium, Microbacterium, Micrococcus and Staphylococcus represent desired smear microorganisms, whereas enterococci which are frequently found in cheese smear are assumed to represent hygiene indicators (Brennan, 2004). The development of cheese smear is characterized by a typical succession of microbial populations and influenced by environmental factors such as pH, water activity, ripening temperature and salt content of the cheese. A balanced smear is a major quality criterion and plays an important role during cheese ripening by preventing spoilage due to growth of undesired microorganisms. For sale, a dry, non-odorous, sparse and nicely colored
smear is required. Our modern lifestyle demands a guaranteed quality and safety of the product over an extended timespan. In addition, the increasing importance of Swiss cheeses as export goods requires long lasting high quality products. As a consequence of consumer habit and retailers logistics more and more fully-ripened red-smear cheeses are wrapped in plastic foil, transported to retailers and sold as foil-prepacked cheese portions from self-service shelves. This wrapping procedure is usually followed by a period of cold storage at the retailers and in the households of consumers which is often associated with a marked deterioration in the smear quality, characterized by an unpleasant off-flavor and a wet, sticky and smudgy cheese smear upon opening of the packed cheese. The problem of sticky smear formation was described by Winkler et al. (2001) and Bachmann et al. (2003) and arises more often since foil-prepacking of small cheese portions is increasingly applied (Bachmann, et al., 2003; Eliskases-Lechner, et al., 1999). Numerous trials to solve the problem by pre-treating cheeses prior to packaging such as drying the rind, removing the rind, retarded smear ripening, different packaging materials and applying water-binding components to the cheese rind have been performed (Winkler , et al., 2008) in co-operation with Swiss cheese retailers. However, none of the treatments provided the desired solution to solve the problem sufficiently.

Hitherto, a thorough scientific investigation of the described problem of development of defect smear due to foil pre-packing of red-smear cheese was not conducted. It is assumed that the exclusion of oxygen through foil-packaging might favor the growth of facultative and strictly anaerobic bacteria and/or a change of the metabolic activity of certain smear microorganisms. This would suggest a microbiological cause for the development of defect smear upon foil-packaging. Therefore, the aim of this study was to analyze and compare the microbial composition of the surface smear of cheeses with defective and non-defective smear. For this purpose two different red-smear cheese varieties were analyzed applying culture dependent methods including cultivation, isolation and identification of pure isolates by 16S rRNA gene sequencing, as well as culture independent techniques such as temporal temperature gradient gel electrophoresis (TTGE) and quantitative real-time PCR (qPCR).
2. Materials and methods

2.1 Cheese samples

Two varieties of fully ripened Swiss red-smear cheeses were studied for microbial differences between non-defective and defective cheese smear: 38 surfaces of variety M and 9 surfaces of variety T produced in 5 plants. All cheeses were provided by the corresponding industrial partner and producer of the cheese. Both varieties are fully surface long-ripened semi-hard Tilsit-like cheeses. Variety M is made with raw and variety T with raw or thermised cow’s milk, with a heat treatment step between 57-68 °C for < 30 s. The cheeses were fully-ripened in the cheese cellar before packaging, for at least 75 days at 13-14 °C in the case of variety M and for 70 to 110 days at 14 °C in the case of variety T. Both cheese types were subsequently stored at 4-8 °C after vacuum wrapping in a gas- and watertight plastic foil. The sampling plan is illustrated in Table 1.

2.2 Microbiological analysis

A 2-3-mm thick rectangle of 8 cm² of the cheese surface was cut with a sterile knife and diluted in 50 mL peptone solution (8.5 % NaCl, 1 % peptone from casein, pH 7) solution. This mixture was homogenized using a stomacher (Colworth 400, Müller + Krempel AG, London, UK) for 4 min. An aliquot of 2 mL of this solution was kept for DNA extraction. Serial dilutions of homogenates were plated on appropriate non-selective and semi-selective media and incubated as described in Table 2. To include strictly anaerobic bacteria the microbiological analysis procedure was performed in parallel in an anaerobic chamber. The cheese portion was opened in the chamber (gas composition: 85% N₂, 10% CO₂, 5% H₂; Coy Laboratory Products Inc., Grass Lake, Michigan, USA), where a rectangle was cut as described before, dissolved in 25 mL of anaerobic peptone solution containing 500 mg L⁻¹ cysteine and 1mg L⁻¹ resazurin in a 50 mL Falcon tube containing 15 g of solid-glass beads (diameter 3 mm) (Sigma-Aldrich, Buchs, Switzerland) and the mixture was thoroughly treated for 7 min on a vortex-shaker. Serial dilutions were prepared with anaerobic peptone solution and plated on
Differential Reinforced Clostridial Medium (DRCM) and Reinforced Clostridial Agar (RCA) plates in the anaerobic chamber, where the plates were also left for incubation as indicated in Table 2.

Table 1
Sampling plan of 47 surfaces from two fully-ripened Swiss red-smear cheese varieties received from five different production plants.

<table>
<thead>
<tr>
<th>Cheese smear</th>
<th>Variety</th>
<th>Plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-defective versus defective</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Foil-prepacked no defect (fpnd)(^a)</td>
<td>2 × M</td>
<td>A, A</td>
</tr>
<tr>
<td>Foil-prepacked with defect (fpwd)(^b)</td>
<td>2 × M</td>
<td>A, A</td>
</tr>
<tr>
<td>Development of defect over storage time</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before packaging (bfp)</td>
<td>4 × M</td>
<td>A, B, C, C</td>
</tr>
<tr>
<td>2 weeks (2w)</td>
<td>4 × M</td>
<td>A, B, C, C</td>
</tr>
<tr>
<td>4 weeks (4w)</td>
<td>4 × M</td>
<td>A, B, C, C</td>
</tr>
<tr>
<td>6 weeks (6w)</td>
<td>4 × M</td>
<td>A, B, C, C</td>
</tr>
<tr>
<td>8 weeks (8w)</td>
<td>4 × M</td>
<td>A, B, C, C</td>
</tr>
<tr>
<td>8 weeks + fluctuation (T-fluc)(^c)</td>
<td>4 × M</td>
<td>A, B, C, C</td>
</tr>
<tr>
<td>Unpacked versus foil-prepacked</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unpacked (up)</td>
<td>2 × M</td>
<td>A, A</td>
</tr>
<tr>
<td>3 × T</td>
<td>D, E, E</td>
<td></td>
</tr>
<tr>
<td>Foil-prepacked no defect (fpnd)</td>
<td>2 × M</td>
<td>A, A</td>
</tr>
<tr>
<td>3 × T</td>
<td>D, E, E</td>
<td></td>
</tr>
<tr>
<td>Foil-prepacked with defect (fpwd)</td>
<td>2 × M</td>
<td>A, A</td>
</tr>
<tr>
<td>3 × T</td>
<td>D, E, E</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Non-defective smear is defined as a dry, non-smeary rind with a typical cheese odor.

\(^b\)Defective smear is defined as a humid to very humid, sticky to very smeary rind with an intensive to very intensive off-odor.

\(^c\)Interruption of cold chain was mimicked by storing cheeses for three days at room temperature (22 °C) after storage for four weeks at 8 °C.
Table 2
Agar media used for cultivation of diverse microorganisms from fully-ripened smear-cheese surface.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Full name</th>
<th>Incubation conditions</th>
<th>Expected organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGYA aerobic</td>
<td>Tryptic glucose yeast agar(^a) supplemented with 1% (w/v) casein peptone(^b)</td>
<td>30 °C, 3 d + 22 °C, daylight, 7 d, aerobic</td>
<td>Total bacterial counts (Total bacteria)</td>
</tr>
<tr>
<td>TGYA anaerobic</td>
<td>Tryptic glucose yeast agar(^a) supplemented with 1% (w/v) casein peptone(^b)</td>
<td>30 °C, 3 d + 22 °C, daylight, 7 d, anaerobic</td>
<td>Total anaerobic bacterial counts (Total anaerobes)</td>
</tr>
<tr>
<td>MB</td>
<td>Marine broth agar(^c)</td>
<td>30 °C, 3 d, aerobic</td>
<td>Heterotrophic marine bacteria (Halotolerants)</td>
</tr>
<tr>
<td>GYPB</td>
<td>Glucose-yeast extract peptone beef extract agar(^d)</td>
<td>30 °C, 4 d, anaerobic</td>
<td>Facultatively anaerobic halophilic and alkaliophilic bacteria (FAHA)</td>
</tr>
<tr>
<td>KFS</td>
<td>KF Streptococcus agar(^c)</td>
<td>42 °C, 3 d, aerobic</td>
<td>Enterococci</td>
</tr>
<tr>
<td>VRBG</td>
<td>Crystal violet neutral-red bile glucose agar(^d)</td>
<td>37 °C, 14 d, aerobic</td>
<td>Enterobacteriaceae (EB)</td>
</tr>
<tr>
<td>DRCM</td>
<td>Differential reinforced clostridial broth(^a) 1 mg L(^{-1}) resazurin(^e), 500 mg L(^{-1}) cysteine(^f)</td>
<td>22 °C, 4 d strict anaerobic in anaerobic chamber</td>
<td>Clostridia and strictly anaerobic bacteria (Strict anaerobes)</td>
</tr>
<tr>
<td>RCA</td>
<td>Reinforced clostridia broth(^a) 1 mg L(^{-1}) resazurin(^e), 8 mg L(^{-1}) novobiocin(^e), 8 mg L(^{-1}) colistin(^e)</td>
<td>37 °C, 4 d strict anaerobic in anaerobic chamber</td>
<td>Clostridia and strictly anaerobic bacteria (Strict anaerobes RCA)</td>
</tr>
<tr>
<td>PY</td>
<td>Phyton yeast extract agar(^c) 20 mg L(^{-1}) chloramphenicol(^f)</td>
<td>30 °C, 3 d aerobic 30 °C, 6 d aerobic</td>
<td>Yeast (Yeast (PY))  Molds (Molds (PY))</td>
</tr>
<tr>
<td>YGCB</td>
<td>Yeast glucose chloramphenicol agar(^a), 10 mg L(^{-1}) bromphenol blue(^g)</td>
<td>30 °C, 3 d, aerobic + 22 °C, light, 3 d aerobic</td>
<td>Yeast (Yeast YGCB)  Molds (Molds YGCB)</td>
</tr>
</tbody>
</table>

\(^a\) Biolife, Milano, Italy
\(^b\) Merck, Dietikon, Switzerland
\(^c\) Becton Dickinson AG, Allschwil, Switzerland
\(^d\) According to Roth, Schwenninger, Eugster-Meier, and Lacroix (2011)
\(^e\) Sigma-Aldrich, Buchs, Switzerland
\(^f\) VWR, Dietikon, Switzerland.

2.3 Colony isolation and identification

Three isolates of each colony type showing a distinct morphology were picked from solid media and streaked three times for purification on the corresponding medium. DNA from single colonies was isolated according to Goldenberger et al. (1995). All coci were screened for identification as enterococci by a multiplex PCR (Jackson et al. 2004). PCR was performed with primer bak 4 (Greisen, Loeffelholz, Purohit, & Leong, 1994) and bak 11w (Goldenberger, Perschil, Ritzler, & Altwegg, 1995) according to the protocol described by
Dasen et al. (1998) for 16S rRNA gene analysis or 2643F and 2341R (Drancourt & Raoult, 2002) for staphylococci rpoB gene sequencing to allocate bacterial isolates to the respective genus and/or species level. For a taxonomic classification of yeast, the ribosomal ITS-2 region was amplified using primers 5.8SF and ITS4R (White, Bruns, Lee, & Taylor, 1990). Sequencing was performed at Microsynth AG (Balgach, Switzerland) and DNA sequences were compared to the GenBank database using the NCBI database Basic Local Alignment Tool (BLAST) analysis in order to identify the closest relatives by 16S rRNA gene or ITS-2 DNA sequence comparison. Isolates with a sequence maximum similarity value of 98.7% or higher were given the name of genus and species whereas isolates with values below 98.7% were given the genus name only. Molds isolates were determined based on morphological characterization.

2.4 DNA extraction from cheese smear
An aliquot of 2 mL cheese smear/peptone solution (described in 2.2) was centrifuged for 3 min at 7000 x g and the pellet was resuspended in 1 mL PBS/EtOH (1:1, Sigma-Aldrich) for total cheese smear DNA extraction. DNA was extracted using Fast DNA Spin Kit for Soil (MP Biomedicals LLC, Illkirch, France) following the manufacture’s protocol and DNA was resolved in DNase free water followed by storage at -20 °C until further analysis.

2.5 Temporal temperature gel electrophoresis analysis of the V2V3 region
A rapid approach based on genetic profiling by temporal temperature gradient gel electrophoresis (TTGE) was used for visualization of microbial community profiles. PCR amplification of variable regions two and three of bacterial 16S rRNA genes (V2V3 region) of total DNA extracted from cheese smear was performed in a T3000 Thermocycler Biometra (Biometra GmbH, Goettingen, Germany) using the following temperature profile: 94 °C for 4 min; 30 cycles of 94 °C for 30 s, 58 °C for 30 s, 68 °C for 60 s; and finally 72°C for 7 min and the primer pair HDA1-GC/HDA2 (Ogier, et al., 2002). PCR products were checked for a size of 200 bp on a 2% agarose gel (GellyPhor LE, Euroclone, Milano, Italy) with a molecular
weight standard of TriDye 100-bp DNA ladder (50 µg mL\(^{-1}\) : BioConcept, Allschwil, Switzerland). TTGE was performed using a DCode universal mutation detection system (BioRad, Reinach, Switzerland) on a 16 x 16 x 1 mm 8.5 % polyacrylamide gel (40 % acrylamide / 37.5 % N,N'-Methylenbisacrylamide, Sigma-Aldrich; 8 M urea, Sigma-Aldrich). Gels were run with 1.5 x TAE buffer pH 8 (60 mMTris (hydroxymethyl)-aminomethane, Sigma-Aldrich; 60 mM acetic acid, Sigma-Aldrich; 1.5 mM EDTA, VWR International AG, Dietikon, Switzerland). Thirty microliters of PCR sample product were mixed with 20 µL loading dye (0.2 % (w/v) Orange G, Sigma-Aldrich; 60 % (v/v) glycerol, VWR), deposited in the slots and separated under defined pre-running (20 V, 66°C, 200°C/h, 15 min) and running (65 V, 66-70 °C, 0.4 °C/h, 16 h) conditions. Gels were stained for 60 min in 2.5 mg L\(^{-1}\) ethidium bromide (Sigma-Aldrich) and discolored for 30 min in distilled water before a picture was taken using a Molecular Imager Gel Doc XR System (Bio-Rad, Reinach, Switzerland). Pictures were imported to GelCompare II software (Bio Maths NV, Sint-Martens-Latem, Belgium) and the patterns were normalized to Marker E previously generated on the basis of isolated microorganisms from cheese smear consisting of *Staphylococcus equorum*, *Marinilactibacillus psychrotolerans*, *Corynebacterium variabile*, *Lactobacillus casei*, *Corynebacterium casei*, *Arthrobacter casei*, *Propionibacterium freudenreichii*.

### 2.6 Quantification of total bacteria by qPCR

A 7500 Fast Real-Time PCR System (Applied Biosystem, Carlsbad, California, USA) was used for qPCR analysis of 16S rRNA gene amplified from all bacteria. The primer Eub338F (Weisburg, Barns, Pelletier, & Lane, 1991) and Eub518R (Muyzer, Dewaal, & Uitterlinden, 1993) formerly used by Fierer et al. (2005) for qPCR of soil probes resulted in a 200-bp product. SYBR Green PCR Master Mix 2x (Applied Biosystem, Warrington, UK) was used, each primer (Microsynth) in a concentration of 0.2 µM and 1 µL of DNA (20-60 µg mL\(^{-1}\) prepared as described in 2.4) in a reaction volume of 25 µL. The temperature profile was as followed: 2 min at 50 °C, 10 min 95 °C and 40 cycles of 15 s at 95 °C followed by 1 min of 60
°C. The specificity of the resulting PCR products was monitored by a melting curve analysis after each run. Quantification of 16S rRNA gene copy numbers was accomplished by amplification of standard (pLME21 plasmid that contained *Bifidobacterium lactis* 16S ribosomal DNA extracted from *Escherichia coli* JM109/pLME21; Laboratory of Food Biotechnology culture collection) dilutions (10^2 to 10^9) containing a predefined template copy number, that were processed in the same manner as the unknown samples. Standards (positive controls), as well as the no template controls, were amplified in triplicates at the same time and under the same conditions as the triplicates of two dilution series of unknown samples. Data were analyzed using the software 7500 Fast SDS (Applied Biosystem). The template copy numbers of the unknown samples were extrapolated from the standard curve and reported as means of triplicates in copy numbers per square centimeter (cn cm^-2) of cheese smear. Copy number variations per genome according to different species were neglected as all species were addressed. 16S rRNA copy numbers were compared to colony forming units.

### 2.7 Statistical Analysis

Data analysis was done with JMP 8.0 software (SAS Institute AG, Wallisellen, Switzerland). One-way ANOVA was applied to evaluate significant (P-value <0.05) differences between unpacked (up), foil-prepacked non-defective (fpnd) and foil-prepacked with defect (fpwd) cheese smear.
3. Results and discussion

3.1 Microbial composition of non-defective versus defective cheese smears

To investigate the difference in the microbial composition of non-defective cheese smear showing a desirable rather dry surface, classified as foil-prepacked non-defective (fpnd) and cheese with defective smear, showing a very humid and smudgy cheese surface, classified as foil-prepacked with defect (fpwd), smear samples of two representative non-defective and defective cheeses of variety M (Table 1) were analyzed by cultivation on a variety of appropriate culture media (Table 2) and quantitative real-time PCR. The results are summarized in Figure 1 which shows the mean of CFU cm$^{-2}$ in comparison to the determined copy numbers for two independent cheese smear samples deriving from different production batches of the same cheese production plant. The differences of microbial CFU between the non-defective versus defective smear are not significant and the overall picture of the microbial composition of non-defective versus defective cheese smear is very similar. The following trend of a slight general decrease of CFU cm$^{-2}$ was observed for samples from defective cheese smear for total cell counts (total bacteria), halotolerant bacteria (halotolerants), facultative anaerobic and alkaliphilic bacteria (FAHA), presumptive staphylococci (staphylococci) and molds. An increase of colony forming units (CFU) on TGYA medium incubated under anaerobic conditions (total anaerobes) was noticed for fpwd cheese samples although it was in a lower range than expected considering a possible problem causing organism being an anaerobic microorganism. Yeast counts in a range of $10^2$ cm$^{-2}$ were very low compared to total CFU ranging around $10^9$ cm$^{-2}$. Quantification of total bacterial counts by qPCR resulted in log 10.36 +/- 0.02 for fpnd and log 10.25 +/- 0.2 for fpwd 16S rRNA gene copy numbers per square centimeter cheese surface which is about 1-2 log higher than the values obtained by culture-based analysis supporting the observed stable composition.
Fig. 1. Microbial cell counts and qPCR copy numbers of 16S rRNA genes of foil-prepacked non-defective (■) and foil-prepacked defective (□) fully ripened red-smear cheese surface. The bars show cell count means of two independent samples. The two last bars indicate on the alternative axis the 16S rRNA copy numbers (cn) for total bacteria measured by qPCR for the same cheese samples as cultivation was performed. The detection limit is indicated by the interrupted line.

