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Biodiversity of anti-listerial microbial cheese ripening consortia and monitoring of a recombinant *Yersinia enterocolitica* reporter strain on soft cheese

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To my wife Michal

“The important thing is not to stop questioning”

Albert Einstein

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*The work described in this section was performed by Geraldine Bresolin and Klaus Neuhaus.

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Summary

First, the temporal stability and diversity of the bacterial species composition as well as the anti-listerial potential of two different, complex and undefined microbial consortia from red-smear soft cheeses were investigated. Samples from two food producers (R and K) were collected two times each at six months intervals and a total of 400 bacterial isolates were identified by Fourier-transform infrared (FT-IR) spectroscopy and 16S rDNA sequence analysis. Coryneform bacteria represented the majority of the isolates, with certain species being predominant. In addition, *Marinolactobacillus psychrotolerans*, *Halomonas venusta*, *Halomonas variabilis*, *Halomonas* sp. (between 10^6 and 10^7 cfu per gram smear) and unknown Gram positive bacteria (between 10^7 and 10^8 cfu per gram smear) are described for the first time in this environment. The species composition of consortium R was quite stable over a period of six months, but consortium K revealed less diversity of coryneform species as well as less stability. While consortium R had a stable, extraordinary high anti listerial potential *in situ*, the anti-listerial activity of consortium K was lower and decreased with time. The cause for the anti-listerial activity of the two consortia remained unknown, but is not due to the secretion of soluble, inhibitory substances by the individual components of the consortium.

Furthermore, the effect of the ripening temperature on the biodiversity and the anti-listerial activity *in situ* of undefined red smear cheese ripening consortia was analyzed. Undefined microbial surface ripening consortia of mature retail red smear cheeses produced in dairies R and K were derived twice (I and II). Each of the four consortia (R I, R II, K I, K II) was used for inoculation of soft cheeses, which were ripened at two temperature regimes (13°C and 16/12°C). The bacterial composition of the consortia was analyzed in the smear waters and the corresponding surface floras derived from mature cheeses at the expiry date. A total of 1200 isolates, was identified by FT- IR spectroscopy, partial 16S rDNA sequence analysis of some isolates and physiological methods. Coryneform bacteria represented 78% of the isolates. As far as we are aware, this is the first report on the presence of *Vagococcus*

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carniphilus, *Psychrobacter* spp. and *Marinobacter* sp. in smear cheese surface floras. Furthermore, the two coryneform bacteria *Brachybacterium tyrofermentans* and *Leucobacter komagatae* are described for the first time on German red smear cheeses. The bacterial composition of consortia R I and R II differed significantly. At a ripening temperature of 13°C, in both consortia, the proportions of coryneforms increased from about 82% in the smear water to 96% and even 100% of the isolates in the corresponding surface floras derived at the expiry date. At 16/12°C, their proportions remained about the same level. With respect to the species level, significant flora successions occurred with both temperature regimes. In case of consortia K I and K II the coryneform content decreased with both temperature regimes compared to the initial content in smear water. In K II at 16/12°C, the coryneforms decreased and, instead, Marinolactobacilli dominated, which had not been detected in the smear water of K II. Despite the structural differences of K I and K II in the smear water the trend of reduction of *A. nicotianane* alongside with an increase of *B. linens* was similar in both consortia at the 16/12°C regime. While in the case of R I and R II the anti-listerial activity was not affected by the different ripening temperatures, with consortia K I and K II this feature appeared to be dependent on the ripening temperature regime.

As a step towards *real time* and *in situ* monitoring of pathogens in cold stored soft cheeses with a real product status, bioluminescent mutants of *Yersinia enterocolitica* were generated by transposon mutagenesis using a promotorless, complete *lux* operon (*luxCDABE*) derived from *Photorhabdus luminescens.* Their production of light in the cheese environment was monitored. Mutant B94, which had the lux cassette inserted into an open reading frame of unknown function was used for direct monitoring of *Y. enterocolitica* cells on cheeses stored at 10°C by quantifying bioluminescence using a photon counting intensified charge-coupled device (ICCD) camera. The detection limit on cheese was 200 cfu/cm^2. Bioluminescence of the reporter mutant was significantly regulated by its environment (NaCl, temperature, cheese), as well as by growth phase, *via* the promoter the *lux* operon had acquired upon transposition. At low temperatures, mutant B94 did

not exhibit the often-reported decrease of photon emission in older cells. It was not necessary to include either antibiotics or aldehyde into the food matrix in order to gain quantitative, reproducible bioluminescence data.

Zusammenfassung

Die zeitliche Stabilität und Vielfalt der Zusammensetzung der bakteriellen Spezies und ebenso das antilisterielle Potential von komplexen und undefinierten mikrobiellen Konsortien von Rotschmiere Weichkäsen wurde untersucht. Proben von zwei Lebensmittelherstellern (R und K) wurden zweimal innerhalb von sechs Monaten gesammelt. Insgesamt wurden 400 Bakterienisolate durch Fourier Transform Infrarot (FT-IR) Spectoskopie und 16S rDNA Sequenz Analyse identifiziert. Coryneforme Bakterien stellten den Hauptteil der Isolate, wobei einige Spezies dominierten. Darüber hinaus wurde *Marinolactobacillus psychrotolerans*, *Halomonas venusta*, *Halomonas variabilis*, *Halomonas* sp. (10^6 bis 10^7 cfu pro gr Schmiere) und unbekannte grampositive Bakterien (10^7 bis 10^8 cfu pro gr Schmiere) zum ersten Mal für dieses Biotop beschrieben. Die Florazusammensetzung des Konsortiums R war sehr stabil über eine Zeit von sechs Monaten, während das Konsortuim K eine geringere Vielfalt an coryneformen Bakterien und eine geringere Stabilität zeigte. Während Konsortium R ein stabiles und besonders hohes antilisterielles Potential *in situ* aufwies, war die anitlisterielle Aktivität von Konsortium geringer und nahm mit der Zeit ab. Die Ursache für die antilisterielle Aktivität der beiden Floren bleibt unbekannt, ist aber nicht auf lösliche Hemmstoffe zurückzuführen.

Darüber hinaus wurde der Einfluß der Reifungstemperatur auf die Biodiversität und die antilisterielle Aktivität *in situ* von komplexen undefinierten Rotschmiere Reifungskulturen untersucht. Dafür wurden Oberflächenfloren von ausgereiften Rotschmierekäsen der Molkereien R und K je zweimal entnommen (I und II). Jedes der vier Konsortien wurde zur Beimpfung von Weichkäse benutzt, welche bei zwei Temperaturen (13°C und 16/12°C) gereift wurden. Die Zusammensetzung der dabei erhaltenen Floren wurde im Schmierwasser und auch in der korrespondierenden Oberflächenflora der gereiften Käse am Ende der Versuche analysiert. Insgesamt wurden 1200 Isolate durch FT-IR Spektroskopie, sowie teilweise durch 16S rDNA Sequenzanalyse und durch klassische Methoden identifiziert. Außerdem

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wurde das antilisterielle Potential jedes Konsortiums für beide Temperaturabläufe bestimmt. Coryneforme Bakterien stellten 78% der Isolate. Soweit uns bekannt ist, ist dies der erste Bericht über das Vorkommen von *Vagococcus carniphilus*, *Psychrobacter* spp. und *Marinobacter* sp. in einer Schmierekäseflora. Weiterhin wurden die Coryneformen *Brachybacteria tyrofermentans* und *Leucobacter komagatae* zum ersten Mal für deutsche Schmierekäse beschrieben. Die Zusammensetzung der Floren RI und RII unterschieden sich beträchtlich. Bei der Reifungstemperatur von 13°C stieg in beiden Konsortien der Anteil der Coryneformen von etwa 82% im Schmierwasser auf etwa 96% der Isolate in der entsprechenden Oberflächenflora am Ende des Versuchs an. Bei einer Reifungstemperatur von 16/12°C dagegen wechselte der Anteil der Coryneformen nicht wesentlich. Hinsichtlich des Speziesanteils ergaben sich erhebliche Verschiebungen bei beiden Reifungstemperaturen. Im Fall der Konsortien K I und K II nahm der Coryneformenanteil bei beiden Reifungstemperaturen im Vergleich zum Schmierwasser ab. Im Fall von KII bei 16/12°C verloren die Coryneformen ihre Dominanz an Marinolactobacilli, welche im Schmierwasser vom K II nicht gefunden worden waren. Unabhängig von den Differenzen von K I und K II beim Schmierewasser hielt der Tend der Reduktion von *A. nicotiana* bei gleichzeitiger Zunahme von *B. linens* in beiden Konsortien bei einer Temperatur von 16/12°C an. Die antilisterielle Aktivität der Konsortien K I und KII hing offenbar von der Reifungstemperatur ab, während bei den Konsortien R I und R II kein Einfluss auf diese Eigenschaft festgestellt werden konnte.

Als Schritt zu einem *real time* und *in situ* Monitoring von Pathogenen in kühlgelagerten Weichkäsen mit unveränderten Produkteigenschaften, wurden Biolumineszenzmutanten von *Yersinia enterocolitica* durch Transposon Mutagenese gewonnen. Hierfür wurde ein promotorloses, komplettes *lux* Operon (*luxCDABE*) von *Photorhabdus luminescens* verwendet. Die Lichtemission auf Käse wurde verfolgt. Die Mutante B49, welche das *lux* Operon in einem offenen Leserahmen unbekannter Funktion trug, wurde für das direkte Monitoring von *Y. enterocolitica* auf Käse verwendet, welcher bei 10°C gelagert wurde. Dabei wurde die Biolumineszenz mit einer Photonen

zählenden ICCD (intensified charge coupled device) Kamera quantifiziert. Die Nachweisgrenze lag bei 200 KBE/cm^2. Mittels des Promotors, den das *lux* operon durch Transposition erworben hatte, wurde die Biolumineszenz der Reportermutante durch Umweltbedingungen (NaCl, Temperatur, Käsematrix) signifikant reguliert, ebenso wie durch die Wachstumsphase. Die für niedrige Temperaturen häufig berichtete Abnahme der Photonen Emission älterer Zellen trat bei Mutante B49 nicht auf. Es war weder der Zusatz von Antibiotika, noch von Aldehyd zur Lebensmittelmatrix notwendig, um die Mutante stabil zu halten und um quantitative, reproduzierbare Biolumineszenzdaten zu erhalten.

1 Introduction

1.1 The bacterial composition of red smear soft cheese surface floras

Red smear cheeses are economically important in Austria, Germany, France, Scandinavia and Switzerland (Eppert *et al*., 1997). The surface of these cheeses is coated with a red-orange smear during ripening (Reps, 1987). This smear determines the organoleptic properties of these cheeses. Soft red smear cheeses are ripened from the surface to interior, mainly through the participation of enzymes secreted by microorganisms present in the smear (Reps, 1987). The microbial succession during the surface ripening starts with the growth of yeasts, which metabolize the lactic acid present and raise the pH on the cheese surface from about 5.0 to about 6.0. When the pH is about 6.0, a salt–tolerant, usually very complex and undefined bacterial consortium begins to develop (El-Erian, A.F.M., 1972; Reps, 1987; Carminati *et al*., 1999) and eventually covers the entire surface of the cheese. The composition of bacterial red smear cheese surface consortia is exceedingly complex. Several studies show that the bacterial surface microflora of red smear cheeses consists of a large number of coryneform species belonging to genera of *Arthrobacter*, *Brevibacterium*, *Corynebacterum*, *Microbacterium*, *Micrococcus* and *Rhodococcus* spp. (Seiler, 1986; Valdés-Stauber and Scherer, 1994; Eliskases-Lechner and Ginzinger, 1995; Brennan *et al*., 2001). Also members of the genera *Staphylococcus* (Bockelmann *et al*., 1997; Carnio *et al*., 1999; Bockelmann and Hoppe-Seyler, 2001; Brennan *et al*., 2001) and *Enterococcus* are sometimes found (Ryser *et al*., 1994; Bockelmann *et al*., 1997a; Burri, 1999). Additionally, some Gram negatives were found on red smear cheese surfaces (Seiler, 1986; Eppert *et al*., 1997; Burri, 1999; Gianotti, 1999). The interaction of the microorganisms on such cheeses during ripening is unknown and, most probably very complex.

1.2 Identification of coryneforms

Identification of coryneforms is usually performed by physiological, phenotypic and biochemical characteristics (Seiler, 1983; Seiler, 1986; Lindenmann *et al*., 1995; Funke *et al*., 1997b; Funke *et al*., 1998;). Not always, however, these methods provide satisfactory results (Brennan *et al*., 2002; Oberreuter *et al*., 2002) and they are both, too laborious and time consuming to be performed on a routine basis. In contrast, molecular methods like the 16S rDNA sequence analysis yield usually precise identifications, but the costs per sample are quite high. Alternatively, the coryneform core part of bacterial red smear surface floras can now be identified by Fourier-transform infrared (FT-IR) spectroscopy. This cost efficient technique allows a rapid and simple identification of microorganisms (Helm *et al*., 1991; Naumann *et al*., 1991; Kümmerle *et al*., 1998) and has been established for identification of coryneform bacteria recently (Oberreuter *et al*., 2002a; Oberreuter *et al*., 2002b).

The principle of this technique is based on the fact that molecular bonds can be excited to vibration via the absorption of energy quanta of impinging infrared radiation (Günzler and Heise, 1996). The absorption spectrum is computed by a Fourier transform from an interferorgram originally collected by the measurement equipment and converts it into a plot of intensity as a function of frequency (wavelength) (Oberreuter, 2001). This plot is compared to the library plots collection of known bacteria.