3.2 Development of cheese smear quality during storage

For investigation of cheese smear development during storage four cheese batches of variety M were selected during a quality assessment in the cheese production plant before packaging. Two of the selected cheese batches were classified as excellent and good by the cheesemaker and were hypothesized not to develop a defect smear. Two other cheese batches were classified as less good (medium) and were hypothesized to develop a wet, smudgy and off-odourous smear. One cheese of each batch was analysed before packaging (bfp), whereas the others were vacuum foil-prepacked and subsequently analysed after 2, 4, 6 and 8 weeks of storage at 8 °C until reaching of expiry date. To study the effect of temperature fluctuation on the cheese smear quality, an interruption of the cold chain was
simulated by incubation of one cheese of each batch at room temperature (22 °C) for three
days after four weeks of cold storage at 8 °C (T-fluc). After a total of 8 weeks cold storage all
four batches revealed a defective smear with wet, smudgy and off-odourous characteristics.
The development of the cell counts for different microbial populations determined on various
culture media is shown in Figure 2. In general, the similar development over time of both
cheese qualities, good and medium, for all microorganism groups can be observed. A
decrease of cell counts per square centimeter surface over time of about 1 log can be
noticed for total bacterial cell counts $10^9$-$10^8$ CFU cm$^{-2}$ (Fig. 2 B), halotolerant bacteria $10^9$-
$10^8$ CFU cm$^{-2}$ (Fig. 2 D) and FAHA $10^9$-$10^8$ CFU cm$^{-2}$ (Fig. 2 D). An increase of anaerobic cell
counts of 1-2 log after wrapping the cheese into plastic foil followed by a decrease after
reaching the timepoint of 6 weeks is noted (Fig. 2 B). Presumptive staphylococci remained
stable over time in a range of $10^8$ CFU cm$^{-2}$ (Fig. 2 A). Molds and yeast were detected in a
similar range on both growth media used (YGCB, Fig. 2 C and PY, data not shown). Yeast
counts (YGCB, Fig. 2 C) were again low and fluctuating between detection limit (<$10^1$ CFU
cm$^{-2}$) and $10^3$ CFU cm$^{-2}$. Quantification of the total bacterial genome copy numbers
performed by qPCR for all samples (Fig. 2 B) revealed a comparable tendency as the cell
counts determined by cultivation. An increase directly after packaging and a decrease toward
the end of storage time was observed for good and medium cheese batches. This setup
revealed a very stable behavior of the surface smear microbiota during storage.
Development of a negative smear characteristic could be observed for all investigated
samples. Starting between week two and four the smear defects increased until reaching
week 8. Interruption of cold chain led to a dramatic reinforcement of defect development.
Hence, foil packaging contributes to defect development and it is impossible even for cheese
specialists to predict whether a cheese smear will develop a defect or stay dry within the foil-
prepackage. Therefore, we conclude that every cheese surface independent of its overall
quality classification before packaging is harboring the potential to develop a defect.
Fig. 2. Development of the surface smear microflora on good (closed line) versus medium (interrupted line) quality cheese under storage conditions. The graph shows the mean of two samples judged to be good or medium, respectively. The time points chosen for analysis of the microflora were bfp (before packaging) and after 2, 4, 6, 8 weeks (we) of storage at 8 °C. For one cheese sample of each quality group interruption of cold chain was mimicked (T-fluc) indicated after the double line. A) Cell counts for staphylococci (□) and enterococci (∆). B) Total bacteria 16S rRNA gene copy numbers indicated on the alternative axis (■), cell counts for total bacteria (◊) and total anaerobes (△). C) Cell counts for molds (●) and yeasts (x). D) Cell counts for halotolerants (○) and FAHA (●). The detection limit is indicated by the interrupted line.

3.3 Comparison of the surface smear microbiota of unpacked versus foil-prepacked non-defective and foil-prepacked defective smear

To investigate potential influence of vacuum foil-prepacking, the composition of the surface smear microbiota of unpacked cheeses devoid of smear defects (up) as well as foil-prepacked non defective cheese (fpnd) and foil-prepacked cheeses with defective smear (fpwd) (triplets described in Table 1) were investigated and compared. The cheese makers were assigned to optically select representative samples for the up, fpnd and fpwd group. Figure 3 shows the mean values obtained from two triplets of variety M (A) and three triplets of variety T (B). For both cheese varieties a stable composition revealing no significant
Fig. 3. Microbial cell counts and qPCR copy numbers of total bacteria 16S rRNA genes of unpacked (■) versus foil-prepacked non-defective (▲) and foil-prepacked defective (□) red-smear cheese surface. A) Mean of two independent triplets of variety M. B) Mean of three independent triplets of variety T. The detection limit is indicated by the interrupted line.
differences for samples up, fpnd and fpwd was observed by the culture-based approach. Comparison of the results from cheese variety M and T revealed also a rather similar microbial composition. Total plate counts (total number of bacteria) as well as the counts for certain microbial populations obtained on the semi-selective media turned out to be rather similar for the different cheese surface samples. No major shift for a single group of microorganism could be observed. Low yeast counts ($\leq 10^1$) were observed for all samples of cheese variety M and T. Molds were already detected after three days of incubation on agar plates in foil-prepacked cheese samples. The stable microbial composition observed by the culture-based approach was confirmed quantitatively by qPCR (Fig. 3). Thus, the applied culture-independent analysis revealed again a rather similar and stable microbial composition of the investigated smear samples showing no significant differences between unpacked cheeses versus the foil-prepacked variants with non-defective and defective smear for both investigated cheese varieties. The quantification of total bacterial genome copy numbers by qPCR using universal 16S rDNA primers Eub338F and Eub518R that were hitherto used for the analysis of soil samples (Fierer, et al., 2005) and faeces (Guo, et al., 2008; Zimmermann, et al., 2010) was for the first time performed for complex cheese smear samples. The obtained genome copy numbers of $10^{10}-10^{11}$ for defective as well as non-defective smear samples confirmed the stable microbial composition of the cheese surface previously observed by culture-based methods. The determined copy numbers per cm$^2$ correlated but were 1-2 log higher than the CFU cm$^{-2}$ obtained on TGYA (total bacteria) and MB (halotolerants) agar plates. Deviations are in an expected range considering a slight overestimation of qPCR data due to the presence of more than one rRNA operon in many bacterial genomes (Jany & Barbier, 2008) as well as an underestimation of CFU cm$^{-2}$ due to the presence of cell clusters that form a single colony, viable but non-culturable (VBNC) bacteria or dead cells and the out-competing of organisms and micro niches on the cheese surface. The numbers of colony forming units determined in this study are in good correlation to the values of $10^8-10^9$ CFU cm$^2$ for cheese smear bacteria reported by other groups (Bockelmann, Willems, Neve, & Heler, 2005; Rademaker, et al., 2005; Valdés-Stauber, et al.,
The cell numbers were found to be higher on agar media containing higher concentrations of NaCl than for TGYA plates typically used for the determination of total plate counts (Roth, Schwenninger, Hasler, Eugster-Meier, & Lacroix, 2010). This might be explained by the adaptation of the respective microorganisms to the salty cheese environment (Roth, Schwenninger, Hasler, Eugster-Meier, & Lacroix, 2010). Therefore, culture media such as MB containing 19.5% (wt/v) NaCl or GYPB containing 7% (wt/v) NaCl seem to be more suitable to cultivate the representative surface smear microflora of cheeses with a salt content of about 9% (Winkler, et al., 2008).

3.4 Microorganisms isolated from red-smear cheese surface

An overview of the microbial diversity of the red-smear cheese varieties M and T is given in Table 3. All isolated organisms were identified by species specific multiplex PCR for enterococci, 16S rRNA or rpoB gene sequencing for bacterial and ITS sequencing for yeast or morphological identification for molds. The table is divided into typical microorganisms that represent well-known colonizer of the surface of red-smear cheeses, and atypical microorganisms that were hitherto not associated with red-smear cheese.

The absence of microorganisms in one type of samples that were present in numbers <10^1 in other sample types should be interpreted as an isolation bias rather than a clear difference in the surface microflora. For samples of variety M and variety T, 27 and 29 different microorganisms were identified on a genus or species level, out of which 16 and 17 represented atypical microorganisms for variety M and T, respectively.

Typical cheese surface bacteria like *Arthrobacter casei*, *Brevibacterium* spp., *Corynebacterium casei*, *Corynebacterium variabile*, *Microbacterium gubbeenense* and *Staphylococcus equorum* were found in both cheese varieties investigated in this study. Others, such as *Alkalibacterium* sp., *Lactobacillus delbrueckii* and *Marinilactibacillus psychrotolerans* were only found in variety M, whereas *Brychobacterium tyrofermentans*, *Corynebacterium stationis* and *Halomonas* sp. were exclusively detected in variety T.
Table 3
Overview of organisms isolated from surface smear of red-smear cheese varieties M and T divided into typical and atypical cheese smear microorganisms.

<table>
<thead>
<tr>
<th>Cheese smear microorganisms</th>
<th>Viable cell counts (cfu cm(^{-2})) in packaging type</th>
<th>Variety (M/T)</th>
<th>Isolation media(^b)</th>
<th>Cheese smear microorganisms</th>
<th>Viable cell counts (cfu cm(^{-2})) in packaging type</th>
<th>Variety (M/T)</th>
<th>Isolation media(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typical</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Alkalibacterium sp.</td>
<td>ni &gt;10(^4) &gt;10(^8) x/- GYPB</td>
<td></td>
<td></td>
<td>Corynebacterium variabile</td>
<td>&gt;10(^4) &gt;10(^8) ni x/x GYPB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arthrobacter sp.</td>
<td>&gt;10(^4) &gt;10(^8) -/x TGYAa</td>
<td></td>
<td></td>
<td>Debaryomyces Hansenii</td>
<td>&gt;10(^4) ni ni x/- YGCB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arthrobacter casei</td>
<td>ni &gt;10(^4) &gt;10(^8) x/x TGYAa</td>
<td></td>
<td></td>
<td>Halomonas sp.</td>
<td>&gt;10(^8) ni ni -/x MB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brachybacterium</td>
<td>&lt;10(^4) &gt;10(^8) ni -/x TGYAa</td>
<td></td>
<td></td>
<td>Marinilactibacillus</td>
<td>ni v &gt;10(^8) x/- TGYAa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tyrofermentans</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Brevibacterium sp.</td>
<td>&gt;10(^8) &gt;10(^8) ni x/x TGYAa/MB</td>
<td></td>
<td></td>
<td>Microbacterium gubbeenense</td>
<td>&gt;10(^8) &gt;10(^8) x/- TGYAa/MB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C.psychrophila</td>
<td>ni &gt;10(^4) x/- YGCB</td>
<td></td>
<td></td>
<td>Scopulariopsis brevicaulis</td>
<td>ni ni &gt;10(^8) x/- VRBG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corynebacterium sp. casei</td>
<td>&gt;10(^8) &gt;10(^8) ni x/x TGYAa/GYPB/MB</td>
<td></td>
<td></td>
<td>Staphylococcus sp.</td>
<td>ni ni &gt;10(^8) x/- GYPB</td>
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<tr>
<td>Atypical</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Advenella incenata</td>
<td>&gt;10(^8) ni ni x/- VRBG</td>
<td></td>
<td></td>
<td>Morganella sp.</td>
<td>ni ni &gt;10(^8) x/- VRBG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus sp.</td>
<td>ni &gt;10(^4) &gt;10(^8) ni -/x TGYAa</td>
<td></td>
<td></td>
<td>Morganella morganii</td>
<td>ni &gt;10(^8) &gt;10(^8) ni x/- VRBG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus amyloliquefaciens</td>
<td>&gt;10(^4) &gt;10(^8) ni -/x TGYAa</td>
<td></td>
<td></td>
<td>Peptonophilus sp.</td>
<td>&gt;10(^8) ni &gt;10(^8) -/x RCB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>ni &gt;10(^8) ni x/x TGYAa</td>
<td></td>
<td></td>
<td>Peptonophilus iromi</td>
<td>ni &gt;10(^8) ni -/x RCB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candida parapsilosis</td>
<td>&gt;10(^8) ni &gt;10(^8) ni x/- YGCB</td>
<td></td>
<td></td>
<td>Peptostreptococcus sp.</td>
<td>&gt;10(^8) ni ni x/- TGYAaan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrobacter sp.</td>
<td>ni &gt;10(^8) ni -/x VRBG</td>
<td></td>
<td></td>
<td>Pichia norwegensis</td>
<td>ni &gt;10(^8) x/- YGCB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterobacter sp.</td>
<td>ni &gt;10(^8) ni -/x VRBG</td>
<td></td>
<td></td>
<td>Propionibacterium sp.</td>
<td>ni &gt;10(^8) &gt;10(^8) x/- TGYAa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterococcus sp.</td>
<td>&gt;10(^8) &gt;10(^8) ni -/x GYPB</td>
<td></td>
<td></td>
<td>Propionibacterium freudenreichii</td>
<td>ni ni &gt;10(^8) x/- TGYAa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>ni &gt;10(^8) &gt;10(^8) x/- KFS/ TGYAa</td>
<td></td>
<td></td>
<td>Proteus sp.</td>
<td>ni &gt;10(^8) &gt;10(^8) ni x/- VRBG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterococcus malodoratus</td>
<td>&gt;10(^8) &gt;10(^8) x/- YGCB/MB</td>
<td></td>
<td></td>
<td>Proteus vulgaris</td>
<td>&gt;10(^8) &gt;10(^8) x/- VRBG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Facklamia tabacinasalis</td>
<td>&gt;10(^8) &gt;10(^8) x/- YGCB/MB</td>
<td></td>
<td></td>
<td>Providencia heimbachae</td>
<td>ni &gt;10(^8) ni x/- VRBG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>ni &gt;10(^8) ni -/x VRBG</td>
<td></td>
<td></td>
<td>Sporophyphydia lactititora</td>
<td>ni &gt;10(^8) ni x/- YGCB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactobacillus brevis</td>
<td>&gt;10(^8) &gt;10(^8) ni -/x RCA</td>
<td></td>
<td></td>
<td>Staphylococcus saprophyticus</td>
<td>&gt;10(^8) &gt;10(^8) ni x/- RCA/YGCB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactobacillus casei</td>
<td>&gt;10(^8) &gt;10(^8) ni -/x TGYAa/DRCM</td>
<td></td>
<td></td>
<td>Vagococcus sp.</td>
<td>ni ni &gt;10(^8) x/- TGYAa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactobacillus delbrueckii</td>
<td>ni &gt;10(^8) &gt;10(^8) ni -/x RCA</td>
<td></td>
<td></td>
<td>Vagococcus carniphilus</td>
<td>ni &gt;10(^8) &gt;10(^8) x/- TGYAa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luteococcus japonicus</td>
<td>&gt;10(^8) ni &gt;10(^8) ni -/x RCA</td>
<td></td>
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</table>

\(^a\) Abbreviations are: up, unpackaged; fpnd, foil-prepacked non-defective smear; fpwd, foil-prepacked defective smear; ni, not isolated under selected conditions; x, microorganism isolated; -, no microorganism isolated.

\(^b\) TGYAa, TGYA aerobic;TGYAan, TGYA anaerobic (see Table 2 for details).
Atypical microorganisms isolated from the smear samples comprised *Advenella incenata*, *Bacillus* spp., *Enterococcus* spp., *Lactobacillus casei*, *Morganella morganii*, *Peptoniphilus* spp., *Peptostreptococcus* sp., *Propionibacterium* spp., *Proteus vulgaris*, *Providencia heimbacheae* as well as *Staphylococcus saprophyticus* and *Vagococcus* spp.. The fact that no *Corynebacterium* spp. were isolated from fpwd cheese surfaces (Fig. 3) was rather considered as an isolation bias due to morphological similarity of different microorganisms on certain media than a clear difference of microbial composition. The following TTGE analysis clearly discloses (Fig. 4) *Corynebacterium variabile* in up, fpnd and fpwd for both cheese types and *Corynebacterium casei* in up, fpnd and fpwd for variety M in clear and for variety T in slightly weaker bands. Furthermore, the determination of *Corynebacterium casei* genome copy numbers by qPCR of up, fpnd and fpwd cheese surfaces (data not shown) confirm the presence of this microorganism even quantitatively. Many of the atypical microorganisms harbour the potential to contribute to the smear defect development. *Bacillus* spp. have the ability to produce long chain polysaccharides. Polysaccharides are known to exhibit smeary features and could therefore contribute to the smudgyness of the cheese smear. Enterococci are regarded as hygiene indicators. Thus their occurrence in high numbers is not desired in any food (Brennan, Cogan, Loessner, & Scherer, 2004). Growth of lactobacilli might be enhanced under anaerobic conditions in foil pre-packed cheese (Table 3). They are typically associated with cheese dough. *Lactobacillus delbrueckii* is a commonly used starter culture microorganism and *Lactobacillus casei* can be regarded as a representative of non starter lactic acid bacteria (NSLAB) but it is also frequently used as a starter culture. This organism could be clearly detected by TTGE (Fig. 4) for up, fpnd and fpwd cheese surface. The detection of lactobacilli in unpacked cheese smear might have originated from the cheese dough by cutting the cheese. However, it can not be excluded that lactobacilli grow under the anaerobic conditions within the foil. *Peptoniphilus* sp. and *Peptostreptococcus* sp. are butyric acid producers which can contribute to off-flavor. *Propionibacterium* spp., in particular *Propionibacterium freudenreichii* are known as an exopolysaccharide producer (Deutsch, Falentin, Dols-Lafargue, LaPointe, & Roy, 2008) which therefore can contribute to
smudgyness of the cheese surface and to off-flavor due to propionate production as well as to blowing of the foil-package through gas formation. *Proteus vulgaris* is a Gram-negative opportunistic pathogen with swarming ability and a potential contributor to off-flavor due to indole production and its potential to produce volatile compounds in large variety (Deetae, Bonnarme, Spinnler, & Helinck, 2007). Further *Enterobacteriaceae* detected in the cheese smear samples comprised *Advenella incenata*, *Citrobacter* spp., *Morganella morganii* and *Providencia heimbacheae*. These species are regarded rather as hygiene indicators providing evidence for the importance of the environmental surrounding for the microbial composition of the cheese surface microflora. Our findings are indicating to a rather complex origin of the cause of the smear defect suggesting that many different microorganisms may contribute to the smear defect problem rather than a single microbial species or microbial group being favored by the conditions within the foil and overgrowing the whole ecosystem on the cheese surface.