1.3 *Listeria monocytogenes* and red smear cheese surface flora

Listeria monocytogenes is a facultative anaerobic Gram-positive, psychrotropic, motile, non-spore forming rod-shaped bacterium, growing from 1 to 44°C, with optimal growth occurring at 37°C (Erkmen, 2001). Due to its ubiquitous nature, its ability to grow at refrigeration temperatures, and its tolerance towards low pH (< pH 5.0) as well as high sodium chloride levels up

to 10% (Farber and Peterkin, 1991), it is very difficult to control *L. monocytogenes* in a cheese environment. Using the traditional "old young smearing" procedure for the production of red smear cheeses, pathogenic microorganisms such as *L. monocytogenes* may also be transferred from the mature to the fresh cheeses. As a result, its incidence in red smear cheeses has not decreased significantly during the last 14 years (Rudolf and Scherer, 2001). To increase the safety of these products, several attempts were carried out to investigate and use the anti-listerial activity of red smear ripening floras. Often, single bacterial strains were isolated from red smear cheese surface floras and evaluated for their anti-listerial activity *in vitro* (Valdés-Stauber *et al*., 1991; Ryser *et al*., 1994; Carnio *et al*., 1999). Furthermore, *in situ* evaluation of the anti-listerial activity of complex bacterial surface floras and single strains derived from red smear cheeses has been performed (Eppert *et al*., 1997; Carminati *et al*., 1999; Carnio *et al*., 2000).

1.4 Ripening temperature regimes during the production of red smear cheeses

Temperature is a major factor in microbial growth and enzyme activity and therefore greatly influences the course of cheese ripening (Weber and Ramet, 1987). Beside direct impact on growth rates it also influences the microbes indirectly, as together with relative humidity it also controls the migration of substrates and growth factors from inner cheese to the rind (Leclercq-Perlat *et al*., 2000). With respect to surface ripened cheeses it has been shown that different ripening temperatures may result in different compositions of bacterial red smear consortia (Reps, 1987).

Temperature regimes used for production of smeared soft cheeses vary significantly. E.g., one-step ripening with constant temperature may be performed at 15°C with a relative humidity of 95% for 28 days (Ryser and Marth, 1989) and 21 days (Ennahar *et al*., 1998), but also at a temperature of 3-5°C for 42 days with 90% relative humidity (Gobbetti *et al*.,1997). Additionally, two-step ripening procedures, e.g. at 16°C for 13 days, followed

by 7 days at 12°C with 95% relative humidity can be applied (Eppert *et al*., 1997). Furthermore, less strict regimes allowing irregular temperature shifts, e.g. between 15°C and 20°C (Brennan *et al*., 2002) have been described. Hence, a purchasable red smear-ripening consortium may be used under very different conditions, depending on the ripening procedures established in the respective dairies.

Since a high incidence of *Listeria monocytogenes* still can be observed in red smear cheeses (Rudolf and Scherer, 2001), for food safety reasons, there is a need for protective ripening cultures with anti-listerial potential.
In situ evaluation of the anti-listerial activity of complex bacterial surface floras and single strains derived from red smear cheeses has been performed in one step (Carminati *et al*., 1999) and two step ripening regimes (Eppert *et al*., 1997; Carnio *et al*., 2000). However, until now nothing was known about their stability over time and their sensitivity towards different ripening temperatures.

1.5 *Y. enterocolitica* and soft ripened cheeses

Yersinia enterocolitica is a facultative anaerobic Gram-negative rod-shaped bacterium, which is widely distributed in the environment (Pritchard *et al*., 1995). It is able to grow over a wide temperature range, from -5 to 42°C (Bergann *et al*., 1995), and therefore thrives well on food kept at refrigeration temperature (Little and Knochel, 1994). This pathogen occurs mainly in meat products (Fukushima *et al*., 1987; Falcao, 1991; Johannessen *et al*., 2000). It has also been recovered from raw milk cheese (Schiemann, 1978; Johnson *et al*., 1990;), as well as from cheese made from pasteurized milk, such as Camembert and Turkish Feta (Nootigedagt and Hartog, 1988; Moustafa, 1990; Erkmen, 1996). Soft surface mould-ripened cheese, such as Camembert and Brie, are of particular risk for transmitting pathogens (Little and Knochel, 1994). Rapid growth of undesirable microorganisms such as *Y. enterocolitca* is supported by high pH, high water activity values and the availability of nutrients in maturing cheeses (Nooitgedagt and Hartog, 1988).

1.6 Monitoring of pathogens by bioluminescence in food

The standard method to enumerate pathogens in food is to sacrifice the samples and analyze serial dilutions for the respective organisms. In case of *Y. enterocolitica* plating on Cefsulodin-irgasan-novobiocin agar produces results after 24 h (Karaioannoglou *et al*., 1985; Little and Knochel, 1994; Erkmen, 1996). A non invasive rapid alternative is to monitor pathogens in food by bioluminescence. The bioluminescence reaction, catalyzed by the enzyme luciferase, involves the oxidation of a long-chain aldehyde and reduced riboflavin phosphate ($FMNH_2$) and results in the emission of a blue green light with a peak at 490 nm (Baker *et al*., 1992) (see appendix C). The synthesis of light is encoded by five essential genes that are orgenized in an operon such as *luxCDABE*. The subunit *luxAB* is coded for the luciferase reaction and the genes coding for aldehyde synthesis by a fatty acid reductase complex are *luxCDE* (Baker *et al*., 1992; Francis *et al*., 2000). In order to monitor pathogens in food by bioluminescence, various *lux*AB reporter genes (encoding the luciferase enzyme), predominantly those derived from *Vibrio* spp. have been used to engineer bacteria (Chen and Griffiths, 1996; Hudson *et al*., 1997; Tomicka *et al*., 1997; Ramsaran *et al*., 1998). For example, bioluminescent *Escherichia coli* O157:H7 have been used to monitor the growth of this pathogen in selected yogurt and cheese varieties. Quantification of the bacteria was determined by surface plating of samples on BHI agar plates followed by incubation up to 18 hours at 37°C and subsequent counting of the bioluminescent colonies (Hudson *et al*., 1997). Photon emission was induced by addition of n-decylaldehyde to the lids of agar plates, since these recombinant pathogens did not contain the complete *lux* operon and were therefore unable to produce the aldehyde, the synthesis of which is encoded by *luxCDE*. The same approach has been used to investigate the survival of *E. coli* O157:H7 and *Listeria monocytogenes* in soft cheeses. In the case of *Listeria*, the aldehyde substrate was sprayed directly onto the colonies to induce light. Bioluminescent *E. coli* and *L. monocytogenes* colonies were detected on selective media after incubation at 30°C for 24 hours and 48 hours, respectively (Ramsaran *et al*., 1998).