### 3.5 Temporal temperature gradient gel electrophoresis (TTGE) analysis

TTGE is a method that was already described for use of molecular fingerprinting analysis of microbial ecosystem in dairy products and cheese (Ogier, et al., 2004; Roth, Schwenninger, Hasler, Eugster-Meier, & Lacroix, 2010). In order to monitor the bacterial composition of the cheese smear and to detect differences in dry versus defect smear, unpacked, foil-prepacked non-defective and foil-prepacked defective samples of both cheese varieties were analyzed by TTGE. The TTGE analysis resulted in very stable and reproducible patterns. Only marginal differences were observed for the investigated sample triplets. Even between the two varieties no consistent differences were detected by this method with the exception that *Arthrobacter casei* was detected in all samples of variety T but not in samples of variety M, although the bacteria have been previously isolated by the culture-dependent approach. In the first triplet of variety M (M36-M38) the foil-prepacked samples showed a higher microbial diversity. However, this finding could not be confirmed with the second triplet (M39
The microbial composition of defect smear (M41). The lowest microbial diversity was observed for the sample foil-prepacked without defect in variety T (T17).

The culture-independent TTGE analysis of the V2V3 region of total DNA extracted from the complex microflora of the cheese smear samples revealed a very similar microbial diversity pattern for unpacked, foil-prepacked non-defective and foil-prepacked defective cheese smear within the same cheese variety (Fig. 4). Differences in the intensity of identical DNA fragments were observed but cannot directly be connected to a higher quantity of the respective microorganisms due to the known bias of PCR amplification. Rather similar patterns were observed after the analysis of the two cheese varieties T and M indicating to only small differences. The results from the TTGE analysis provide evidence that the microbial composition of the different cheese varieties is rather similar, but shows still a higher diversity than the cheese smear samples from unpacked and foil-prepacked cheeses of the same variety. In conclusion, the TTGE patterns supported the previous finding of an unexpected stable microbial composition showing only slight variations between different production batches than between cheese with defective and non-defective smear.

Fig. 4. TTGE analysis of 16S rRNA gene V2V3 region of complex cheese surfaces of unpacked (up) versus foil-prepacked non-defective (fpnd) and foil-prepacked with defect (fpwd) variety M (M36 - M41) and T (T13 - T21). Marker E consisting of six microbial TTGE bands previously isolated microorganisms of the cheese smear differing in G+C contents from low (33 mol%, Staphylococcus equorum) to high (60 mol%, Propionibacterium freudenreichii).
4. Conclusion

This comparative study of foil-prepacked defective and non-defective red-smear cheese smear by a polyphasic approach applying culture-dependent as well as culture-independent methods revealed an unexpected stable microbial composition for all surface smear samples investigated, independent from the cheese production plant, packaging technology and time point of storage for both cheese varieties M and T. The cause of defective smear could not be allocated to a particular microorganism or microbial group. Furthermore, it could be consistently demonstrated by the investigations of this study that the cheese smear composition exhibits a rather stable microbial composition over the storage time. Various microbial species with the potential to contribute to the smear defect development during storage of foil-prepacked cheeses were identified such as *Bacillus* spp., *Propionibacterium* spp., *Peptoniphilus* spp. and *Proteus vulgaris*.

In conclusion, this study supports the hypothesis of a complex etiology of the observed smear defect. More than a single factor seems to contribute to the development of the defect during storage, where the presence of more than one problem causing organism is doubtless. However, environmental factors such as pH, accumulation of water and gas within the package that exert an influence on the microbiota are also not neglectable. In regard of the results obtained in this study, an alteration of the metabolic properties of the present microflora due to vacuum foil-packaging seems to be more likely than dramatic changes of the microbial composition. To further investigate the development of a wet, smudgy, sticky and off-odorous surface smear during storage of foil-prepacked red-smear cheeses future studies will focus on the analysis of the changes in the metabolic activity of the microorganisms present in unpacked and foil-prepacked cheese.
Acknowledgments

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Chapter 3

Effect of packaging materials, environmental factors and rRNA transcriptional activity of surface microflora on red-smear cheese defect development

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Abstract

Two Swiss cheese varieties were investigated to elucidate how microbial growth and rRNA transcriptional activity leads to a defective cheese surface when red-smear cheese portions are wrapped in plastic films. Four alternative films with different water and gas permeabilities were used for packaging experiments. A defective smear was associated with anaerobic conditions, increases in water activity and decreases in pH over a period of 8 weeks at 8°C. Decreases of log 0.7-1.6 total bacteria, log 0.6-1.2 Corynebacterium casei and log 0.6-1.6 Bacillus spp. 16S rRNA copy numbers measured by RT-qPCR were obtained for both varieties under packaging conditions suggesting a decrease in transcriptional activity. One alternative film, which was porous to O₂, CO₂ and water vapor, created a less humid smear and higher pH during storage. In conclusion, cheese smear development is mainly dependent upon packaging film’s permeability and is associated with a decrease in the rRNA transcriptional activity of cheese smear microorganisms.
1. Introduction

Post-ripening storage of red-smear cheeses (Brennan, 2004; Larpin-Laborde, et al., 2011; Mounier, et al., 2005; Mounier, et al., 2009) in film-prepacked portions undergoes dramatic changes to negative consumer perception due to off-odorous and smudgy surface (Amato, et al., 2012). These sensorial defects might be related to metabolic activities of cheese surface microbes in response to the new environmental conditions created between the film and the smear and diffusional barriers. Important factors that exert influence on the smear ecosystem under storage conditions are redox potential, the pH, water activity, salt and interactions with the cheese dough. The influence of wrapping and physicochemical factors on the rRNA transcriptional activity of cheese smear microflora after ripening was to our knowledge little investigated.

Reverse transcription qPCR (RT-qPCR) is a powerful method to estimate the transcriptional activity of microorganisms and has been widely used to analyze gene expression in pure cultures (Deutsch, Falentin, Dols-Lafargue, LaPointe, & Roy, 2008; Reimann, Grattepanche, Rezzonico, & Lacroix, 2010). It is also a suitable method to monitor the fate of microorganisms during cheese making and ripening (Falentin, et al., 2010, Falentin et al., 2012). Determination of gene expression on the surface of retail cheese samples is challenging compared to pure cultures due to the complex matrix and the presence of uncharacterized microbiota (Monnet, et al., 2013). The RNA pool represents the global transcription activity of the cheese microbiota, and normalization of RT-qPCR data can be performed with the cheese weight (Monnet, et al., 2013). In this study, the global transcription activity of cheese smear microflora during storage was measured by 16S rRNA gene transcripts normalized to the surface of the cheese.

Cheeses are dynamic products in which processes do not stop immediately after controlled ripening, many of them may continue within the packaging and storage until consumption.
For certain varieties, particularly those with active surface microflora such as bacterial surface-ripened or mold-ripened cheese, packaging has a strong effect on the microbial processes of the cheese and is increasingly recognized as an important factor in protecting and controlling the quality of cheese (Kelly, 2007). Factors that must be considered in selecting a cheese packaging material include permeability to water vapor, oxygen, NH₃, CO₂ and light (Kelly, 2007). To our knowledge, only a few studies have addressed packaging material in the case of red-smear cheeses. Picque et al. (2011) investigated the impact of three films that differed in gas and water vapor permeability on the quality of Saint-Nectaire cheese, a pressed, uncooked, mold-ripened French cheese. Conte et al. (2013) investigated a bio-plastic film based on polylactic acid (PLA) with antimicrobial properties provided by copper nanoparticles for Italian Fiordilatte cheese packaging.

The aim of our study was to investigate physicochemical factors such as pH, water activity and redox potential of the cheese smear and microbial factors that contribute to cheese smear defects in plastic films before packaging and during storage at 8°C. *Corynebacterium casei* was chosen as a typical representative of red-smear cheese surface microorganisms, and *Bacillus* spp. was chosen as a previously isolated atypical cheese smear microorganism that produces exopolysaccharides (Amato et al. 2012).

Complete DNA and RNA transcripts of total bacteria, *C. casei* and *Bacillus* spp. were measured by qPCR and RT-qPCR to determine cell copy numbers and rRNA transcriptional activity, respectively. To investigate the effect of packaging material on the development of cheese surface defects, portions of two Swiss red-smear cheese varieties (M and T) were wrapped in their standard and four alternative films that possess different antimicrobial, gas and water vapor properties.
2. Materials and methods

2.1 Cheese selection, packaging and defect definition

All 21 portions of the fully ripened Swiss red-smear cheese variety M (see Table S1 in the supplementary information) were obtained after ripening in the cheese cellar for at least 75 days at 13-14°C (relative humidity (RH) at approximately 90%) while those of variety T spent 70-110 days at 14°C (RH 92%) (Amato, et al., 2012). All cheese portions used as controls were wrapped in gas- and water-tight standard film (Csf) with the standard packaging process used by industry before being stored at 8°C. The storage temperature corresponds to the average temperature of the door of a domestic fridge (Koutsoumanis, Pavlis, Nychas, & Xanthiakos, 2010; Laguerre, Hoang, & Flick, 2013). The cheese portions for the experiment were packaged in four alternative films (Table 1) before being stored at 8°C for 8 weeks. Film A was a nanosilver-coated film with antimicrobial activity that was selected to inhibit cheese surface microorganisms. The influence of O\textsubscript{2}, CO\textsubscript{2} and water vapor permeabilities on defect development was studied with films of high (film B), medium (film C) and very high (film D) gas and water vapor permeability. Cheese surface samples were taken before packaging (bfp) and after 2, 4, 6 and 8 weeks and were analyzed for appearance, pH, water activity value, redox potential, microbial composition and rRNA transcriptional activity.

Table 1
Technical data for standard films (Csf M and T) and alternative films (A-D) used in this study.

<table>
<thead>
<tr>
<th>Code</th>
<th>O\textsubscript{2}</th>
<th>CO\textsubscript{2}</th>
<th>water vapor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[cm\textsuperscript{3} m\textsuperscript{-2} d bar]</td>
<td>[cm\textsuperscript{3} m\textsuperscript{-2} d bar]</td>
<td>[g m\textsuperscript{-2} d]</td>
</tr>
<tr>
<td>Csf M</td>
<td>&lt; 30</td>
<td>&lt; 50</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>Csf T</td>
<td>&lt; 30</td>
<td>&lt; 105</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>A</td>
<td>0.7</td>
<td>- \textsuperscript{a}</td>
<td>0.5</td>
</tr>
<tr>
<td>B</td>
<td>&lt; 375</td>
<td>&lt; 1750</td>
<td>&lt; 25</td>
</tr>
<tr>
<td>C</td>
<td>&lt; 175</td>
<td>&lt; 750</td>
<td>&lt; 14</td>
</tr>
<tr>
<td>D</td>
<td>&lt; 1100</td>
<td>&lt; 30500</td>
<td>&lt; 27</td>
</tr>
</tbody>
</table>

\textsuperscript{a} - means "not specified"
The humidity, smudginess and odor of each cheese surface was judged upon opening the package, and overall appearance was defined as no, small or clear defect according to the defect definition scheme (Table 2).

### Table 2
Defect classification scheme of cheese smear surfaces based on humidity, smudginess and odor.

<table>
<thead>
<tr>
<th>Humidity</th>
<th>Smudginess</th>
<th>Odor</th>
</tr>
</thead>
<tbody>
<tr>
<td>I&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>dry</td>
<td>not smeary typical cheese</td>
</tr>
<tr>
<td>II&lt;sup&gt;c&lt;/sup&gt;</td>
<td>little humid sticky</td>
<td>little off-odor</td>
</tr>
<tr>
<td>III&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>humid smeary</td>
<td>intensive off-odor</td>
</tr>
<tr>
<td>IV&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>very humid very smeary</td>
<td>very intensive off-odor</td>
</tr>
</tbody>
</table>

<sup>a</sup> no defect: ≥ 2 criteria from column I
<sup>b</sup> clear defect: ≥ 2 criteria from column III and IV
<sup>c</sup> small defect: other criteria combinations

### 2.2 Determination of weight, redox potential, water activity and pH of the cheese surface

Each cheese portion was weighed (Mettler Toledo PB602-S, Greifensee, Switzerland) immediately after being wrapped in the film and before opening to calculate storage weight loss as a function of different packaging materials. The graph depicts one measurement at each time point that is, in fact, the mean of four measurements each for standard film and films B, C and D after 8 weeks of storage. The redox potential of the cheese surface was checked with anaerobic atmosphere indicator strips (bioMérieux, Marcy l’Etoile, France) that were added during packaging. If the color of the strip changed from blue (ae) to white (an), anaerobic conditions occurred wherein the redox potential dropped below the transition point of +11 mV. Water activity ($a_w$-value) of the cheese surface was measured with an AquaLab CX3 meter (Gerber Instruments AG, Effretikon, Switzerland) at 25°C on a round surface slice (4 cm diameter and 2-3 mm thick) taken from the upper side of the cheese portions. The
smear pH was determined by inserting an electrode into the smear/peptone solution (8 cm², 50 mL⁻¹) as described previously (Amato et al., 2012). Both the $a_w$-value and the pH are expressed as the mean values of at least three measurements performed on the same sample.

2.3 DNA and RNA extraction from the cheese smear and reverse transcription of purified RNA

A cheese smear volume (8 cm² rectangle x 2-3 mm thickness) was cut with a sterile knife, diluted in 50 mL of peptone solution and homogenized in a stomacher. Then, DNA was extracted using a Fast DNA Spin Kit for Soil (MP Biomedicals LLC, Illkirch, France) as described previously (Amato et al., 2012).

A second sample of rectangular area 8 cm² and corresponding to a weight of approximately 2.3 ± 0.2 g was used for RNA isolation. It was aseptically cut into small pieces and mixed with 4 mL of sterile filtered 4 M guanidine thiocyanate (VWR International AG, Dietikon, Switzerland)-0.1 M Tris-HCl (Chemie Brunschwieg AG, Basel, Switzerland; pH 7.5) and 250 µL of sterile filtered 10% N-laurylsarcosine (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) (Bonaïti, Parayre, & Irlinger, 2006). Approximately 3 mL of sterile solid glass beads of diameter 3-4 mm (Sigma-Aldrich Chemie GmbH) were added, and the assay was homogenized for 5 min with a vortex until the cheese smear had dissolved. This raw extract was stored at -80°C until further use. A volume of 500 µL was used for RNA extraction according to Monnet et al. (2008) with some adaptations. One mL of Trizol (Life Technologies Europe, Zug, Switzerland) and 500 µL of zirconia beads (0.1 and 0.5 mm in diameter mixed 1:1 v/v; Carl Roth GmbH, Karlsruhe, Germany) were added to the raw extract, and 2.03x 10⁸ copies of luciferase mRNA (Promega, Dürendorf, Switzerland) were used as an internal reference to estimate loss of RNA during extraction and purification after cell lysis (Reimann, et al., 2010). Mechanical disruption was performed three times in a
Retsch mill for 60 s at the maximal frequency of 30 s⁻¹ with a 3 min cooling step on ice between cycles. This treatment was followed by centrifugation at 4°C for 10 min at 12'000 g. The supernatant was treated with 230 µL of chloroform (Sigma-Aldrich Chemie GmbH) for 3 min at room temperature (22°C) and for 2 min on ice before centrifugation at 12'000 g and 4°C for 15 min. The RNA in the upper water phase was precipitated with 700 µL of 2-propanol (Sigma-Aldrich Chemie GmbH) at RT for 15 min before centrifugation at 4°C for 15 min at 10'000 g. The pellet was resuspended in 85 µL of RNase-free water for DNase treatment with 10 µL of RDD buffer and 5 µL of DNase (containing 30 Kunitz units) from a RNase-Free DNase Set (Qiagen AG, Basel, Switzerland); the solution was mixed and incubated for 15 min at room temperature. RNA cleanup was performed using RNeasy Mini Kit (Qiagen AG) according to the manufacturer’s protocol. RNA was eluted in 85 µL of RNase-free water after the first cleanup to perform a second DNase treatment as described; the final solution was eluted in 30 µL of RNase-free water.

The total RNA concentration and purity were determined spectrophotometrically using a Nanodrop ND-1000 (Peqlab, Erlangen, Germany). To check for DNA contamination of the extracted RNA, a PCR targeting 16S rRNA gene with primers Eub 338 F and Eub 518 R (Table 3) was performed in duplicate for each sample.