Addition of an aldehyde can be circumvented in monitoring bacterial biluminescence by cloning the complete lux operon (*luxCDABE*) into these cells. Bioluminescent bacteria containing the *P. luminescens* full *lux* operon have several advantages to being used over strains containing *luxAB* only. Not only are bacteria carrying the full *lux* operon easier to monitor in food environments, they can also be used to monitor and track disease processes in living animals by measuring bioluminescence directly through the tissues of the animal using highly sensitive CCD cameras. Such an approach has been used to monitor bioluminescent *Salmonella enterica* serovar *Typhimurium*, *Staphylococcus aureus, Streptococcus pneumoniae* and *Escherichia coli* infections in animals (Contag *et al*., 1995; Francis *et al*., 2000; Francis *et al*., 2001; Rocchetta *et al*., 2001). The whole *lux* cassette of *P. luminescens* has also been used for real-time monitoring of the adherence of bioluminescent *Escherichia coli* O157:H7 to beef carcass surface tissue *in situ* (Siragusa *et al*., 1999). However, in the latter case the bioluminescent reporter strain harbored the *lux* operon on a plasmid. Hence, all experiments had to be performed in the presence of an antibiotic to maintain the *lux* mutant.

In order to fully benefit from bioluminescence as a non-invasive tool for research in all aspects of food safety, the system must be applicable under natural conditions. This means that the particular ecosystem investigated should be influenced as little as possible. By using the entire *lux* operon and stably integrating this onto the bacterial chromosome, (i) the necessity to penetrate the contaminated food matrix with an aldehyde can be avoided, and (ii) the competitive flora is not influenced by either the aldehyde, which can be toxic at high concentrations, or an antibiotic added to maintain the bioluminescent character of the reporter strain. Since food is often stored refrigerated, it is desirable to monitor pathogens *in situ* at low temperature without temperature shift for bioluminescence measurements. For this purpose, the reporter gene cassette needs to be controlled by a promoter that is active at cold storage temperatures.

1.7 Aims of this work

Traditional production of various foodstuffs such as Kefir, sourdough and red smear cheeses includes the transfer of complex undefined microbial consortia from mature products to freshly prepared matrices. Until now, it is unknown for red smear cheeses, whether the repeated transfer to fresh substrates, or their indigenous microflora, influence the composition and the anti-listerial activity of the respective consortia over time. Therefore, the first aim of this work was to assess the temporal stability of the biodiversity and the anti-listerial activity of microbial cheese ripening consortia. We examined these characteristics of two surface floras of German red smear soft cheeses.

Red smear ripening consortia exhibited an inhibitory effect against *Listeria monocytogenes* (see section 3.1). Until now, however, it was unknown, whether a certain red smear consortium maintains its anti-listerial activity when applied at different ripening temperatures. Hence, the second aim of this work was to investigate the influence of different temperature regimes during soft cheese ripening on the anti-listerial activity of two different complex red smear consortia and the composition of their bacterial subflora.

There is a need for a non-invasive monitoring method for pathogens in order to avoid sacrificing the samples. Successful monitoring of pathogens in dairy products using bioluminescent bacteria has already been demonstrated (e.g., Hudson *et al*., 1997; Ramsaran *et al*., 1998). These reporter strains were not able to synthesize the aldehyde needed for luciferase activity. Due to the addition of aldehyde, monitoring in a real product status was not possible. Real time *in situ* monitoring without addition of aldehyde was only reported for using a plasmid containing strain which adhered to beef carcass surface tissue (Siragusa *et al*., 1999), but this had to be done in the presence of ampicillin. Therefore, the third goal of this work was to develop a bioluminescent reporter strain without the need to add aldehyde or antibiotics to the food stuff that allows *in situ* and *real time* monitoring of a pathogen even at low temperature.

2. Material and Methods

2.1 Temporal stability and biodiversity of two complex, anti-listerial cheese ripening microbial consortia

2.1.1 Media

The media which were not commercially available were prepared according to the recipes presented below.

PC3+ broth (Plate count with 3% NaCl) (per liter)

Pepton from casein	5.0	g
Glucose	1.0	g
Yeast extract	2.5	g
NaCl	30.0	g

pH 7.0 ± 0.1

For agar plates add 15.0 g agar-agar.

TSB-ANC broth (per liter)

Pepton from casein	17.0	g
Phytone – Peptone	3.0	g
Glucose	2.5	g
Yest extract	6.0	g
NaCl	5.0	g
K_2HPO_4	2.5	g
Supplements		
Acriflavine	2.3	mg
Nalidixic acid	0.92	mg
Cycloheximide	2.875	mg

pH 7.3 ± 0.2

The three supplements were added to a sterilized media at 50°C.

All media were sterilized at 121°C, 15 min.

2.1.2 Bacterial strains and ripening consortia

Undefined surface floras (microbial consortia) from two different red smear soft cheeses produced from pasteurized cow milk in factories R and K were harvested two times, separated by a six months interval. The consortia of the first and the second sampling were used for the subsequent cheese ripening experiments A and B, respectively. The consortia were scraped off the cheese surfaces and about 10 g of smear were homogenized in 100 ml sterile NaCl solution (5%). After centrifugation (8000 rpm, 25 min), the supernatant was discarded and the cells were resuspended in 20 ml sterile NaCl solution (5%) plus 4.2 ml sterile glycerol (87%). The suspensions were then dispensed into micro tubes (2 ml/tube) and subsequently stored at –70°C. The preparations were conducted under sterile conditions. A defined consortium derived by Maier and Rudolf (our laboratory, unpublished results) from red smear soft cheese of factory M was used as a control flora. It consists of 12 bacterial strains and 4 yeast strains and was also stored at –70°C.

L. monocytogenes WSCL 1364 from Vacherin Mont d' Or cheese (Bille, 1989) was used as an indicator strain for *in situ* testing of the anti-listerial activity of the various ripening consortia. It was cultivated for 24 h at 30°C in brain heart infusion broth (BHI; Merck) and diluted in quarter strength Ringer solution (Merck) to achieve appropriate cell counts for brine contamination.

2.1.3 Cheese inoculation and ripening

All experiments for the evaluation of the anti listerial activity were performed with independent repetitions. Viable bacterial cell counts of the frozen consortia were determined on $PC3^+$ agar after incubation for 72 h at 30°C. Viable yeast cell counts (in case of flora M) were determined on YGCB (Yeast glucose-chloramphenicol; Merck; supplemented with 10μg of bromophenol blue/liter) agar, after incubation for 48 h at 30°C. The undefined consortia (R and K) and the defined consortium (M) were suspended in sterile 5% NaCl solution to obtain at least 5×10^7 CFU/ml brine.

This solution was used to inoculate soft cheeses (45% fat content in dry matter), which were obtained right after dry salting from a German dairy.

Fresh cheeses were checked for absence of *Listeria* (see 2.1.4). The smearing procedure was started 1 day after salting. The first day of smearing was designated as day 1 of the ripening period. All in all, smearing was performed five times at intervals of 2 or 3 days by dipping the cheeses into the smear water in order to moisten their entire surface. Sterile gloves were used to manually rub the liquid over the cheese surface. At every day of smearing 50 ml of smear water were freshly prepared for each flora and one pair of gloves were used to treat all cheeses with the respective smear water. Cheeses were incubated under laboratory conditions in glass dessicators as described previously (Sulzer, 1991) at 16°C from day 1 to 13 and subsequently at 12°C until day 20 with 95% relative humidity. Cheeses were packed in aluminum foil at day 20 and then stored at 10°C for 3 weeks. Contamination with *L. monocytogenes* WSLC 1364 was performed by contaminating the brine at day 1, just before smearing. The contamination levels ranged from 10^1 to 10^3 CFU/ml smear water.

2.1.4 Detection of *Listeria monocytogenes* in cheese

Detection of *Listeria* was performed according to the standard 143A:1995 of the International Dairy Federation (IDF). A 25 g sub-sample of the cheese surfaces was homogenized with 50 ml of 1.75% (wt/vol) tri-sodium citrate-dehydrate buffer ($C_6H_5Na_3O_7$ x $2H_2O$, pH 7.5; Merck) in a Stomacher (model Lab-Blender 400; Kleinfeld Labortechnik, Hannover, Germany). Then added to 175 ml tryptose soy enrichment broth supplemented with the inhibitors Acriflavin, Nalidixin acid and Cycloheximid (TSB-ANC). After incubation of 48 h at 30°C a loopful ($\varnothing \cong 4$ mm) of the enrichment culture was streaked on Oxford agar (Oxoid). After incubation of 48 h at 37°C presence of a typical listeria colonies was examined.

2.1.5 Enumeration of *Listeria monocytogenes* in cheese

When the detection of *Listeria* yielded a positive result on oxford agar, the next *Listeria* analysis was performed as cell count. The two main surfaces of 5 mm-thick slices of cheese (20 g; 50 cm^2) were homogenized in 180 ml 1.75% (wt/vol) tri-sodium citrate dehydrate buffer ($C_6H_5Na_3O_7$ x $2H_2O$, pH 7.5; Merck). Serial 10-fold dilutions of these suspensions were plated on Oxford

agar and incubated at 37°C for 48 h. Cell counts were calculated per cm^2 of cheese surface (see appendix A).

2.1.6 Evaluation of anti-listerial activity

The tested 400 isolates (100 per factory R+K and sampling A+B) were maintained as glycerol stocks at –70°C (see section 2.1.7). The indicator strain (*L. monocytogenes* WSCL 1364) and the control strain (*Staphylococcus equorum* WS 2733), which had been found to produce a bacteriocin with antagonistic effect against the former (Carnio *et al*., 2000), were grown at 30°C on tryptose agar (Merck) and plate count agar for 24 h, respectively, and stored at 4°C as stock cultures for one week. To screen the anti-listerial activity, the coryneform isolates were grown in $PC3^+$ broth as described by Valdés-Stauber et al. (1991). The Gram negative isolates were propagated accordingly for 48 h. The Gram positive and catalase negative bacteria were grown in M17 (Merck) broth at 30°C for 48 h. The control strain was grown in BHI (Merck) broth at 30°C for 24 h while shaking at 150 rpm (Carnio, 1999). The total flora of each consortium was scrapped off from countable PC3+ agar plates as used for isolation of bacteria (see section 2.1.7). The anti-listerial activity of the isolates, surface floras and the control strain were examined according to Valdés-Stauber et al. (1991) by spotting 10 µl of sterile-filtered culture supernatants on tryptose soft agar (Tryptose broth, Merck with 8 g agar/l) containing the indicator strain. 6.5 ml of the soft agar (47°C) was mixed with 0.1 ml of a 24-h culture of the indicator strain grown at 30°C, and the mixture containing 3 x 10^6 to 5 x 10^6 indicator cells per ml, was poured into a petri dish (diameter 85 mm). The plates were incubated at 30°C and examined for inhibition zones after 24 h, 48 h and 72 h using Henry's illumination.

2.1.7 Isolation of bacteria from surface floras

Microtubes of the microbial consortia R and K, were thawed and plated as 10-fold dilutions on $PC3^+$ plates. One hundred colonies per consortium and sampling were selected randomly from countable $PC3^+$ agar plates after incubation at 30°C for 3 days, followed by 4 days at room temperature, and

were purified by re-streaking on $PC3^+$ agar. The purified isolates were stored as glycerol stocks at –70°C.

2.1.8 Identification of bacterial isolates

The purified isolates were subjected to a KOH test (Buck, 1982) and checked for the presence of catalase with 3% H_2O_2. The cell morphology was determined by phase-contrast microscopy. Due to their cell morphology, the Gram and catalase positive isolates were considered as putative coryneforms and related species and identified by FT-IR spectroscopy (Oberreuter *et al*., 2002). Additionally, representative strains of similarity clusters yielded by FT-IR spectroscopy were subjected to 16S rDNA sequence analysis. Strain lysis, 16S rDNA sequence amplification and purification of the PCR product have been performed according to Oberreuter et al. (2002). After purification, PCR products were sequenced by SequiServe (Vaterstetten, Germany). Cycle sequencing was performed by using the universal 16S rDNA binding primer 5' f [5'- AGAGTTTGACCTGGCTCA-3'] position 8-26 in the *E.coli* numbering system (Brosius *et al*., 1978).

Gram positive and catalase negative isolates were characterized by biochemical tests. These included gas formation from glucose (2% wt/vol) at 30°C for 48 h in De Man-Rogosa-Sharpe (MRS; Oxoid) broth, and gas formation from citrate at 30°C for 48 h in M17 broth from Terzaghi (Merck) with 2.5% (wt/vol) diammoniumcitrate ($C_6H_{14}N_2O_7$; Merck). Growth was tested at 42°C for 48 h in All Purpose Tween® (APT; Merck) broth at 37°C for 48 h in M17 broth with 6.5% NaCl, and on Kanamycin Esculin Azide agar (Merck) at 37°C for 48 h. Additionally, acid formation from lactose was determined in M17 broth (Merck) at 30°C for 48 h. For identification by FT-IR spectroscopy, cells were incubated at 30°C for 48 h on APT agar under anaerobic conditions and then treated according to Oberreuter et al. (2002), using a lactic acid bacteria reference library (Herbert Seiler, this laboratory, unpublished data). Since the Gram positive and catalase negatives could not be identified by these methods, subsequently they were also subjected to 16S rDNA analysis as described above.

The gram-negative isolates were checked for the presence of cytochrome oxidase using ready to use test strips (Bactident ®; Merck), and subsequently identified using the biochemical test systems *BBL™ Enterotube ™ II* or by *BBL® Oxi/Ferm ™ Tube II* (Becton Dickinson), respectively. Due to unclear results, the final identification of the Gram negatives was performed by 16S rDNA analysis as described above.

2.1.9 pH measurements

The pH of the cheese surfaces was determined as mean of five measurements per cheese and time point of measurement with a surface electrode Inlab 426 (Mettler Toledo, Steinbach, Germany). The pH of liquid cultures of various isolates was determined with a POLY-PLAST electrode (Hamilton AG; Bonaduz, Switzerland).