Reverse transcription (RT) was performed with four µL of purified total RNA (20-200 ng µL⁻¹) from above in a volume of 40 µL as described before (Reimann et al., 2010).
Table 3
Oligonucleotide primers targeting 16S rRNA and luciferase genes used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target</th>
<th>Application</th>
<th>Sequence (5'-&gt; 3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eub 338</td>
<td>Eubacteria&lt;sup&gt;a&lt;/sup&gt;</td>
<td>qPCR and DNA contamination</td>
<td>ACTCCTACGGAGGAGGCAGCAG</td>
<td>Weisburg et al. (1991)</td>
</tr>
<tr>
<td>Eub 518</td>
<td>Eubacteria&lt;sup&gt;a&lt;/sup&gt;</td>
<td>DNA contamination</td>
<td>ATTACCCCGGGCTGGCTGG</td>
<td>Muyzer et al. (1993)</td>
</tr>
<tr>
<td>Fs15</td>
<td>Corynebacterium casei&lt;sup&gt;a&lt;/sup&gt;</td>
<td>qPCR</td>
<td>CCGCAAGGCTAAAACCTCAAAAGGAAT</td>
<td>Monnet et al. (2006)</td>
</tr>
<tr>
<td>Fs 17</td>
<td>Corynebacterium casei&lt;sup&gt;a&lt;/sup&gt;</td>
<td>qPCR</td>
<td>ACCGACCACAAGGGAAAGACT</td>
<td></td>
</tr>
<tr>
<td>pA</td>
<td>Eubacteria&lt;sup&gt;a&lt;/sup&gt;</td>
<td>qPCR Standard</td>
<td>AGAGTTTGATCCTGGCAG</td>
<td>Edwards et al. (1989)</td>
</tr>
<tr>
<td>pH</td>
<td>Eubacteria&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C. casei</td>
<td>AAGGAGGTGATCCAGCCGCA</td>
<td>Han et al. (2012)</td>
</tr>
<tr>
<td>YB-F</td>
<td>Bacillus spp. &lt;sup&gt;a&lt;/sup&gt;</td>
<td>qPCR</td>
<td>GCAACGAGCGCAACCCCTTGGA</td>
<td></td>
</tr>
<tr>
<td>YB-R</td>
<td>Bacillus spp. &lt;sup&gt;a&lt;/sup&gt;</td>
<td>qPCR</td>
<td>TCATCCCCACCTTCCCTCCGCT</td>
<td>Mosoni et al. (2007)</td>
</tr>
<tr>
<td>F8</td>
<td>Eubacteria&lt;sup&gt;a&lt;/sup&gt;</td>
<td>qPCR Standard</td>
<td>AGAGTTTGATCMTTGGCTC</td>
<td></td>
</tr>
<tr>
<td>1492 R</td>
<td>Eubacteria&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Bacillus spp.</td>
<td>GNTACCTTGTACGACTT</td>
<td></td>
</tr>
<tr>
<td>reflucF</td>
<td>luciferase gene&lt;sup&gt;b&lt;/sup&gt;</td>
<td>qPCR</td>
<td>TACAAACACCCCAACATCTTTCGA</td>
<td>Johnson et al. (2005)</td>
</tr>
<tr>
<td>reflucR</td>
<td>luciferase gene&lt;sup&gt;b&lt;/sup&gt;</td>
<td>qPCR</td>
<td>GGAAGTTCACCGGCGTCAT</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> 16S rRNA gene

<sup>b</sup> Internal reference mRNA
2.4 Quantitative real-time PCR (qPCR) of the DNA and cDNA of 16S rRNA genes

DNA samples were measured to quantify the bacterial population; cDNA was measured to monitor bacterial rRNA transcriptional activity; and RNA was used as a negative control for DNA contamination and non-specific amplification by qPCR (data not shown).

The 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, California, USA) was used for qPCR analysis of 16S rRNA genes amplified from total cheese surface bacteria, C. casei and Bacillus spp. for total DNA and cDNA (reverse-transcribed RNA) to quantify the microbial population and rRNA transcriptional activity, respectively. Quantification of total bacterial 16S rRNA gene copy numbers for each cheese smear surface was performed for DNA, cDNA and RNA samples in the same run as previously described for DNA samples by Amato et al. (2012).

Quantification of C. casei DNA and cDNA copy numbers was achieved using the primers fs15 and fs17 (Table 3). Templates for the generation of qPCR standard curves were produced by amplification of C. casei DSM 44701 T in a standard PCR with primers pA and pH (Table 3), and dilutions (10^{-2}-10^{-9}) containing a predefined template copy number were processed at the same time and in the same way as the tested samples. The annealing temperature was set to 65°C instead of 60°C for C. casei.

Quantification of Bacillus spp. DNA and cDNA were performed using primers YB-F and YB-R (Table 3), and standards were produced with Bacillus subtilis Wiesby 168 (Laboratory of Food Biotechnology culture collection) as a template with primers F8 and 1492 R (Table 3) that targeted the 16S rRNA gene. Dilutions (10^{-4}-10^{-9}) containing predefined template copy numbers were amplified at the same time as tested samples.

The total RNA recovery rate of each sample was calculated after reference luciferase cDNA quantification was performed using primer refLuc F and refLuc R (Table 3) and cDNA derived from the reverse transcription of the luciferase mRNA in dilutions (10^{-1}-10^{6}) to generate standard curves with predefined copy numbers.
2.5 Data calculation from qPCR

Data analysis was performed by the 7500 Fast System Sequence Detection software Version 1.4 (Applied Biosystems, Carlsbad, USA) using automatic cycle threshold (ct). Standard curves were generated by plotting ct against the log quantity of the 10-fold dilutions for specific standards in each run. Standard curves with linear ct values ($R^2 > 0.99$) and amplification efficiencies above 90% ($\log E = \text{slope}^{-1}$; slope close to -3.3) were accepted. For each run, a dissociation curve was generated to check for the product specific melting temperature. Two dilutions of DNA and cDNA were measured in triplicate and the means are displayed in the graphs normalized per square centimeter of the cheese surface. Average RNA recovery rates of 87.8± 4.5 % for M and 86.12± 3.8 % for T were achieved for the 21 cheese surfaces as measured by internal reference Luciferase qPCR (see Fig. S1 in supplementary information). As RNA extraction and recovery rate were reproducible over all 42 samples and the data for quantifications over storage time are comparable among all samples, the RNA recovery coefficients were neglected in the calculation.
3. Results and discussion

3.1 Intrinsic factors of cheese smear defects in standard films

To monitor the effects of wrapping on red-smear cheese surface defects, redox potential, water activity value and pH were measured before wrapping the cheese portions into standard film and again every second week after packaging and storage at 8°C. Anaerobic conditions were achieved for all cheese portions of both varieties in standard film within one day after packaging, which confirmed other observations; no respiratory activity was measured in the film with low water vapor permeability (Picque et al. 2011). Water activity increased from 0.937 to 0.948 for variety M and from 0.943 to 0.948 for variety T (Fig. 1 A and B). Variety M displayed a delayed increase with similar kinetics compared to variety T. Water diffuses from the cheese center to the surface, which is drier than in dough, and has a reverse salt gradient when cheese is ripened in the cellar (Pavia, Trujillo, Guamis, & Ferragut, 2000). As long as the cheese is not wrapped in film, water evaporates and the surface dries. When the cheese portions are packed in a water-vapor-tight film, the water accumulates on the cheese surface and contributes to cheese surface humidity and increased water activity. The pH dropped strongly after wrapping the cheese portions in standard film from 8.3 to 7.0 for variety M and from 7.8 to 7.3 for variety T over 8 weeks of storage (Fig. 1 A and B). The pH decrease could be caused by CO$_2$, which is produced by smear microorganisms through lactate degradation and decarboxylation of amino acids at the cheese surface (Bennet & Johnston, 2004) and by diffusion of the solute (lactate) with water. CO$_2$ accumulation may occur for the CO$_2$-tight standard film.
3.2 Microbial populations and rRNA transcriptional activity during defect development in standard film

A clear and progressive defect development over storage time was observed after 6 weeks of storage for both varieties wrapped in standard films (Fig. 2). Under these conditions, total bacteria DNA copy numbers dropped slightly from the initial packaging to week 8 from log 10.7 to log 10.3 for variety M (Fig. 2 A) and from log 10.8 to log 10.6 for variety T (Fig. 2 B). *C. casei* DNA copy numbers were detected at a magnitude of $10^9$ for both varieties over the storage period; this represented 6.1 % and 6.3 % of the number of copies for the total population for varieties M and T, respectively. *Bacillus* spp. DNA copy numbers were of the magnitude of $10^8$ for variety M and $10^7$ for variety T, which account for 0.4% and 0.03% of the total cheese surface population, respectively. A slight decreasing tendency over the storage period was also observed in DNA copy numbers of *C. casei* and *Bacillus* spp. (Fig. 2). The cDNA (RNA transcripts) representing 16S rRNA copy numbers were measured for varieties M and T (Fig. 2) to quantify rRNA/protein synthesis ability in the cheese smear. RNA transcripts of total bacteria were detected at log 13.1 for M and log 12.5 for T before wrapping the cheese in plastic film. A clear decrease of log 0.7 in variety M and log 1.6 in variety T was observed after packaging. The number of copies of RNA transcripts of *C. casei* and *Bacillus* spp. also showed a clear decrease of 0.6-1.2 log and 0.6-1.6 log over the
storage period for varieties M and T, respectively. The decrease in the number of 16S rRNA transcripts over the storage period for all target groups and cheese varieties suggests a strong decrease of bacterial protein synthesis ability in the cheese smear after packaging. It has been argued that targeting the 16S rRNA gene, even at the RNA level, does not create a true picture of the active species of an ecosystem because 16S RNA molecules are much too stable (Sheridan, Masters, Shallcross, & Mackey 1898). It has been shown that 16S rRNA copies are among the more stable molecules but did clearly decrease 24 hours after bacterial death (Cenciarini, et al., 2008). Furthermore, total RNA is still regarded as being continuously transcribed when detected over a time span of 2 months in a cheese matrix (Falentin, et al., 2012). The quantification of cDNA copy numbers targeting 16S rRNA genes is considered a valuable approach for assessing the viability of bacterial cell to perform one kind of metabolic activity in a complex cheese smear system over elongated time spans such as 8 weeks. The decrease of rRNA transcriptional activity observed for all tested groups (including total bacteria, a typical cheese smear representative and a potentially problem-causing organism) suggests that defect development cannot be allocated to only one microorganism species or microorganism group but rather to a general shift of the smear microflora after wrapping the cheese portion in plastic film. These findings are in agreement with our previous observations using culture-dependent methods (Amato, et al., 2012) for which a very similar composition of the smear microflora was found independent of the presence or absence of a defective smear. There is, so far no evidence whether reduced ability to synthesize rRNA and proteins is accompanied with dying cells which in turn could contribute to defect development due to cell lysis and release of intracellular enzymes. Decreases in bacterial cell numbers due to autolysis have been associated with increased peptidolytic activity in the cheese (Ndoye et al 2011).
Fig. 2. 16S rRNA gene copy numbers (cn) of cheese varieties M (A) and T (B) before packaging (bfp) and after 2, 4, 6 and 8 weeks of cold storage. Bacterial composition and rRNA transcriptional activity were measured by DNA and cDNA (rRNA transcripts) copy numbers, respectively, for total bacteria, C. casei and Bacillus spp. Total bacteria DNA (●) and cDNA (■), C. casei DNA (▲) and cDNA (▲), and Bacillus spp. DNA (●) and cDNA (●) are depicted for two red-smear cheese surface (A and B). Defect classification is illustrated beneath the graphs according to the defect definition scheme (Table 2).

3.3 Weight reduction of cheese in standard and alternative packaging films

Cheese portions were wrapped in films A (contains nanosilver), B (high gas and water vapor permeability), C (medium permeability) and D (very high permeability) for subsequent storage at 8°C for 8 weeks (Table 1). Because cheese weight during storage is important to cheese quality and profitability and should be tightly controlled for each cheese variety, this parameter was monitored during cheese storage in the different films (Fig. 3). Weight losses over the 8-week storage time for portions packed in standard films were 0.3±0.09 % and 0.4±0.04 % for varieties M and T, respectively. The weight losses for samples in all alternative films were higher with a maximum of 1.2±0.01 % after 8 weeks, which was recorded for film C (Fig. 3 A). The measured weight losses for standard film for varieties M and T are in agreement with values measured for low water vapor permeability packaging for St. Nectaire cheese (Picque et al., 2011).
Fig. 3. Weight loss over storage time for film-prepackaged samples of variety M (A) and T (B) wrapped in standard film (●), film A (■), film B (▲), film C (●) and film D (X), respectively. Each point in the graphs demonstrates the difference in percentage of its original portions weight at the time of wrapping.

3.4 Redox potential, water activity and pH of the cheese surface wrapped in alternative films compared to standard films

Similar to standard films, anaerobic conditions were achieved inside films B and C (Fig. 4 A and B) within the first day after packaging. In contrast, aerobic conditions were measured on the surface of cheese wrapped in films A and D; this result was not expected for film A but may be explained by the stiffness of the film, which resulted in poor sealing ability and the development of cracks in the seal during the storage time. The aerobic conditions measured in film D were consistent with that film’s high oxygen permeability, and aerobic conditions were further confirmed by mold growth on the cheese smear and dough surface (see Figure S2 in supplementary information). The water activity of the cheese surface increased after packaging for variety M (Fig. 4 C) from 0.937 to 0.948 and for variety T from 0.943 to 0.948 (Fig. 4 D) for all films. A clear relation between the film type and water activity value could not be obtained as the rather high values observed for film A with variety T were very similar to that observed for the standard film with variety M. The pH of the cheese surface wrapped in film C dropped similarly to standard film for both varieties (Fig. 4 E and F) but decreased less than standard films for films A and B. Most notable was the pH stability of the cheese surface wrapped in film D; the value remained at a high pH of 8 for variety M and increased from 7.8 to 8 for variety T. The high pH on the cheese surface was correlated with aerobic conditions...
and visible mold growth. A constant or slightly increasing pH has already been reported for the surface of St. Nectaire cheese wrapped in films of medium and high water vapor permeability; mold growth and respiratory activity were also observed (Picque, et al., 2011). The combination of lactate consumption by the molds and evaporation of CO$_2$ through the highly permeable film could contribute to retain a pH over the time of storage that was similar to the pH measured before packaging.

**Fig. 4.** Redox potential, water activity value and pH over an 8-week storage time for red-smear cheese surfaces of variety M (A, C and E) and T (B, D and F) wrapped in standard film (●), film A (■), film B (▲), film C (●) and film D (X).
Redox potential depicted on top for each variety, anaerobic conditions are labeled as (an), aerobic conditions as (ae). (−) indicates that measurement was not performed. Standard films measurement (Fig. 1) are repeated here as references for the alternative films.
3.5 Appearance, microbial population and rRNA transcriptional activity of cheese smear wrapped in alternative films

The appearances of the cheese smears are depicted in Fig. 5. Films B and C and the standard film produced small defects after two and clear defects after 6 weeks for both M and T varieties. Film A produced the same defects as the standard film for variety T (see Fig. S3 in supplementary information) in the supplementary information), but only small defects were observed for variety M (Fig. 5) at the end of the storage time. The cheese smear of both varieties wrapped in film D showed only small defects with a nice, sparse, dry smear. However, these positive attributes were accompanied by visible mold growth and a moldy smell, which do not fulfill the appearance requirements of a red-smear cheese (see Fig. S2 in supplementary information).

Numbers of DNA copies for total bacteria, *C. casei* and *Bacillus* spp. were very similar to those observed for standard films for all chosen alternative films and for both varieties. A general decrease of protein synthesis ability was detected corresponding to a drop of RNA transcription over the storage period for all alternative films; this was similar to that in standard films. A strong decrease in 16S rRNA copy numbers was observed for film A after 2 weeks for total bacteria (Fig. 5 A), *C. casei* (Fig. 5 C) and *Bacillus* spp. (Fig. 5 D). This decrease was not as pronounced for variety T but was a similar development to that standard films (see Fig. S3 A, C and E in supplementary information). Film A contains silver nanoparticles with proven antimicrobial activity (Loher, Schneider, Maienfisch, Bokorny & Stark, 2008). The initial decrease observed for variety M can be explained by the films’ antimicrobial properties even though the data suggest an unexpectedly low antimicrobial efficacy on the cheese surface. Additional experiments demonstrated the limitations of the films with respect to high bacterial load (10⁸ cfu mL⁻¹) and high protein content (5-20% bovine serum albumin) (M. Schuppler, unpublished data). The diminished antimicrobial activity of film A on the red-smear cheese surface can be explained by the fact that the cheese surface contains a high bacterial load of 10⁹ cfu cm⁻² and high protein content. Films B and C
produced clear defects in both varieties. The decrease in number of cDNA copies over the storage time was observed to be even stronger for the cheeses wrapped in films B and C than in standard film for total bacteria (Fig. 5 A and B) and *Bacillus* spp. (Fig. 5 E and F) and comparable to standard film for *C. casei* (Fig. 5 C and D). Therefore, films B and C could not prevent red-smear cheese surface defects and confirm that defect development is accompanied by a general decrease in rRNA transcriptional activity during storage. The nice, sparse, dry smear observed in film D was classified as only slightly defective due to its undesirable visible mold growth and moldy smell (see Fig. S2 in the supplementary information). The rRNA transcriptional activity of total bacteria (Fig. 5 B), *C. casei* (Fig. 5 D) and *Bacillus* spp. (Fig. 5 F) on cheese smears wrapped in film D decreased over the storage time similar to standard film. These findings were rather unexpected and take into account aerobic conditions within the film (Fig. 4 A and B), the high steady pH compared to standard film (Fig. 4 E and F) and the preserved appearance of the smear. The inhibition of the bacterial flora could be partly due to the strong visible mold growth. Although this film could not completely prevent red-smear cheese surface defects, it harbors the potential for applications in combination with mold growth inhibition.
Fig. 5. Red-smear cheese defect development over storage time for cheese wrapped in standard film compared to alternative films. The appearance of the cheese surface is shown on top for the films shown in the graphs in the corresponding columns. DNA and cDNA 16S rRNA copy numbers of total bacteria (A and B), C. casei (C and D) and Bacillus spp. (E and F) are depicted for standard film (closed lines) and the four alternative films (interrupted lines) for cheese variety M. Standard films were shown in Fig. 2 A and are repeated here to allow easy comparison of the films.