2.2 Ripening temperature effect on biodiversity and anti-listerial activity of two complex undefined microbial red smear cheese ripening consortia

2.2.1 Media

The non-commercial media used in this project are described in section 2.1.1.

2.2.2 Bacterial strains and ripening consortia

Undefined surface floras (microbial consortia) from two different red smear soft cheeses produced from pasteurized cow milk in factories R and K were harvested two times (I and II). The consortium R II was harvested one and half year later than R I. The consortia K I and K II were harvested with one-week in between. The preparation of those consortia and a defined consortium M were done as described in section 2.1.2. *L. monocytogenes* WSCL 1364 Vacherin Mont d' Or cheese (Bille, 1989) was used as an indicator strain for *in situ* testing of the anti- listerial activity of the various ripening consortia. It was cultivated for 24 h at 30°C in brain heart infusion

broth (BHI; Merck) and diluted in quarter strength Ringer solution (Merck) to achieve appropriate cell counts for brine contamination. *Staphylococcus aureus* WS 1754 was used as positive control in the coagulase assay (see section 2.2.7).

2.2.3 Cheese inoculation and ripening

Each ripening consortium (R I, R II, K I, K II) was evaluated for its anti-listerial activity *in situ* with two different ripening temperature regimes, both of which have been observed in the dairy industry. The whole procedures including times of smearing, packing and storage until the expiry date (day 41) are displayed in Fig. 2.1. All fresh cheeses were obtained from the same dairy

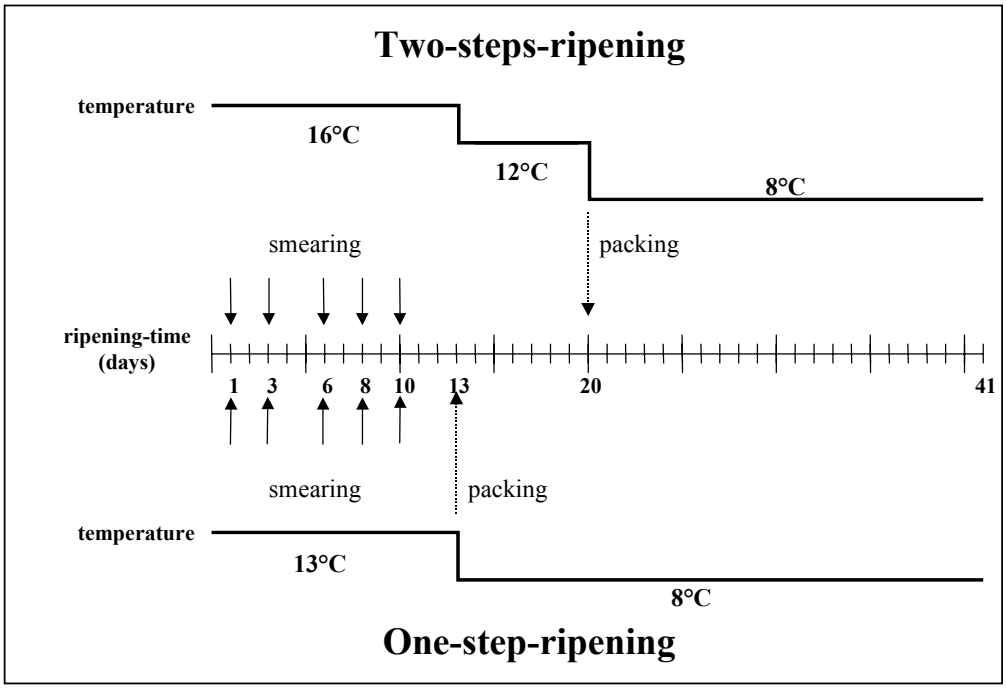

Fig. 2.1 Two ripening procedures of soft red smear cheeses. One step ripening at a constant temperature of 13°C until packing at day 13. Two step ripening at 16°C for 13 days, followed by 7 days at 12°C until packing at day 20. The relative humidity

of both ripening procedures was 95%. Cheeses were packed in aluminum foil and subsequently stored at 8°Cuntil the expiry date (day 41).

after salting and checked for absence of *Listeria* (see section 2.1.4). The cheeses were inoculated according to Eppert et al. (1997). Viable cell count of the frozen consortia was performed as described in section 2.1.3. Cheeses were incubated under laboratory conditions in glass dessicators as described previously (Sulzer, 1991). After packing in aluminum foil the cheeses were stored at 8°C until the expiry date (day 41). Contamination with *L. monocytogenes* WSCL 1364 was performed by contaminating the brine at day 1, just before smearing. In previous experiments (see section 3.1.4) consortium R had showed a stable anti listerial activity with a contamination level of 10^2 CFU/ml brine and consortium K with a contamination level of 10^1 CFU/ml brine. Therefore, the contamination levels in this study were 10^2 CFU/ml brine and 10^1 CFU/ml smear water, respectively.

2.2.4 Detection of *Listeria monocytogenes* in cheese

The detection of *L. monocytogenes* was performed as described in section 2.1.4.

2.2.5 Enumeration of *Listeria monocytogenes* in cheese

The enumeration of *L, monocytogenes* was performed as described in section 2.1.5.

2.2.6 Isolation of bacteria from smear water and from inoculated cheeses at the end of the ripening

The frozen consortia that had been harvested from retail cheeses were thawed and suspended in brine solution to prepare the smear water for inoculation the green cheeses. The smear water (SW) of each consortium (R and K) and sampling (I and II), were plated as 10-fold dilutions on $PC3^+$ plates. One hundred colonies per consortium and sampling (Σ=400 isolates) were selected randomly from countable $PC3^+$ agar plates after incubation at 30°C for 3 days, followed by 4 days at room temperature, and were purified by re-streaking on $PC3^+$ agar. The purified isolates were stored as glycerol

stocks at –70°C. The surface floras from inoculated cheeses at the end of the storage time of the two ripening procedures were scraped off and suspended with tri-sodium citrate buffer (1:10) and were homogenized in a lab blender (model Lab-Blender 400; Kleinfeld Labortechnik, Hannover, Germany). Serial 10-fold dilutions of these suspensions were plated on $PC3^+$ plates, which were incubated as mentioned above. One hundred colonies per consortium, sampling time and ripening procedure (13°C and 16/12 °C) (Σ=800 isolates) were selected, purified and stored as described above.

2.2.7 Identification of bacterial isolates

Gram positive and catalase positive isolates were regarded as putative coryneforms or related organisms and identified by FT-IR spectroscopy according to Oberreuter et al. (2002). The FT-IR spectra were also used to create dendrograms that displayed the similarity of the isolates of the respective consortium. Within the FT-IR dendrograms, the spectral distance of 1.0 was chosen as a limit to distinguish between major similarity clusters at the species level. Additionally, representative strains of similarity clusters yielded by FT-IR spectroscopy were subjected to 16S rDNA sequence analysis as described in section 2.1.8. Furthermore, the putative *Corynebacterium casei/ammoniagenes* strains were tested for anaerobic growth on PC3+ agar and production of acid from glucose in PC3+ broth supplemented with 2% glucose under aerobic conditions. Both tests were performed at 30°C for 72 h. Gram positive and catalase negative isolates, as well as gram-negative isolates were also analyzed for similarity by FT-IR spectroscopy. For identification purpose, representatives of the resulting clusters were subjected to 16S rDNA analysis as described in section 2.1.8. Culture preparation for FT-IR spectroscopy of Gram negative isolates was identical to the method for coryneforms (Oberreuter *et al*., 2002). Gram positive catalase negative isolates were cultured anaerobically at 30°C for 48 h on M17 agar. Additionally, the latter were tested for acid formation from lactose as described in section 2.1.8.