Standard film (DNA: ◆ and cDNA ○) are depicted in each graph as a reference. Alternative films A (DNA ■ and cDNA ○) and B (DNA ▲ and cDNA △) are shown in the first column (A,C and E). Alternative films C (DNA ● and cDNA ○) and D (DNA X and cDNA X) are depicted in the second column (B, D and F).
4. Conclusions

Our analysis of red-smear cheese surface defect development in film-prepackaged portions revealed that defect development in two independent fully ripened Swiss varieties is associated with anaerobic conditions, increases in water activity value and decreases in pH. Our data suggest that defect development is correlated with diminished rRNA transcriptional activity of the microbiota including total bacteria, *C. casei* and *Bacillus* spp. The tight plastic film used as the industry standard may cause an increase in the amount of water on the cheese’s surface, but it also allows the accumulation of gases such as CO$_2$, NH$_3$ and short-chain fatty acids (SCFA) in the smear and within the package (Fig. 6). Water accumulation increases the water activity on the surface; CO$_2$ accumulation decreases the pH on the surface; and anaerobic conditions could decrease the rRNA transcriptional activity of the cheese smear microflora within standard film.

The cheese storage tests with four alternative films (three of which produced defects) demonstrate that packaging cheese portions into films always confronts the possibility of a smudgy, humid and off-odorous smear. We demonstrated that the film properties do influence the physicochemical cheese smear characteristics and, consequently, the cheese smear microflora. Aerobic conditions and higher pH combined with a healthy microflora seem to be favorable for a high quality cheese smear. This study suggests a future approach would involve testing a water vapor permeable film in combination with either mold growth inhibition or oxygen control using O$_2$-scavengers or O$_2$-tight film in order to prevent cheese smear defect.
Fig. 6. Model of red-smear cheese surface under different packaging conditions.
Acknowledgments

This project was performed with an industrial partner of the Swiss cheese industry and is supported by the Swiss innovation promotion agency (CTI, project number 10273-3). We kindly thank Perlen Converting AG, Sealed Air Cryovag, BVS Verpackung and Suedpack for providing the different packaging materials.
Table S1
Overview of all red-smear cheese surface samples of two Swiss cheese varieties M and T taken before (unpacked) and after 2, 4, 6 and 8 weeks (w) of storage after wrapping them in either the standard films (Csf) or in one of the four alternative films A-D.

<table>
<thead>
<tr>
<th>Week</th>
<th>unpacked</th>
<th>Csf</th>
<th>Film A</th>
<th>Film B</th>
<th>Film C</th>
<th>Film D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>M42</td>
<td>M43</td>
<td>M44</td>
<td>M45</td>
<td>M46</td>
</tr>
<tr>
<td>2 w</td>
<td></td>
<td>T46</td>
<td>T47</td>
<td>T48</td>
<td>T49</td>
<td>T50</td>
</tr>
<tr>
<td>4 w</td>
<td></td>
<td>M48</td>
<td>M49</td>
<td>M50</td>
<td>M51</td>
<td>M52</td>
</tr>
<tr>
<td>6 w</td>
<td></td>
<td>T52</td>
<td>T53</td>
<td>T54</td>
<td>T55</td>
<td>T56</td>
</tr>
<tr>
<td>8 w</td>
<td></td>
<td>M53</td>
<td>M54</td>
<td>M55</td>
<td>M56</td>
<td>M57</td>
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</table>

Fig. S1. Internal standard reference Luciferase cDNA copy number recovery rate as an indication of RNA loss during extraction after bacterial cell disruption.
Fig. S2. Cheese surface after 8 weeks of variety M wrapped in standard film (A) and film D (B). Redox strip indicating anaerobic conditions (white) for standard film (A) and aerobic conditions (blue) for films D (B, C and D). Mold growth on cheese surface of variety T on the dough (C) and the smear (D) accompanied by nice, sparse and dry smear wrapped in film D (B, C and D) compared to standard film (A).
Fig. S3. Red-smear cheese defect development over storage time for cheese wrapped in standard film compared to alternative films. The appearance of the cheese surface is shown on top for the films shown in the graphs in the corresponding columns. DNA and cDNA 16S rRNA copy number of total bacteria (A and B), C. casei (C and D) and Bacillus spp. (E and F) are depicted for standard film (closed lines) and the four alternative films (interrupted lines) of cheese variety T. Standard film was shown in Fig. 2 B before and is repeated for an easier reading and comparison of the films.

Standard film (DNA ◆ and cDNA ○) are depicted in each graph as a reference. Alternative films A (DNA ■ and cDNA □) and B (DNA ▲ and cDNA △) are shown in the first column (A, C and E). The alternative films C (DNA ● and cDNA ○) and D (DNA X and cDNA X) are depicted in the second column (B, D and F).
Chapter 4

Different strategies to combat red-smear cheese defect

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Abstract

Five different approaches for red-smear cheese surface defect prevention in film-prepacked portions like salting, drying, application of protective cultures and antifungal component as well as alternative packaging materials were performed with Swiss variety T. The challenge tests during 8 weeks at 8°C demonstrated that neither salting nor drying or application of bacteriocin producing *Pediococcus* strain combined with standard film could prevent defect formation. The application of salt on the cheese surface even obviously enhanced the cheese smear defect. Protective cultures in application of brush or spray method and antifungal component in combination with previously promising film D beside mold growth was not sufficient to completely prevent defect formation. The pH in film D increased 0.2-0.3 in either application. Nevertheless, defect development mechanism like a clear pH drop of 0.5 over storage time, decrease of microbial transcriptional activity and living bacterial cells over storage time in defect development could be repeatedly shown in standard film. Finally, the challenge test of a further alternative film E with and without antifungal component could completely prevent defect formation.
1. Introduction

Red-smear cheese surface defect in film-prepacked portions is a problem of modern society demanding long lasting products accessible in self-services shelves (Chapter 2). A complex etiology of the cause for red-smear cheese defect has been postulated and a high impact of packaging material on the cheese surface microflora has been shown (Chapter 3). Various trials to enlighten different aspects of cheese smear defect development in search for a global solution of a complex problem are summarized in this chapter.

The use of salt (NaCl) as a food preservative dates from prehistoric times and together with fermentation and dehydration (air/sun), is one of the classical methods of food preservation (Guinee & Fox, 2004). Salt together with the desired pH, water activity and redox potential, contributes to minimization of spoilage and prevention (Guinee & Fox, 2004). This ancient method of preventions could be applied on whole loaf cheese surface before cutting the cheese loaf to portions and wrapping them into plastic film for distribution and sale. Drying the cheese loafs before packaging could prevent the cheese surface to develop a defect because of the drier smear at time of packaging. This practice is used by the Suisse cheese industry and was communicated to be efficient due to the water loss before packaging (Industrial partner, personal communication). Biopreservation with the aid of biologically derived antimicrobials of the producer cultures active against bacteria and fungi is a helpful tool to avoid an overuse of food additives (Schwenninger & Meile, 2004). Protective cultures and bacteriocin producing strains are important in food industry and find its origin in the growing consumers’ demands for foods that are ready to eat, fresh-tasting, nutrient and vitamin rich, but at the same time minimally-processed and preserved (Galvez, et al., 2007).

Bacteriocins, are ribosomally synthesized antimicrobial peptides, produced by Gram-positive and Gram-negative bacteria. Lantibiotics and pediocin-like bacteriocins, two bacteriocins of Gram-positive bacteria, have attracted a lot of attention because of their broad range of action against other Gram-positives including some important pathogens such as staphylococci and Listeria (Nes, et al., 2007). Nisin (E234) is the most well-known bacteriocin...
with a broad range used for processed cheese, salad dressings, canned foods and meats to extend shelf live (Muppalla, et al., 2012). Pediocin of *Pediococcus acidilactici* was demonstrated to have a strong inhibitory activity against *Listeria monocytogenes* in various meats such as fermented sausages and vacuum-packaged products as well (Holzapfel, et al., 1995). Some *Pediococcus* strains are of particular importance in the food industry for use as biopreservation tools or probiotic strains through the production of the bacteriocin with high anti-*Listeria* activity (Mathys, et al., 2007). Pediocin is proposed for inhibition of food borne pathogens and food spoilage organisms and food contaminating molds (Narayanan, et al., 2013). The addition of a pediocin producing strain *Pediococcus acidilactici* for cheese surface defect prevention was tested in standard film. Furthermore, the concept of biopreservation was applied by a protective culture to use against mold growth in combination with the high gas- and water vapor permeable film D previously shown to be promising in defect prevention (Chapter 3). The commercially available protective culture Holdbac YMC containing a *Propionibacterium jensenii* and *Lactobacillus paracasei* subsp. *paracasei* strain has been shown to have antifungal properties in yoghurt and on cheese surface models (Schwenninger & Meile, 2004). The improvement of promising film D by addition of an antifungal component containing natamycin needs to be evaluated. Natamycin is a fungicide that occurs naturally and is produced during fermentation by the bacterium *Streptomyces natalensis*, commonly found in soil (Vierikova, et al., 2013). It has a broad spectrum of activity for most yeasts and fungi that come into consideration in food (EFSA, 2009). Presence of natamycin has not any influence on taste or appearance and is approved for the surface treatment of semi-hard and semi-soft cheese and dry, cured sausages at a maximum level of 1mg dm$^{-2}$ surface (EFSA, 2009) Natamycin is permitted as an antimicrobial preservative in European Union (EU) countries, mainly for processed meat and cheese products. The Swiss cheese association (KOS) decided not to use natamycin (Switzerland Cheese Marketing, 2007).

The aim of these various approaches was to enlighten different aspects of defect development to find a possible solution. Therefore, the cheese surface defect was tried to be
Different strategies to combat red-smear cheese defect

prevented by addition of dry salt on the surface, drying the cheese loafs and application of a bacteriocin producing strain to inhibit growth or activity of possible defect causer before packaging the cheese portions into standard film. Further, film D, the so far best performing packaging material with the drawback of mold growth (Chapter 3) is tested in combination with a protective culture, an antifungal component and wrapped in a thicker version of film D to overcome the drawback of mold growth. In a last step, a water vapor permeable, alternative packaging material was tested with and without Delvocid. All these approaches for defect prevention performed in challenge tests over storage time at 8°C will contribute to better understanding of cheese surface defect mechanism and its prevention.
2. Materials and methods

All cheese loafs and cheese portions used in these experiments were fully ripened Swiss red-smear cheese of variety T provided by the corresponding cheese partner as previously described (Amato, et al., 2012). Various strategies like application of additives, pretreatment of the cheese portions and varying packaging material for defect prevention of red-smear cheese surface defect in film-prepacked portions were performed. All cheese portions used as controls were wrapped in gas- and watertight standard film (Csf) by the industrial partner to keep original packaging process before they were stored at 8°C (Chapter 3).

2.1 Addition of salt on the cheese surface

Whole cheese loaf was dry salted by turning the whole loaf in pure dry NaCl immediately before cutting it into eight portions and wrapping them in standard film. A control from the same production was cut and film-prepacked without the application of salt. Salted cheese portions and controls were opened and analyzed for appearance, water activity and pH after one and six weeks as previously described (Chapter 3).

2.2 Drying the cheese surface before packaging

Whole cheese loafs were dried at 14°C, at relative humidity (RH) of 70% for 24 or 48 hours before cutting and packaging into plastic standard film. Weight loss during drying period was monitored by weighing the cheese loafs at the beginning and after 24 and 48 hours. Controls from the same production batch, portions dried for 24 and 48 hours were analyzed for optical appearance, water activity and pH, microbial composition and living bacterial cells (described in Chapter 5) before packaging (bfp), as well as after 2, 4, 6 and 8 weeks of storage at 8°C.

2.3 Application of an antimicrobial producing microorganism

Pediococcus acidilactici UVA-1-UL101 (Laboratory of Food Biotechnology culture collection) was cultured in deMan, Rogosa and Sharp broth (MRS, Carl Roth GmbH + Co., Karlsruhe,
Germany) with cysteine (MRSC 0.05% L-cystein hydrochlorid, Merck, Dietikon, Switzerland) overnight under anaerobic conditions at 37°C. The cells were centrifuged and resuspended in 0.85% NaCl solution (Merck) before 2.5 x 10⁹ cells/cm² were applied by brushing 5 x 0.1 mL on each cheese surface. The cheese portions were left to dry for an hour before vacuum packing them into standard film and storage at 8°C.

2.4 Application of protective culture by brush and spray technique in combination with film D, film D19 and Delvocid an antifungal in combination with film D and E

A protective culture (Holdbac YMC, Danisco Cultor AG, Kreutzlingen, Switzerland) for growth control of yeasts and molds and some heterofermentative lactic acid bacteria was applied on the cheese surface. Therefore the freeze dried protective culture was mixed with 0.85% NaCl solution (Merck) to a stock solution containing 5 x 10¹¹ cells mL⁻¹ which was either brushed or sprayed on the surface with the goal to result in approximately 2.5 x 10⁹ cells cm⁻². For application by brush technique five dots of 0.1 mL stock solution were spread on the surface and equally distributed with a silicon brush. For spraying technique stock solution was filled in a spray bottle and one dosage of 1 mL was applied on each cheese surface side. A loss of 50% of solution volume was calculated for spraying based on previous tests. All samples were left to dry for an hour before they were wrapped into high gas and water vapor permeable film D (Chapter 3).

The little thicker Film D₁₉ (19 instead of 16 µm) with slightly reduced oxygen (9500 instead of 11000 cm³ m⁻² d bar), carbon dioxide (26500 instead of 30500 cm³ m⁻² d bar) and water vapor (23 instead of <27 g m⁻² d) permeability was tested.

The antifungal solution containing natamycin was prepared shortly before application by dilution of Delvocid containing 4% of natamycin (Delvocid, DSM Nutritional Products, Kaiseraugst, Switzerland) 1:40 v/v with tap water. A spray bottle was used to apply the antifungal solution on the cheese surface and to ensure good wetting. The surface was left for drying for one hour before wrapping the cheese into high gas and water permeable film D (Chapter 3) and low gas and high water permeable film E (Chapter 5).
A further alternative film E (DSM Nutritional Products) with reduced oxygen and water vapor permeability (Chapter 5) compared to film D (Chapter 3) was tested in combination with and without the antifungal component. The component was applied on the cheese surface as described above. All cheese portions treated and untreated wrapped in either film were stored together with the controls at 8°C.

2.6 Cheese surface analysis

Controls and cheese portions of all approaches were opened and analyzed before packaging, as well as after 2, 4, 6 and 8 weeks. The cheese surface appearance was rated according to the defect definition scheme (Chapter 3). The pH and partly water activity value was monitored as previously described (Chapter 3). Molecular independent methods to measure microbial populations and transcriptional activity were performed as described previously (Chapter 3) and PMA-qPCR for living cells copy number determination targeting the 16S rRNA genes as described in the next chapter (Chapter 5).
3. Results and discussion

3.1 Addition of salt

A very wet and very smudgy smear rated as clear defective (Fig. 1) which was distributed all over the cheese dough and within the packaging was observed when the surface of variety T was dry salted previous to packaging. The addition of dry salt was not successful in defect prevention. In contrast the salt provoked water to diffuse from the cheese dough to the surface and the optical appearance was even worse than the control already 5 days after packaging. A pH reduction of 0.2 by the salt could be observed compared to the control of the same cheese production without addition of salt at the time of measurement one week after packaging (Fig. 1). The pH of controls showed a drop of 0.35 reaching pH 7 over storage time until week 6 the same tendency as previously observed (Fig. 1). The observation of lower pH of the salted surfaces is rather surprising considering that the pH raised with higher salt content of the brine in cheddar cheese (Guinee & Fox, 2004). Furthermore, a decreasing salt in moisture gradient from the surface (~9%, w/w) to the center (~0.2%, w/w) of freshly brine-salted Manchego cheese was paralleled by a pH gradient in the same direction (Pavia, Trujillo, Gu amis, & Ferragut, 1999). Considering that this behaviour was at a pH 5.5-6.0 and pH reduction was associated with lactate production of starter culture of young cheese. The NaCl in watery solution reaches a neutral pH of around 7. The cheese surface pH measured before packaging was in average at 7.4±0.2 for variety T and at 7.6±0.5 for variety M (Data not shown). The quite high standard deviation of variety M was interpreted as an indication that the pH of the cheese surface measured before packaging is influenced by the time of last brining. Water activity value for the control was observed at 0.95 which was in accordance with previous values after wrapping the cheese portions into plastic films. The water activity was decreased dramatically by the salt addition by 0.11 and increased to 0.90 until reaching week 6 (Fig 1). The dramatic reduction of $a_w$-value by salt additions was expected as the oldest preservation methods act by lowering the water activity through addition of water soluble component such as sugar or salt.
(Coppola, et al., 2008). The extremely wet, smudgy and diluted smear that was observed all over the cheese surface and within the package can be explained by the double amount of water lost by NaCl uptake of the cheese. This mechanism was in accordance with the very salty taste of the cheese dough when eaten (data not shown) and the effect of dry salting explained by Guinee (2004). When the cheese surface is dry salted, a counter flow of moisture from the cheese will create a supersaturated brine layer on the cheese surface and salt uptake then occurs by an impeded diffusion process.

**Fig. 1.** Cheese surface pH (full symbols), water activity value (empty symbols) (A) and appearance (B) of control (■, □) and salted (▲, △) cheese portions.
3.2 Drying cheese loaves before packaging

Whole cheese loafs displayed a weight loss of 0.4% after 24 and 0.8% after 48 hours of drying. Cheese portions dried for 24 hours were rated as clear defective after 6 weeks of storage just the same as controls. Cheese portions dried for 48 hours were rated as little defective after 6 and 8 weeks according to a less smeary and bad smelling surface. The defect revealed in a smaller dimension, but could not completely be prevented by drying. The water activity was not influenced by drying and was between 0.94-0.96 similar as the controls. In contrast, the pH of dried samples for 24 and 48 hours before packaging was lowered by log 0.3 but decreased to a pH of 6.9 as the control of the same batch until reaching week 8 (Fig. 2). The DNA copy numbers were measured at 11.0± 0.2 log for total bacteria, at 8.5± 0.1 log for *Bacillus* spp. and at 10.1± 0.2 log for *C. casei* with a decreasing tendency (Fig. 3 A, B, C). A clear decreasing tendency of living bacterial cells correlating with defect development over storage time could be observed in standard film for all targeted group in this trial. Total bacteria decreased by 1 log (Fig. 3 A), *Bacillus* spp. by 2 logs (Fig. 3 B) and *C. casei* decreased most pronounced with 2.5 logs (Fig. 3 C) living bacteria 16S rRNA gene copy numbers per square centimeter measured by PMA-qPCR. The living cell copy number decrease was a bit less pronounced for *Bacillus* and *C. casei* on cheese surface dried for one day, but nevertheless a clear decrease could be still observed. Additionally, the decreasing living cell copy numbers of the control and dried cheese portions were accompanied by a reduction of transcriptional activity (data not shown). This reduction conducted 0.2 log or 21% for total bacteria, 47% for *Bacillus* spp. and even 71% for *C. casei* reaching 8 weeks of storage compared to before packaging. These observations are strongly supporting the hypothesis of a strong impact of drying cells to defect development.
Fig. 2. Cheese surface pH (full symbols), water activity (empty symbols) (A) and appearance (B) development over storage time for controls (■, □) cheese portions dried for 24 (▲, △), and 48 hours (●, ○).
**Fig. 3.** Total DNA (full symbols) and living cells DNA (empty symbols) 16 S rRNA gene copy numbers for total bacteria (A), *Bacillus* spp. (B) and *C. casei* (C) over storage time of controls (■, □), cheese portions dried for 24 (▲, △) and 48 (●, ○) hours.
3.3 Application of an antimicrobial *Pediococcus* strain

The empirical application of the pediocin producing strain *Pediococcus acidilactici* could not prevent the cheese surface to derive wet, smudgy and off-odorous. The *Pediococcus* treated cheese surface developed a clear defect already after six weeks exactly the same as observed for control samples in the same experiment (data not shown). The defect’s cause could not be allocated to one microorganism species or group (Amato, et al., 2012) and the pediocin did not hit the right species spectrum to prevent cheese smear defect, so a more general defect prevention strategy needs to be addressed. Furthermore, the inhibition of the surface microflora was not promising. Therefore, the opposite to generate conditions in which the surface microflora can remain intact gain importance (Chapter 3).

3.4 Film D in combination with a protective culture and an antifungal component

All cheese portions wrapped in film D and D19 revealed a small defect until reaching the end of storage time (Fig. 4). This was a reduced defect compared to the portions in standard film which depicted a small defect starting at week 4 and ended in a clear defect until week 8 (Fig. 4). The mold prevention measurement of applying a protective culture was not effective in the brushing and spraying modes of application. Mold growth was observed starting from week 4. Also film D19 with slightly reduced permeabilities started to develop visible mold growth. The antifungal component containing natamycin only was efficient and could prevent visible mold growth on the cheese smear and dough until reaching the end of storage time. The cheese surface pH dropped by 0.4 for standard film and increased by 0.2-0.3 for all version tested in film D. The pH increase of cheese surface within film D in combination with the antifungal component did show that pH increase is not directly correlating with mold growth as previously possibly assumed (Chapter 3). It can therefore rather be explained by its high CO₂ permeability. In analogy the acidifying role of carbon dioxide in milk was shown by Barbano (Barbano) (2007), who patented the technology of acidified pasteurized milk for fat reduced cheddar cheese production. Nevertheless, satisfying defect prevention could not be achieved with none of the optimization approaches used for film D.
Different strategies to combat red-smear cheese defect

**Fig. 4.** Cheese smear surface pH development over storage time in standard film compared to film D in combination with various treatments. The control standard film (■) and film D (▲) are depicted in full symbols, film D in combination with the protective culture sprayed (Δ), with protective culture brushed (□), with the antifungal component (☒) and the thicker film D19 (○) are depicted in empty symbols and are connected with interrupted lines.
3.5 Promising alternative film E in combination with an antifungal component

The challenge test of water vapor permeable film E did show no defect of the cheese surface until the end of storage period. Independently of Delvocid pretreatment cheese surfaces wrapped in film E remained dry, not smeary, non-off-odorous and free of visible mold growth whereas cheese portions wrapped in standard film developed a clear defect (Fig. 5). A reduced pH decrease over storage of 0.3 compared to 0.5 for standard film was observed for cheese portions wrapped in film E (Fig. 5). A decreasing tendency of living bacteria for total bacteria was shown for all cheese portions wrapped in standard film and film E (Fig. 6 A). A clear reduced decrease of living bacteria cell copy numbers could be depicted for *Bacillus* spp. (Fig. 6 B) and *C. casei* (Fig. 6 C) for all cheese surfaces wrapped in film E. The antifungal component treated cheese surfaces depicted even a reduced decrease of *Bacillus* spp. compared to non-treated cheese surface in film E but the tendency compared to standard film remained the same. A reduced dying of aerobic cheese smear organisms illustrated by *Bacillus* spp. and *C. casei* over storage period could be shown in film E compared to standard film. Film E could successfully prevent red-smear cheese surface defect by revealing a dry, not smudgy smear with a typical cheese odor independent of the treatment with antifungal component. The cheese surface pretreated with antifungal component was judged exactly the same as untreated cheese surface wrapped in film E by 19 potential consumers (Fig. 7). These findings suggested film E as a possible solution for film-prepacked red-smear cheeses defect prevention without the necessity of an antifungal component containing natamycin which is according to interest and agreement of the Swiss cheese producers (Switzerland Cheese Marketing, 2007).

The films water vapor permeability can be made responsible for the dry appearance of the smear. The observed reduced pH decrease, as well as the clearly reduced decrease of aerobic bacteria over storage time are strong indications of better surviving conditions for the cheese surface microflora. These data are also giving evidence that the inhibition of the cheese smear microflora is not to be achieved, but furthermore a balanced cheese surface microflora can contribute to a sparse, nicely colored and odorous smear.
Fig. 5. Cheese smear surface pH and appearance (below the graph) development over storage time in standard film (■) compared to water vapour permeable film E (△) and film E with a Delvocid pre-treatment (○).
Fig. 6. Development of 16S rRNA gene copy numbers of cheese smear surface wrapped in standard film (■, □) compared to water vapour permeable film E (●, ○) and film E in combination with antifungal treatment (▲, △). DNA copy numbers are depicted in full symbols, DNA copy numbers of living bacterial cells are depicted in empty symbols.
Fig. 7. Cheese tasting variety T wrapped in standard film and film E after 8 week of storage time at 8 °C. One control (closed line) and two cheese portions wrapped in film E with (dotted line) and without (interrupted line) addition of antifungal component were judged by 19 people. The dough was rated for taste (A), the cheese smear for humidity (B), smearyness (C), odor (D) and optical appearance (E).
4. Conclusions

Different strategies with the intention to inhibit cheese surface microflora growth and/or metabolic activity failed to prevent cheese surface defect formation. Adding salt as ancient additive to inhibit microbes by reducing water activity value, application of protective culture and a film with antimicrobial properties (Chapter 3) were not sufficient to prevent the problem. The drawback of mold growth in film D could not be satisfying solved by application of the protective culture. The application of Delvocid containing natamycin could prevent the mold growth but the appearance of the cheese smear was not satisfying. Drying the cheese loaves before packaging could show that water loss has an impact of defect development due to the smear’s humidity. Nevertheless, drying even for 48 hours was not enough to prevent defect development completely. These findings combined with the fact that defect development was observed to be accompanied by a decrease of transcriptional activity and living bacterial cells are strongly giving evidence that inhibiting the cheese surface microflora is not the right measure for red-smear cheese defect prevention. Therefore a solution needs to be found where a balanced metabolic active cheese surface microflora can be kept for as long as possible under storage conditions. We demonstrated in a challenge test that red-smear cheese defect can be prevented by the water vapor permeable film E. Furthermore, our study showed that an antifungal treatment is not even needed.
Acknowledgments

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Chapter 5

Red-smear cheese surface defect development in plastic films affects viability and nitrogen metabolism of the surface microflora

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Abstract

Film-prepackaging of fully bacterial ripened red-smear cheeses have been associated with a defect development over storage time. To prevent defect formation an alternative packaging film E was tested compared to standard film in two and three independent storage trials for variety M and T, respectively. A high impact of packaging on metabolic activity of the cheeses surface microflora was observed by the protein coding \( mqa \) gene transcripts of the representative member \( Corynebacterium \) casei. A significant decrease in living bacterial cells of 1.2 log for \( C. \) casei and 0.9 log for \( Bacillus \) spp. measured by viability quantitative PCR (v-qPCR) containing a propidium monazide (PMA) staining previous to DNA extraction combined with an enhanced proteolysis over storage time was detected in cheese smear wrapped in standard film. The alternative, low \( O_2 \), water vapor permeable packaging film E showed a dry, sparse and non-odorous smear until the end of storage. Micro-aerophilic conditions, a significant decrease of water activity of 0.03 and a slightly reduced pH drop was observed for the alternative film E compared to standard film where anaerobic conditions, water activity increase of 0.016, a significant pH drop of 0.4 and an accelerated proteolysis in the cheese smear during 8 weeks at 8°C were measured. The promising alternative packaging material was tested for two Swiss varieties and arose to be a possible solution for defect prevention allowing a convenient handling of the cheese tested in a potential consumer test.
1. Introduction

Fully ripened semi-hard red-smear cheeses are often cut in portion and film-prepacked for distribution and sale in self-service shelves. Within the packaging film the cheese surfaces develop a defective smear during cold storage. The cause of defect development could not be allocated to a particular microorganism or a microbial group. Therefore, a complex etiology of the smear defect of film-prepacked portions including the smear microorganisms as well as environmental factors and microbial metabolic activity was proposed (Amato, et al., 2012). Defect development over storage time could be clearly associated with anaerobic conditions, a slightly increasing water activity value, a pH drop over storage time and a clear decrease of rRNA transcriptional activity of total bacteria, *Corynebacterium casei* and *Bacillus* spp. (Chapter 3).

The metabolic activity of microorganisms can be measured, alternatively to 16S rRNA, by quantification of more labile mRNA transcripts of protein encoding genes that are probably better viability markers as for their instability after the death of bacteria (Cenciarini, et al., 2008). The malate: quinone oxidoreductase (MQO) encoded by the *mqo* gene is a membrane-bound enzyme essential for *Corynebacterium glutamicum* (Bott & Niebisch, 2003) and in many cases necessary for growth and especially important for the adaption to changing growth environments (Kabashima, et al., 2013). For this reason the expression of *mqo* gene may be a good indicator for metabolic activity of bacteria and also good indicator for the vitality of the cheese smear microflora. In retail cheese, a good correlation between *mqo* transcripts detection and colony morphotype assignment and a good agreement between the abundance of mRNA and rRNA was found for *C. casei* (Monnet, et al., 2013).

The decrease of metabolic activity measured in 16S rRNA transcripts was interpreted as an indication of the importance of dying cells which can contribute to defect development by cell lysis and release of intracellular enzymes (Chapter 3). In Emmental cheese, bacterial cell numbers decrease was associated with cell lysis releasing intracellular peptidases involved
in cheese proteolysis during cheese ripening (Deutsch, Ferain, Delcour, & Lortal, 2002; Falentin, et al., 2010; Falentin, et al., 2012). The bacterial cell lysis can add to cheese surface humidity and the released enzymes conductive to proteolysis. Proteolysis contributes to cheese surface smudginess and possible off-odor in film-prepacked cheese portions. Enhanced proteolysis and its monitoring at the surface of Tilsit cheese has been previously shown (Churchill, Hannon, & McSweeney, 2003). To disclose the living cells on red-smear cheese surface over storage time propidium monoazide (PMA) treatment combined with quantitative PCR (PMA-qPCR) (Nocker, et al., 2006) is a powerful method. PMA enters compromised cells only and binds double stranded DNA by photo-induction. The PMA-DNA product is not being amplified by PCR (Elizaquível, et al., 2012) and therefore PMA-qPCR is giving an accurate picture of living bacteria compared to conventional PCR where no distinction between dead and living bacterial DNA could be made (Nocker, et al., 2006). The suitability of a PMA-qPCR or also called viability qPCR (v-qPCR) assay has been confirmed by several studies using different bacterial groups in food such as foodborne pathogenic organisms like *E.coli* O157:H7, *Listeria monocytogenes* and *Salmonella* in spinach and mixed salad (Elizaquível, et al., 2012) and probiotics in cheddar cheese (Desfossés-Foucault, et al., 2012). But to date it has not been described for the detection of living bacterial cells in cheese smear.

Packaging is increasingly recognized as an important factor in protecting and controlling the quality of cheese (Kelly, 2007) and our previous research disclosed packaging materials do influence the physicochemical properties and therefore the cheese smear microflora of semi-hard red-smear cheese surface (Chapter 3). The aim of this study is therefore to investigate red-smear cheese surface defect development and prevention over storage of cheese portions wrapped in standard film and alternative film E. Intrinsic factors such as redox potential, pH and water activity were monitored; bacterial metabolic activity and viability were quantified by application of combined qPCR, RT-qPCR addressing 16S rRNA gene and *mqo* gene transcripts and v-qPCR. Proteolysis was monitored by nitrogen and free amino acids
measurement and sensory consumer acceptance tests of cheese portions wrapped in alternative packaging film E compared to standard film were performed.
2. Materials and methods

2.1 Storage test and cheese surface analysis

The water vapor permeable alternative film E (Table 1) was tested in storage tests for variety T and M by comparison of cheese portions wrapped in their corresponding standard film Csf T and Csf M, respectively (Table 1). Cheese portions were provided by industrial partners after ripening as previously described (Amato, et al., 2012). Cheese portions used as controls were wrapped in gas- and watertight standard film (Csf M and Csf T) and all cheese portions of the industrial trial wrapped in water vapor permeable film E were packaged at the industrial partner to keep original process before they were stored at 8°C. Cheese surface samples were taken before packaging (bfp), after 2 and 8 weeks. Appearance, redox potential, pH and water activity ($a_w$-value), were monitored as previously described (Chapter 3).

Table 1

<table>
<thead>
<tr>
<th>Permeability</th>
<th>Code</th>
<th>$O_2$ [cm$^3$m$^{-2}$ d bar]</th>
<th>$CO_2$ [cm$^3$m$^{-2}$ d bar]</th>
<th>Water vapor [cm$^3$m$^{-2}$ d bar]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Csf M</td>
<td>&lt; 30</td>
<td>&lt; 50</td>
<td>&lt; 2</td>
<td></td>
</tr>
<tr>
<td>Csf T</td>
<td>&lt; 30</td>
<td>&lt; 105</td>
<td>&lt; 2</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>&lt; 35</td>
<td>-</td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>

- means “not measured”

2.2 PMA treatment of single cultures and cheese smear for live/dead bacterial cells determination by viability-qPCR (v-qPCR)

Accuracy of v-qPCR in cheese smear was evaluated by combined cultivation on Marine Broth agar plates (Becton Dickinson AG, Allschwil, Switzerland) at 22°C for 48-72 hours and determination of copy numbers by q-PCR of PMA treated and untreated single culture and
cheese smear. For single cultures C. casei DSM 44701 T was used, to test complex samples the cheese smear stored in glycerol and the cheese surfaces of two commercially bought cheeses were examined. Living C. casei single cultures (cultivated in Marine Broth medium over night at 22°C), were mixed with dead bacteria (heat treatment for 10 min at 100°C) in different ratios. One mL of the single culture or two mL of cheese smear/peptone solution (8 cm²/50 mL) were centrifuged at 7000 g for 3 min and resuspended in 1 mL peptone solution where propidium monoazide (PMA)-treatment was performed with 2.5 µL of PMA (Biotium, Hayward, Canada) stock solution (20 mM in dimethyl sulfoxide (DSMO), VWR International AG, Dietikon, Switzerland). This solution was incubated in the dark at room temperature for 5 min. It was then exposed for 10 min to light from a 500 W halogen lamp at 20-30 cm distance on a shaker in an ice box. After 5 min of incubation the Eppendorf tube was shaken additionally by hand to insure proper mixing. Bacterial cells were pelleted by centrifugation at 7000 g for 3 min, resuspended in 1 mL PBS/EtOH (1:1) and divided into two tubes of 0.5 mL for storage at -20°C until DNA extraction was performed as previously described (Amato, et al., 2012).

2.3 Quantification of bacterial DNA, PMA-DNA and cDNA copy numbers by quantitative real-time PCR

Total DNA, PMA-DNA and cDNA (reverse transcribed RNA) copy numbers of total bacteria, Corynebacterium casei and Bacillus spp. were measured by qPCR analysis of 16S rRNA gene in a 7500 Fast Real-Time PCR System (Applied Biosystem, Carlsbad, California, USA) as previously described. Copy numbers quantification was performed for DNA, PMA-DNA and cDNA simultaneously as previously described for total bacterial DNA samples (Amato et al., 2012). Bacillus spp. and C. casei (Chapter 3) were quantified using primers YB-F and YB-R (Han, et al., 2012) for Bacillus spp. and the primers fs15 and fs17 (Monnet et al., 2006) for C. casei. RNA recovery rate was measured by reference Luciferase cDNA quantification (Johnson, et al., 2005) as described before (Chapter 3).
The malate quinone oxidoreductase (mqo) gene transcripts of C. casei were quantified by qPCR in cDNA samples of the cheese smear using the primers CcmF (mqo_Cc1075-1094) and CcmR (mqo_c1182-1163R) (Monnet, et al., 2013). Therefore RNA extraction and reverse transcription was performed as previously described (Chapter 3). qPCR standard curves were generated with templates produced by amplification of C. casei DSM 44701T (German collection of microorganisms and cell cultures, Germany) in a standard PCR with primer CcmF and CcmR. Dilutions (10^{-4}\text{-}10^{-10}) containing a predefined template copy number were processed at the same time and in the same way as tested samples. The cycle program consisted of an initial step of 95°C for 10 min and 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s and polymerization at 60° for 1 min.

For each run a dissociation curve was done to check for the product specific melting temperature. Three independent replications of two dilutions of DNA, PMA-DNA and cDNA were measured in triplicates and means normalized per square centimeter of cheese surface are displayed in the graphs. Data analysis was performed with JMP 10 software (SAS Institute AG, Wallisellen, Switzerland). Bivariate Fit was used to evaluate correlation between PMA treated and untreated colony forming units (cfu) and correlation of PMA treated cn and cfu. One-way analysis of variance (ANOVA) was used to evaluate significant (P <0.05) differences for different time and between standard film and film E. Comparison for each pair using Student’s t was conducted (alpha=0.05).