The *Staphylococcus* isolates were tested for coagulase activity, using the commercial Bacto Coagulase plasma EDTA kit (Difco). The isolates were

grown in BHI broth (Merck) for overnight at 37°C. 2-3 drops of each overnight culture were added to a tube of rehydrated coagulase plasma and then incubating for 24 h at 37°C. The formation of clot in the plasma indicates coagulase production.

2.2.8 pH measurements

The pH measurements were performed according to section 2.1.9.

2.3 Sensitive *in situ* monitoring of a recombinant bioluminescent *Yersinia enterocolitica* reporter mutant in *real time* on Camembert cheese

2.3.1 Media

The media which was not commercially available was prepared according to the recipe presented below.

LB broth (per liter)

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g

pH 7.4

For agar plates add 15.0 g agar-agar.

The media were sterilized at 121°C, 15 min.

2.3.2 Bacterial strains and cheese

Mutants carrying the *luxCDABE* reporter construct were created using *Yersinia enterocolitica* strain NCTC 10460 (see section 2.3.3). Stock cultures were maintained on brain heart infusion agar (BHIA, Merck Germany) incubated at 30°C, stored at 4°C and subcultured at monthly intervals. Camembert cheeses (80 g, 45% fat in dry matter) were purchased from a local supermarket three weeks before the expiry date and were kept at 10°C overnight in the laboratory.

2.3.3 Construction of bioluminescent reporter strains*

A pool of random *luxCDABE* fusions was generated in *Y. enterocolitica* NCTC 10460 by mating this strain with *E. coli* S17λ-1 pir containing a pUT-mini-*Tn5 luxCDABE*-Km^2 plasmid, as described by Winson et al. (1998). *E. coli* S17-1λpir containing pUT- mini-*Tn5 luxCDABE* Km^2 was grown at 37°C in LB broth (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, pH 7.4), supplemented with 50 μg/ml ampicillin and *Y. enterocolitica* NCTC 10460 was grown in LB broth at 30°C without any antibiotics. Both strains were grown to an optical density of 0.5 at 600 nm. For mating, 100 μl of each culture were mixed and supplied with 800 μl 10mM $MgSO_4$ solution, followed by a 5 min incubation at room temperature. The cells were pelleted (5 min, 3000 rpm at RT), then resuspended in 100μl LB broth before being spread onto LB agar plates (without antibiotics) and incubated overnight at 30°C. The following morning, each lawn of cells was removed from the plate surface by being carefully scrapped off. These cells were then resuspended in 13% glycerol, shock frozen in five aliquots of 1ml, each in liquid N_2, and stored at –70°C. One frozen aliquot was resuspended in approximately 10 ml LB broth to an OD_{600} of 0.3. Subsequently, 100 μl of this suspension were plated on LB selection plates (145 mm diameter) containing 200 μg/ml kanamycin and 20 μg/ml chloramphenicol. Kanamycin selects for transpositional events, and the chloramphenicol discriminates for *Y. enterocolitica*. After 24 h at 30°C the plates were examined for colonies that produced high levels of bioluminescence.

2.3.4 Contamination of Camembert cheese

Bioluminescent mutants were grown (2.5% NaCl, 10°C, 48 h) in brain heart infusion broth (BHI, Merck). 0.3 ml of a 100- fold dilution was applied to the surface of the cheese under sterile conditions, corresponding to about 10^4 CFU/cm^2. The cheeses were stored at 10°C overnight to let the surface dry and were then packed in aluminium foil. *Y. enterocolitica* was enumerated by homogenizing 20 g of the camembert surface with 180 ml of 1.75% tri-sodium citrate-dehydrate solution (pH 7.5) using a Stomacher (model Lab-Blender 400; Kleinfeld Labortechnik, Hannover, Germany). Serial 10-fold dilutions of

* The work described in this section was performed by Geraldine Bresolin and Klaus Neuhaus.

these suspensions were plated directly on Cefsulodin-irgasan-novobiocin (CIN) agar (Oxoid) (30°C, 24 h) for the total *Yersinia* count (see appendix B).

2.3.5 Correlation of bioluminescence and CFU/cm^2 on BHI agar plates

The mutant B94 was grown at 10°C in BHI broth with 2.5% salt and without salt to a titer of 6 x 10^7 CFU/ml. 0.1 ml of a decimally diluted culture were inoculated on pre-cooled BHI agar (10°C). The bioluminescence measurements were performed during the first 5 days at 10°C. Immediately after measuring the bioluminescence, *Y. enterocolitica* was enumerated by homogenizing the whole agar of the plate (20 ± 1 g) with 180 ml of 1.75% tri-sodium citrate-dehydrate solution (pH 7.5) using a Stomacher (model Lab-Blender 400, Kleinfeld Labortechnik, Hannover, Germany). Serial 10-fold dilutions of these suspensions were plated directly on BHI agar, incubated (30°C, 24 h) and the total *Y. enterocolitica* count recorded (see appendix B).

2.3.6 Quantification of bioluminescnce

The samples were screened for bioluminescence by a photon counting intensified charge-coupled device (ICCD) camera (model C2400-75H, Hamamatsu Photonics, Hamamatsu City, Japan). After exposure for 1 minute (restrictor position at 0.95), the images were processed with an Argus 20 image processor (Hamamatsu) and were transferred to a Macintosh Power PC. Photon emission was quantified by the Living-Image™ Software Package V. 4.0 (Xenogen Corporation, Alameda, California).

3 Results

3.1 Temporal stability and biodiversity of two complex, anti-listerial cheese ripening microbial consortia.

In order to evaluate the temporal stability of undefined microbial consortia and their corresponding anti-listerial activity, two different, complex and undefined microbial consortia from red smear soft cheeses were collected from two producers (R and K) two times each in six months intervals. A total of 400 bacterial isolates were identified by Fourier-transform infrared (FT-IR) spectroscopy and 16S rDNA sequence analysis. The consortia of the first and the second sampling were used for the subsequent cheese ripening experiments A and B, respectively and listeria growth was evaluated *in situ*.