2.4 Determination of nitrogen and free amino acids in the cheese smear

The cheese was divided into the smear, the rind, the dough close to surface and the remaining dough which were analyzed before packaging and after 7 weeks. Water content was measured according to the Karl-Fischer method (ISO 5536). Total nitrogen (TN), water soluble nitrogen (WSN) and non-protein nitrogen (NPN) were performed by potentiometric Kjeldahl method (ISO8968-1; ISO8968-2). A suspension of 5.0 g cheese sample in water and separation of water phase was done before WSN determination. The non-water soluble part of the cheese sample was mixed with 24% of trichloracetic acid for NPN precipitation...
and measurement. Free amino acids were measured photometrically in 1.25 g cheese sample at 340 nm by a Gallery Analyzer (Thermo scientific, Germany). All free amino acids, as well as di- and tripeptides were precipitated with 0.5 mol/L perchloric acid (Sigma-Aldrich Chemie GmbH) and mixed with o-phthaldialdehyde (Merck, Darmstadt, Germany) and 2-mercaptopoethansulfonic acid (Merck) with which photometric measurable product of 1-Alkylthio-2-alkylisoindol-binding were built and measured (Rohm, Tschager, & Jaros, 1996).

2.5 Evaluation of cheese quality by consumers

Consumer acceptance of cheese portion wrapped in film E compared to standard film was tested for three independent trials of variety T and one of variety M. The first and the second potential consumer group consisting of 17 subjects judged the same portions, one control and one and two test portions for the challenge test and the repetition trial, respectively. The subjects did not know in which film the portions have been previously packed. The cheese portions of the industrial trial were rated by 56 persons, all untrained cheese consumers. Each subject received two film-prepacked cheese portions, one in standard film and one in alternative film E, with randomized three digit numbers which they had to handle and rate in parallel.

The subjects received a preprinted table to cross the corresponding attribute fitting best with the samples being either the cheese dough or the cheese smear. The cheese dough was rated for taste, the cheese smear for humidity, smearyness, odor and optical appearance as indicated in Table 2. The attributes were given the numbers one to four for statistical analysis.

JMP 10 software (SAS Institute AG, Walisellen, Switzerland) was used for statistical data analysis. One-way analysis of variances (ANOVA) followed by a nonparametric comparison for each pair using Wilcoxon method was applied for determination of significant (p-value < 0.05) differences.
Table 2
Attributes given for cheese taste, cheese smear humidity, smearyness, odor and optical appearance for sensory rating.

<table>
<thead>
<tr>
<th>Value for statistical analysis</th>
<th>4</th>
<th>3</th>
<th>2</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taste dough</td>
<td>very good</td>
<td>good</td>
<td>bad</td>
<td>very bad</td>
</tr>
<tr>
<td>Humidity smear</td>
<td>dry</td>
<td>little humid</td>
<td>humid</td>
<td>very humid</td>
</tr>
<tr>
<td>Smearyness smear</td>
<td>not smeary</td>
<td>sticky</td>
<td>smeary</td>
<td>very smeary</td>
</tr>
<tr>
<td>Odor smear</td>
<td>typical</td>
<td>little</td>
<td>intensive</td>
<td>very intensive</td>
</tr>
<tr>
<td>Appearance smear</td>
<td>cheese</td>
<td>off-odor</td>
<td>off-odor</td>
<td>off-odor</td>
</tr>
<tr>
<td></td>
<td>normal</td>
<td>tolerable</td>
<td>acceptable</td>
<td>acceptable</td>
</tr>
</tbody>
</table>
3. Results and discussion

3.1 Alternative film E compared to standard film

Three independent storage experiments, a challenge test (chal), a repetition (rep) and an industrial trial (ind), were performed with the water vapor permeable alternative packaging Film E (Table 1). Packaging was performed in real industrial packaging process in the case of industrial trial for both Swiss varieties T and M. Cheese portions wrapped in standard film progressively developed a clear defect (Fig. 1B, Fig. S1B in supplementary information). All cheese portions wrapped in alternative film E displayed a dry, sparse, non-off-odorous and nice colored cheese smear until the end of storage for both varieties T (Fig. 1B) and M (see Fig. S1B in supplementary information). The pH dropped significantly on cheese surface of variety T in standard film before packaging from 7.4 to 7 after 8 weeks (Fig. 1A), the pH drop was slightly reduced on cheese surface wrapped in film E. The water activity value on cheese surface in standard film increased from 0.94 to 0.95, while in film E a significant drop from 0.94 to 0.91 was observed for variety T (Fig. 1A). The same tendencies were observed for variety M (see in Fig. S1A supplementary information). The $a_w$-value drop observed within film E correlates with its seven fold higher water vapor permeability compared to standard film (Table 1). Redox potential of cheese smear in standard film always indicated anaerobic conditions (Chapter 3); the redox potential of film E was measured twice for each variety resulting in neither clear blue nor clear white coloration (data not shown). These unclear colorations were interpreted as redox potential being very close to the transition point of +11 mV. These micro-aerophilic conditions could prevent mold growth, a problem that had been observed with the previously best performing film D (Chapter 3). A treatment with antifungal component has been tested and revealed not to be necessary (Chapter 4) which is in agreement of Swiss cheese producers who decided not to use natamycin (Switzerland Cheese Marketing, 2007). Slightly higher pH and micro-aerobic condition within film E are in agreement with higher pH in “medium-water vapor permeable” film and higher metabolic
activity in “medium- and high-water vapor permeable” film measured for St. Nectaire cheese (Picque, et al., 2011).

Fig. 1. pH (■, ○) and water activity (●, ○) (A) and appearance (B) development of cheese portions variety T wrapped in standard film (full symbols) and film E (empty symbols) over storage time. Appearance of the cheese smear of the challenge test (chal ▲), the repetition (rep ▼) and the industrial (ind ▼) trial were judged according to the defect definition scheme (Chapter 3, Tab. 2). The pH value derived from three (chal, rep, ind), the water activity value from two (rep, ind) independent storage tests and the error bars indicate standard deviation of the measurements. Different letters indicate significant (alpha=0.05) differences.
3.2 Microbial composition and metabolic activity of the cheese smear microflora

Copy numbers per square centimeter cheese surface measured by qPCR for total bacteria with $10^{10}$-$10^{11}$ (see Fig. S2 in supplementary information), *C. casei* $10^{6}$-$10^{10}$ (Fig. 2) and *Bacillus* spp. $10^{7}$-$10^{8}$ (see Fig. S2 in supplementary information) were in accordance with previous copy numbers and cell counts (Amato et al., 2012, Chapter 3). Global metabolic activity in cheese smear was measured by cDNA (RNA transcripts) of 16S rRNA genes of total bacteria, *Bacillus* spp. and *C. casei*. The previously reported decreasing trend of 16S rRNA copy numbers in standard film (Chapter 3) could be confirmed and a significant decrease of the protein coding *mqo* gene mRNA transcripts of *C. casei* was observed (Fig. 2). For alternative film E a decrease after 2 weeks of 16S rRNA and *mqo* mRNA transcripts copy numbers was observed followed by a recovering tendency (Fig. 2). A high impact of packaging in either plastic film on cheese surface microflora in general is suggested by these data. The tendency of cDNA recovery from the smear wrapped in alternative film E suggests a higher metabolic activity of the microflora reaching the end of storage time. This observation correlates with the micro-aerophilic conditions on the cheese surface wrapped in film E corresponding to a more suitable environment for the mostly aerobic cheese surface bacteria.

3.3 Decrease of living bacteria over storage time

Viability qPCR (v-qPCR) method was evaluated for its accurate picture of living bacterial cells. Therefore we measured a single culture of *C. casei* DSM 44701T, a former cheese smear sample and a commercial cheese smear with PMA-qPCR method and cultivation in parallel. Colony forming units of untreated and PMA treated *C. casei* overnight cultures correlated well (R2=0.97) indicating that cell viability is not affected by PMA treatment (Fig. 3). The constant detection of 16S rRNA gene copy numbers of untreated samples independent of the live/dead ration (9.86±0.16) indicate the overestimation of qPCR by amplification of dead bacterial DNA. Copy numbers of PMA treated samples correlate well (R2=0.98) with PMA treated cfu showing that PMA-qPCR only amplifies living bacterial DNA.
(Fig. 3). In single cultures a slight overestimation of 0.2 log is observed. This overestimation is about 1.3 log.

![Figure 2](image_url)

**Fig. 2.** Microbial counts, living bacterial copy numbers and metabolic activity of *C. casei* over storage time quantified by 16S rRNA and malate quinone oxidoreductase (*mqo*) gene copy numbers (cn). The symbols in the graphs demonstrate DNA copy numbers of standard film (●) and film E (◊), PMA-DNA copy numbers of living bacterial cells of standard film (■) and film E (□), cDNA copy numbers of 16S rRNA transcripts of standard film (●) and film E (◊) and cDNA copy numbers of *mqo* RNA transcripts of *C. casei* of standard film (▲) and film E (△). Error bars indicate standard deviation of three independent repetitions. Values labeled with different letters are significantly different (alpha=0.05) from each other.

when applied to complex cheese microflora (see Table S1 in supplementary information).

The higher deviation from plate counts can be explained by including viable but not culturable microorganisms (VBNC). PMA-qPCR was demonstrated to be a suitable culture independent method to display an accurate picture of living bacterial cells on red-smear cheese surface. The use of PMA-qPCR is well suited to quantify total cellular viability (Kramer, Obermajer, Matijasic, Rogelj, & Kmetec, 2009). PMA-qPCR allows more specific
differentiation than cultivation. A differentiation on species level is possible depending on the primer chosen for qPCR. This specificity is an advantage of PMA-qPCR and a well-known problem of cultivation due to the lack of very specific cultivation media. In addition of qPCR, PMA-qPCR targeting 16S rRNA gene copy numbers of total bacteria, *C. casei* and *Bacillus* spp. were performed for film-prepacked red-smear cheese surface development over storage time. PMA-qPCR disclosed a decreasing trend of living total bacteria of 0.4 log over storage of 8 weeks for standard film and film E (see Fig. S2 in supplementary information). *Bacillus* spp. copy numbers of living bacteria cells in standard film showed a significant decrease over storage whereas the decrease in film E was reduced (see Fig. S2 in supplementary information). A significant drop of living bacteria cell copy numbers of *C. casei* of 1.2 log over 8 weeks in standard film was observed (Fig. 2). This significant decrease in living bacterial cells was reduced to a drop of 0.6 log in film E (Fig. 2). Falentin et al. (2012) underlined that a decrease of one log of cell lyses during ripening results in the release of the largest part (90%) of the pool of cytoplasmic enzymes. The lysis of cells of lactococci lead to the release of intracellular peptidases capable of hydrolyzing the peptides produced by rennet, plasmin or microbial proteinases in soft cheese (Gripon, 1997). A dozen peptidases were isolated and characterized, all with an optimum pH close to neutrality. An indirect role of *Staphylococcus xylosus* to cheese ripening by release of intracellular proteolytic enzymes by cell-lysis is assumed (Ghosh, et al., 2009). Our data disclose that 40-92 % of cheese smear microflora bacterial cells died within standard film over storage of 8 weeks at 8°C. Film E depicts better condition for red-smear cheese microbiota ending in a clear diminished decrease of aerobic smear organism like *C. casei* and *Bacillus* spp. as well as showing a dry, sparse, nicely colored and non-off-odorous smear. A connection between dying bacterial cells and red-smear cheese defect development is strongly indicated by our study and supports the hypothesis of possible defect contribution of dying bacterial cells by cell-lysis.
Fig. 3. Evaluation of PMA treatment by comparison of untreated (■) and PMA treated (□) cfu mL$^{-1}$ with untreated (□) and PMA (■) treated 16S rRNA gene copy numbers (cn mL$^{-1}$).

3.4 Enhanced proteolysis in cheese smear wrapped in standard film

Proteolysis was analyzed as a contribution to defect development of dead bacterial cells by cell-lysis and release of intracellular enzymes was assumed. Total nitrogen, non-protein nitrogen, water soluble nitrogen and free amino acids were measured for cheese smear, rind, dough close to rind area and dough for cheese portion before packaging and after 7 weeks of storage in standard film and alternative film E. Proteolysis could be observed over storage time with increasing concentration of water soluble nitrogen (Fig. 4 E, F), non-protein nitrogen (Fig. 4 G, H) and free amino acids (Fig. 4 I, J) in all samples stored for 8 weeks (■, □) compared to portion before packaging (■). Total nitrogen value remained stable over storage time for the rind, the dough close to surface and the dough in case of both cheese varieties (Fig. 4 C, D). In case of the cheese smear a drastic decrease of total nitrogen for cheese wrapped in standard film of 23.4 g kg$^{-1}$ for variety M and 16.5 g kg$^{-1}$ for variety T, after 7 weeks was observed. This decrease in total nitrogen on cheese surface was
Viability and nitrogen metabolism

diminished if cheese was wrapped in film E, 10.8 g kg\(^{-1}\) for variety M and 13.1 g kg\(^{-1}\) for variety T (Fig. 4 C, D). Nitrogen, in form of ammonium, can diffuse from the cheese smear to the cheese core (data not shown) and in form of ammonia can evaporate to the surrounding. Free amino acids in cheese smear increased to almost the double in standard film from 336 mmol kg\(^{-1}\) to 765 mmol kg\(^{-1}\) before packaging until 7 weeks of storage for variety M (Fig. 4 I) and from 343 mmol kg\(^{-1}\) to 572 mmol kg\(^{-1}\) for variety T (Fig. 4 J). If cheeses were wrapped in alternative film E the increase to 527 mmol kg\(^{-1}\) for variety M and 460 mmol kg\(^{-1}\) for variety T was not as high as in standard film. Proteolysis taking place over time in the dough, the dough close to the rind, the rind and the smear, but is strongly enhanced in the smear wrapped in standard film. An enhanced proteolysis in the cheese smear in general compared to the cheese core is in accordance with previous studies during ripening of the cheese (Churchill, et al., 2003; Gobbetti, et al., 1997). Pronounced cheese smear defect in standard film is associated with a strong enhanced proteolysis in the smear compared to alternative film E. Waterloss measured with Karl-Fischer method (Fig. 4 A) in smear wrapped in film E was with 122 g/kg for M and 95 g/kg for T higher than in smear wrapped in standard film (51 g/kg for M and 45 g/kg for T) and correlated with a weight loss of 5-6% after 8 weeks of storage when the whole portions was weighted (data not shown).
Fig. 4. Nitrogen, free amino acid and water content of the cheese smear, rind, dough close to surface and dough of cheese portions before packaging (■) and wrapped in standard film (■) or film E (□) after 7 weeks of storage at 8°C. Water content of variety M (A) and T (B), total nitrogen (TN) variety M (C) and T (D), water soluble nitrogen (WSN) variety M (E) and T (F), non-protein nitrogen (NPN) variety M (G) and T (H) and free amino acids variety M (I) and T (J).
3.5 Consumer acceptance of cheese portions wrapped in alternative film E

To investigate the sensory acceptance of cheese portions wrapped in alternative packaging material compared to standard film cheese portions of all three trials of variety T (Fig. 5) and one of variety M (see Fig. S3 in supplementary information) were judged by cheese consumers. For that, the subjects received a questionnaire to rate the cheese smear’s humidity, smearyness, odor and appearance as well as the taste of the cheese dough with the given attributes (Table 2). One cheese portion wrapped in standard film and one portion wrapped in film E of the challenge test was rated by 17 subjects (Fig 5A). One cheese portion in standard film and two portions in film E of the repetition test were judged by 17 subjects (Fig. 5B). For the industrial trial a consumer test with 56 subjects was performed (Fig. 5C). Each subject received a film-prepacked pair of a portion wrapped in standard film and a portion wrapped in film E labeled with a random three digit code. The dough of all cheese portions wrapped was rated as good in either film (Fig. 5, Table S2 in supplementary information). The odor of the cheese surface was rated significantly better for the cheese portions wrapped in film E of the challenge test and the repetition (Fig. 5A, B). The odor of the cheese portions of the industrial trial were rated as little off-odor independent on the packaging material. The cheese smear of portions wrapped in alternative film E were in all three performed tests rated as significantly less humid, smeary and better in appearance (Fig. 5, Table S2 in supplementary information). Three tests of variety T and one of variety M with cheese consumer showed that cheese surface wrapped in standard films are described as humid, smeary, little-intensive off-odor with a tolerable- just acceptable appearance whereas cheese surface wrapped in alternative film E are described as dry, not smeary, typical cheese to little off-odor and a normal to acceptable appearance.
Fig. 5. Cheese portion evaluation judged by cheese consumers for taste of the dough and the cheese smear for humidity, smearyness, odor and total appearance. All cheese portions were rated according to the criteria in Table 2. In each test cheese portions wrapped in standard film (full line —) and cheese portions wrapped in alternative film E (interrupted line ... ) were by each subject. Cheese judged portions of the challenge test (A) were rated by 17 (N=17), the repetition was rated by 17 (N=17) and cheese portions of the industrial trial were rated in analogous proceedings of a hedonic trial by 56 (N=56) persons. Stars (*) are indicating significant differences (P-value <0.05) between standard film and alternative film E.
3.6 Factors of cheese surface defect development

The following mechanism of cheese surface defect development is summarized and illustrated in Fig. 6. A salt and pH gradient in the cheese dough is established during ripening due to salt uptake by brining and activity of the starter cultures (Guinee, 2004; Pavia, Trujillo, Guamis, & Ferragut, 2000). Water diffuses from the center of the dough to the cheese surface against the salt gradient. The cheese surface is drying by water vapor loss in the ripening cellar. The sparse, nicely colored smear consists of a metabolically active microflora, while produced CO$_2$, NH$_3$ and short chain fatty acids can evaporate to the surrounding. By packaging a cheese portion in standard film a cheese smear defect develops over storage time appearing in a wet, smudgy and off-odorous smear (Amato, et al., 2012). The gas and water vapor tight plastic film completely blocks diffusion and therefore leads to accumulation of water, CO$_2$, short chain fatty acids (SCFA) and most probably NH$_3$ (not measured) (Chapter 3). Water activity increases due to water accumulation, pH decreases by CO$_2$ and lactate accumulation. The lack of oxygen and anaerobic conditions affect the aerobic bacteria of the cheese smear. The metabolic activity of the microflora is decreased and bacteria die within standard film as shown for C. casei and Bacillus spp. Dead cells lyse and therefore release their intracellular enzymes, including proteases and lipases, that can contribute to the ongoing proteolysis in the smear and metabolite production. Cheese surface defect does not develop in alternative film E. The smear stays dry due to water vapor permeability of the film and water activity drops significantly (Fig. 1). The pH decrease is slightly reduced due to not complete impermeability to CO$_2$ and oxygen causing micro-aerophilic conditions (Fig. 1). These conditions impact metabolic activity of the smear microflora to a lesser extent than in standard film and bacteria survived better until the end of storage time (Fig. 2). A sparse, dry and nicely colored cheese surface allows a convenient handling when cheese portion are wrapped in alternative film E.
Fig. 6. Model of red-smear cheese surface defect mechanism and prevention by alternative packaging with film E.
1. Conclusion

This study discloses microbial contribution of red-smear cheese surface defect of film-prepacked portions evolving over storage. Cheese smear defect development has been shown to be accompanied by a pH drop, anaerobic conditions and correlated with a decreasing number of living bacterial cells. The assumed contribution to proteolysis in the cheese smear of dead bacterial cells by cell lysis and release of intracellular enzymes was confirmed by analysis of nitrogen and free amino acids. Furthermore, the alternative film represents an applicable solution for red-smear cheese surface defect prevention with high consumer acceptance shown in three independent tests. The cheese smear humidity, smearyness, odor and optical acceptance of cheese wrapped in alternative film have been judged as significantly better than wrapped in standard film. A better environment for cheese smear microbiota is provided by film E compared to standard film exhibited in a diminished pH drop over storage time, as well as in micro-aerophilic conditions instead of strict anaerobic conditions. The more convenient conditions result in a smaller decrease in living bacterial cells as shown for C. casei and Bacillus spp. combined with a diminished proteolysis on cheese smear wrapped in alternative film compared to standard film.

In conclusion this study supports the hypothesis of complex etiology of the observed smear defect. The contribution of multiple factors such as pH or redox potential on the cheese smear microbiota and the impact of cheese smear microbiota on defect development could be shown and an alternative packaging solution could prevent defect formation.
Acknowledgments

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## Supplementary information

**Table S1**

Colony forming units and copy number of untreated and PMA treated *C. casei* single culture (SC), cheese smear of commercially bought red-smear cheese (CCS) and cheese smear stored in glycerol for three month (T46).

<table>
<thead>
<tr>
<th>live/dead ratio</th>
<th>untreated</th>
<th>PMA treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>plate counts</td>
<td>qPCR</td>
</tr>
<tr>
<td></td>
<td>cfu cm(^{-2})</td>
<td>log (cfu cm(^{-2}))</td>
</tr>
<tr>
<td>SC A</td>
<td>100/0</td>
<td>5.82E+08</td>
</tr>
<tr>
<td></td>
<td>41/59</td>
<td>2.37E+08</td>
</tr>
<tr>
<td></td>
<td>34/66</td>
<td>1.96E+08</td>
</tr>
<tr>
<td></td>
<td>4/96</td>
<td>2.18E+07</td>
</tr>
<tr>
<td>SC B</td>
<td>100</td>
<td>4.36E+08</td>
</tr>
<tr>
<td></td>
<td>64/36</td>
<td>2.80E+08</td>
</tr>
<tr>
<td></td>
<td>36/64</td>
<td>1.58E+08</td>
</tr>
<tr>
<td></td>
<td>8/92</td>
<td>3.27E+07</td>
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<tr>
<td>T46</td>
<td></td>
<td>4.10E+08</td>
</tr>
<tr>
<td>CCS A</td>
<td></td>
<td>4.36E+08</td>
</tr>
<tr>
<td>CCS B</td>
<td></td>
<td>5.09E+08</td>
</tr>
</tbody>
</table>
Table S2
Statistical analysis of cheese tasting. Different letters indicate significant differences (p<0.05) of non-parametric comparison for each pair using Wilcoxon method.

<table>
<thead>
<tr>
<th>Film</th>
<th>Variety</th>
<th>N</th>
<th>Taste dough</th>
<th>Humidity smear</th>
<th>Smeary-smear</th>
<th>Odor smear</th>
<th>Optical appearance smear</th>
</tr>
</thead>
<tbody>
<tr>
<td>Csf</td>
<td>T</td>
<td>17</td>
<td>2.94 ± 0.97 a</td>
<td>1.83 ± 0.92 a</td>
<td>2.00 ± 0.77 a</td>
<td>1.80 ± 1.04 a</td>
<td>1.83 ±1.04 a</td>
</tr>
<tr>
<td>E</td>
<td>T</td>
<td>19</td>
<td>2.74 ± 0.65 a</td>
<td>3.42 ± 0.51 b</td>
<td>3.53 ± 0.61 b</td>
<td>3.52 ± 0.61 b</td>
<td>3.79 ±0.41 b</td>
</tr>
</tbody>
</table>

Repetition film E

<table>
<thead>
<tr>
<th>Film</th>
<th>Variety</th>
<th>N</th>
<th>Taste dough</th>
<th>Humidity smear</th>
<th>Smeary-smear</th>
<th>Odor smear</th>
<th>Optical appearance smear</th>
</tr>
</thead>
<tbody>
<tr>
<td>Csf</td>
<td>T</td>
<td>17</td>
<td>2.59 ± 0.71 a</td>
<td>2.41 ± 0.94 a</td>
<td>2.47 ± 0.87 a</td>
<td>2.29 ± 0.84 a</td>
<td>2.24 ±1.03 a</td>
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<tr>
<td>E</td>
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<td>2.94 ± 0.66 a</td>
<td>3.88 ± 0.33 b</td>
<td>3.88 ± 0.33 b</td>
<td>3.18 ± 0.53 b</td>
<td>3.18 ±0.53 b</td>
</tr>
<tr>
<td>E</td>
<td>T</td>
<td>17</td>
<td>3.12 ± 0.60 a</td>
<td>3.82 ± 0.39 b</td>
<td>3.82 ± 0.39 b</td>
<td>3.11 ± 0.86 b</td>
<td>3.12 ±0.86 b</td>
</tr>
<tr>
<td>Csf</td>
<td>M</td>
<td>18</td>
<td>2.50 ± 0.86 a</td>
<td>1.28 ± 0.57 a</td>
<td>1.44 ± 0.86 a</td>
<td>1.89 ± 0.83 a</td>
<td>1.56 ±0.86 a</td>
</tr>
<tr>
<td>E</td>
<td>M</td>
<td>18</td>
<td>2.89 ± 0.47 a</td>
<td>3.78 ± 0.43 b</td>
<td>3.78 ± 0.43 b</td>
<td>3.44 ± 0.62 b</td>
<td>3.72 ±0.75 b</td>
</tr>
<tr>
<td>E</td>
<td>M</td>
<td>18</td>
<td>3.50 ± 0.51 b</td>
<td>3.33 ± 0.77 c</td>
<td>3.28 ± 0.75 c</td>
<td>3.33 ± 0.77 b</td>
<td>3.56 ±0.78 b</td>
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</tbody>
</table>

Consumer acceptance test industrial trial film E

<table>
<thead>
<tr>
<th>Film</th>
<th>Variety</th>
<th>N</th>
<th>Taste dough</th>
<th>Humidity smear</th>
<th>Smeary-smear</th>
<th>Odor smear</th>
<th>Optical appearance smear</th>
</tr>
</thead>
<tbody>
<tr>
<td>Csf</td>
<td>T</td>
<td>56</td>
<td>3.14±0.77 a</td>
<td>1.89±0.78 a</td>
<td>2.08±0.84 a</td>
<td>2.98±0.84 a</td>
<td>2.77±0.97 a</td>
</tr>
<tr>
<td>E</td>
<td>T</td>
<td>56</td>
<td>3.19±0.67 a</td>
<td>3.14±0.60 b</td>
<td>3.39±0.62 b</td>
<td>3.16±0.91 a</td>
<td>3.45±0.65 b</td>
</tr>
</tbody>
</table>
Fig. S1. pH and water activity (A) and appearance (B) development of cheese surface of variety M wrapped in standard film (full symbols) and film E (empty symbols) over storage time. Appearance of the cheese smear of the repetition (rep ▲) and the industrial (ind ◀) trial were judged according to the defect definition scheme (Chapter 3, Tab. 2). The pH and water activity value derived from two (rep,ind), independent storage tests and the error bars indicate standard deviation of the measurements. Different letters indicate significant (alpha=0.05) differences for comparison for each pair using Student’s t.
**Fig. S2.** Microbial composition and metabolic activity of red-smear cheese microflora over storage time measured by 16S rRNA gene gene copy numbers (cn). The symbols in the graphs demonstrate DNA copy numbers of standard film (●) and film E (○), PMA-DNA copy numbers of living bacterial cells of standard film (■) and film E (□), cDNA copy numbers of 16S rRNA transcripts of standard film (●) and film E (○). Error bars indicate standard deviation of three independent repetitions. Values labeled with different letters are significantly different (alpha= 0.05) from each other.
Fig. S3. Cheese portions of variety M wrapped in standard film (full line —) and film E (interrupted lines ---) were stored for 8 weeks and derived from the same production. All three cheese portions were opened and unwrapped before rating by 18 subjects was performed without knowing in which packaging material the cheese portions had been wrapped before.
Chapter 6

General conclusions and perspectives
6.1 General conclusions

The packaging of fully ripened red-smear cheese portion into plastic film for distribution and sale in self-service shelves is a practice increasingly done due to retailer’s logistics and consumer’s convenience. This practice is often associated with the development of a wet, smudgy and off-odorous cheese surface causing an unpleasant opening and therefore losses for the industry. For sale a dry, sparse, nice colored and non-off-odorous smear according to the variety’s prescription is required. The problem of a smear defect evolved and is known in cheese industry since cheese portion are film-prepacked. Many trials to solve the problem were performed in the industry but a satisfying solution could not be found. Therefore, this project was raised with the aim to investigate the mechanism and possible solution of the problem of red-smear cheese defect. The first hypothesis claimed a microorganism or a shift in smear-microflora composition would provoke the cheese smear defect due to the changing conditions trough film-packaging and subsequent storage. To investigate the difference in cheese surface microflora of defective and non-defective smear a polyphasic approach using culture dependent and culture independent methods were used. Thirty-eight cheese surface of variety M and 9 surface of variety T were cultivated on various media, colonies picked and identified by 16S rRNA sequencing and microbial composition was compared using culture independent TTGE method (Chapter 2). For all cheese portions, defective or non-defective, unpacked or film-prepacked and stored for 8 weeks at 8°C and even for both investigated varieties M and T a very similar microbial composition was observed. A list with typical and atypical microorganisms was postulated containing *Alkalibacterium* sp., *Arthrobacter casei*, *Brachybacterium tyrofermentas*, *Brevibacterium linens*, *Corynebacterium casei*, *C. stationis*, *C. variabile*, *Halomonas* sp., *Marinilactobacillus psychrotolerans*, *Microbacterium gubbeenense*, *Staphylococcus equorum*, *Candida psychrophila*, *Debaryomyces hansenii*, *Scopulariopsis brevicaulis*, *Scopulariopsis flava*, *Trichothecium domesticum* and *Yarrowia lipolytica* for the typical microorganisms. An even longer list for less expected microorganisms containing *Advenella*
Conclusions and perspectives

incenata, Bacillus sp., Candida parapsilosis, Enterococcus sp., Facklamia tabacinasalis, Lactobacillus spp., Morganella sp., Propionibacterium sp., Proteus sp, Providencia heimbachaeae, Staphylococcus saprophyticus and Vagococcus was found and giving evidence of the influence of the adventitious microflora on the cheese surface composition. A first storage test showed that every cheese portion harbors the potential to develop a defect and a cold chain interruption enhances defect development. Therefore we postulated that defect development is induced by the changed activity and/or metabolism of the smear microflora due to the switch to anaerobiosis caused through film packaging. Not a certain microorganism or a certain microbial group is responsible for defect development but as the third hypothesis states cheese smear defect is rather caused by multiple factors than by only a single one. Our fourth hypothesis says that a balanced cheese smear microflora can be achieved by controlling physicochemical conditions.

To investigate the environmental factors, microbial composition and rRNA transcriptional activity of the cheese smear microflora and further the effect of packaging material a storage test with standard and four alternative films (A, B, C and D) was performed for both variety M and T (Chapter 3). qPCR targeting the 16S rRNA gene of total bacteria, C. casei and Bacillus spp. was applied to monitor microbial copy numbers and metabolic activity of general smear bacteria, a typical representative of the aerobic or facultative anaerobic cheese smear and an unexpected, atypical representative with a potential to contribute to defect development. Defect development could be defined as a progressive process clearly associated with anaerobic conditions, a water activity increase, a pH drop and a decrease of transcriptional activity and accumulation of metabolites within the gas and water vapor tight standard film over storage. The films A-C were not applicable alternatives but confirmed that defect development is accompanied by the stated conditions. The decrease of transcriptional activity indicated that an inhibition of the microflora is associated with a defective smear and therefore is not to be achieved by prevention measurements. A high gas- and water permeable film D revealed a dry and sparse smear in combination with visible mold growth. A combination of film D with mold prevention was proposed as defect prevention strategy.
Therefore, the film was tested in combination with an antifungal component and a protective culture. The high water and low gas permeable film E was used in a storage test in combination with and without antifungal component (Chapter 4) and revealed a dry, sparse, non-odorous cheese smear until the end of storage even without any additional treatment. As defect development was associated with a metabolic decrease, v-qPCR method was used to distinguish between living and dead microorganisms over storage period (Chapter 5). We confirmed that defect development was associated with anaerobic conditions, a water activity increase and a pH drop. The decreasing trend of metabolic activity could be additionally shown by measuring the $mqo$, a protein coding gene, transcripts and showed a significant decrease in living aerobic bacterial cells shown for *C. casei* and *Bacillus* spp. The dead cells contribute to cheese surface defect by lysis and release of intracellular enzymes which can contribute to proteolysis and lipolysis. A challenge test, a repetition and an industrial scale up test demonstrated that the low gas and high water vapor permeable film could successfully prevent defect formation for both varieties until end of storage time. Film E disclosed micro-aerobic conditions, a diminished pH drop and a significant decrease of water activity value. These conditions seemed to be more favorable compared to standard film as metabolic activity drop was reduced and a diminished decrease of living bacteria and therefore a more balanced smear was observed. Cheese portions wrapped in standard film and alternative film E of each test were judged by cheese consumers to monitor acceptance of the alternative packaging material. The cheese smear of the portions wrapped in film E were in all three test significantly better evaluated according to smear humidity, smearyness and total appearance. Therefore a solution to prevent defect formation of red-smear cheese surface with a high consumer acceptance over 8 weeks of storage at 8°C could be shown.
6.2 Outlook and perspectives

In the industrial scale up trial one hundred portions of each variety M and T were packed in the alternative film and showed that defect development of the cheese surface could be successfully prevented. The used bags were rather big in size and produced some wrinkles which were not appreciated by the potential consumers. The appearance of the film-packed cheese portion has to be improved by fine adjustment of the packaging size and vacuum- and sealing properties of the packaging machine. The high water vapor permeability of the alternative film also allows the dough surface to dry over time when exposed to this film for a long time such as 8 weeks. Therefore a combination of the alternative film E for the cheese smear surface and a lid of standard film in contact with the cheese dough surface is an optimization possibility at the moment under investigation at our industrial partner. The combination of film E and standard film is more difficult to use for portions cut in triangles. For export, whole cheese loaves are film-packed and therefore film E represents an adequate packaging solution to maintain a dry, sparse, nice colored smear during transport to foreign countries. The alternative packaging film E can not only be used for semi-hard red-smear cheeses but could also be applied for hard cheese such as Gruyère. For soft or semi-soft cheese such as Muenster or Arenenberger the film could be applied for short-term storage, long-term storage in film E of a soft cheese could change its consistency as waterloss has an high impact on these type of cheeses.

Ammonia produced by deamination of aminoacids is an important metabolite of cheese ripening as it contributes with its pungent smell to flavor and pH increase in ripening. The measurement of ammonia in cheese smear and the surrounding would be a further component to be added to the model of cheese defect development.

We investigated the microbial composition, living bacteria and metabolic activity of red-smear cheese in an approach using qPCR, v-qPCR and RT-qPCR targeting total bacteria, and *C. casei* a typical representative and *Bacillus* spp. an atypical group of the cheese smear. The approach to use qPCR is promising due to its specificity and if DNA, PMA-DNA and cDNA
are analyzed in combination a better insight of microbial behavior can be obtained than by
targeting the DNA only. A differentiation between cell wall compromised cells and living
bacterial DNA can be made by application of PMA-DNA and metabolic activity is
complementary monitored by cDNA measurement. The cheese smear contains many other
species than the analyzed bacteria. We could clearly demonstrate that metabolic activity and
living bacteria of two aerobic bacteria are significantly decreasing over storage time.
Investigation of all the bacteria found in this complex ecosystem would be interesting and
could contribute to understand the dynamics in cheese smear during ripening and storage.
Addressing the less expected species of the cheese smear and especially anaerobic species
like Lactobacillus spp. and Propionibacterium sp. could be of special interest as the defect
was clearly associated with anaerobic conditions in standard film and an increase after
packaging was observed by cultivation methods. Also yeast and molds are part of the
cheese smear and even if they are only present in lower numbers at the end of ripening it
could be interesting to have a closer look at them under different packaging conditions.
Next generation sequencing are promising methods for the analysis of complex microbial
communities (Monnet, et al., 2013) and are more and more applied for the analysis of
complex matrices in environmental sample such as soil and food. To analyze microbiomes
from food including all the microbial genes in a given samples, metagenomics and
metatranscriptomics have great potential. The current limitation is the database availability
due to many genomes of food-related bacteria still to be sequenced (Ercolini, 2013). A
combined high-throughput (HTS) analysis of DNA, PMA-DNA and cDNA of red-smear
cheese surface of unpacked, standard film-prepacked and alternative film-prepacked cheese
portions could therefore elucidate microbial dynamics including bacterial, living bacterial and
metabolically active species. Post ripening microbial dynamics could add to understand in
depth the impact of film-packaging on the cheese smear ecosystem and elucidate the
microbial process of defect development.
Bibliography


Bibliography


**Bachelor and Master Thesis performed during this project:**


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