3.1.1 Identification of 400 representative bacterial isolates

According to the results of the preliminary characterization, the total of 400 isolates was subdivided into three groups, (i) Gram positive and catalase positive non-sporeformers (378 isolates), (ii) Gram positive and catalase negative non-sporeformers (15 isolates) and (iii) Gram negatives (7 isolates) (see Fig. 3.2 on page 29). All 378 isolates of the first group were identified as coryneforms at the species level by FT-IR spectroscopy, representing 94.5% of the total isolates. As an example, the FT-IR dendrogram of flora R (sampling A) is displayed in Fig. 3.1, where isolate designations in bold letters indicate additional 16S rDNA sequence analysis. In this case, the identification of 94 coryneform isolates resulted in 8 major FT-IR clusters. Clusters 1 and 2 are clearly separated from the others and comprise *Brevibacterium linens*. Clusters 3, 4, 6 and 8 include *Corynebacterium variabile*. Next to these, clusters 5 and 7 comprise *Corynebacterium casei*. All three species formed more than one independent cluster.

3.1.2 Composition and temporal stability of consortium R

The composition of the undefined microbial consortium derived from

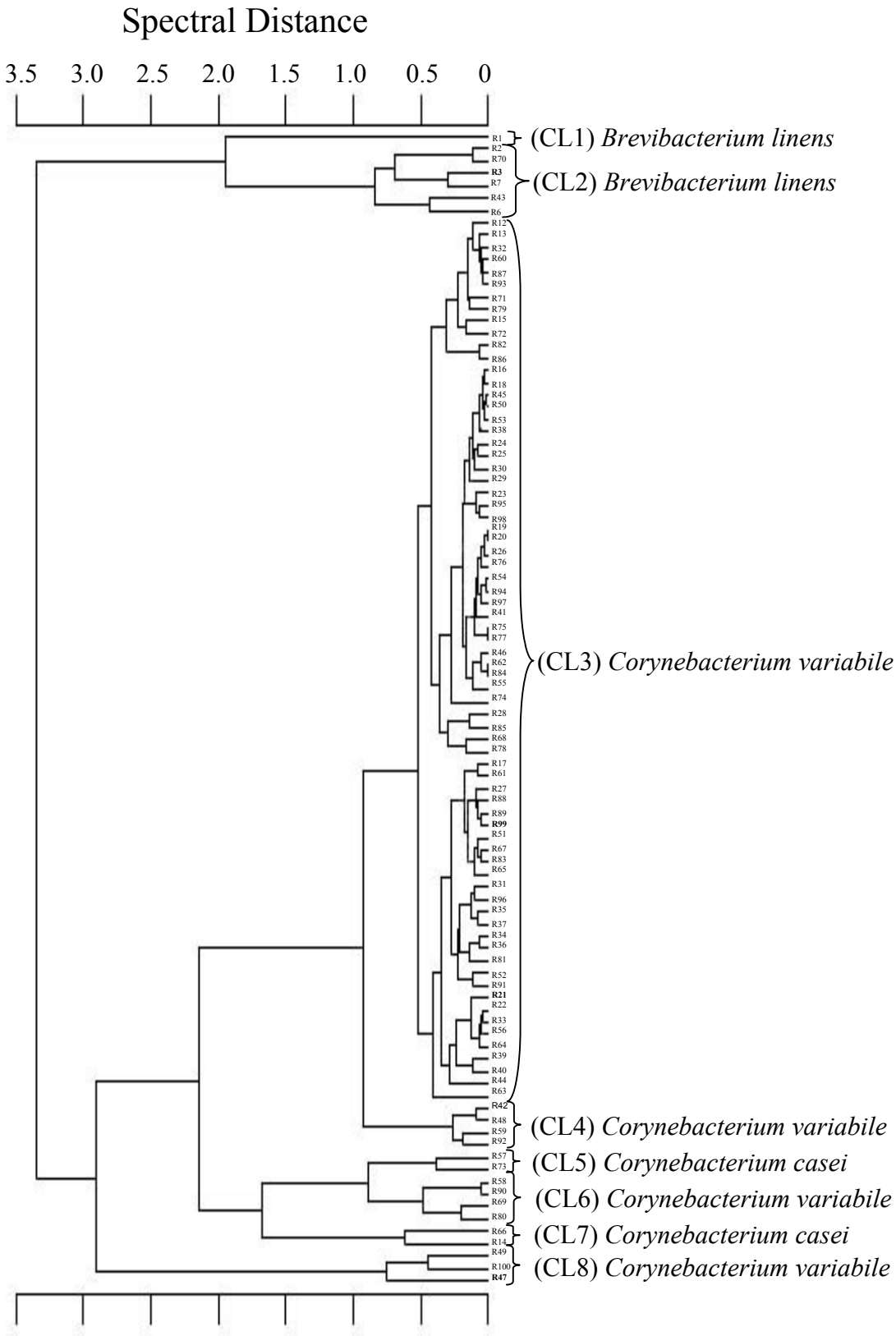

Fig. 3.1 Dendrogram of 94 coryneforms from a complex red smear surface flora (dairy R, sampling point (A) in Fig. 3.2) identified by FT-IR spectroscopy. Bold letters indicate additional 16S rDNA sequence analysis.

mature red smear soft cheeses of factory R at the beginning (A) and at the end (B) of a six-month period is given in Fig. 3.2. At both samplings the majority of the consortium was formed by coryneform bacteria, representing 94% of the isolates. In both analyses, *C. variabile* was the dominant species with almost identical proportions of 83% and 80% of the total isolates, respectively. The proportions of all other species ranged between 1% and 7% with *B. linens* and *C. casei* contributing also to the coryneform subflora in the first analysis. At the second sampling (B) additional coryneforms, namely *Arthrobacter nicotianae* (4%) and *Microbacterium gubbeenense* (5%) were found. 12 Gram positive and catalase negative bacteria were the only non-coryneform isolates from consortium R. They neither formed gas from glucose or citrate, nor did they grow at 42°C or in the presence of 6.5% NaCl or on Kanamycin Esculin Azide agar. Two isolates formed acid from lactose (see Tab. 3.1). Since none of these 12 isolates could be identified by FT-IR spectroscopy, all were subjected to 16S rDNA analysis. The two lactic acid bacteria (LAB) revealed 99% sequence similarity to an uncultured bacterium from feedlot manure, while the other 10 isolates could not be related to any described species.

3.1.3 Composition and temporal stability of consortium K

Coryneform bacteria at both samplings (A and B) formed the majority of consortium K, representing 92% and 99% of the isolates, respectively (Fig 3.2). In both samplings, *A. nicotianae* was the predominant species with 90% and 76% of the isolates, respectively. The only other coryneform bacterium was *B. linens*. It increased significantly from 2% to 22% of the isolates at sampling (A) and (B), respectively (Fig 3.2). Additionally, a few Gram positive catalase negative isolates and Gram negatives were found in both analysis. Two of the three Gram positive catalase negative isolates formed acid from lactose (see Tab. 3.1), but were negative in all other physiological tests (see section 2.1.8) and could not be identified by FT-IR spectroscopy. By 16S rDNA analysis all the three could be identified as *Marinolactobacillus psychrotolerans.* The Gram negative isolates could not be identified by by

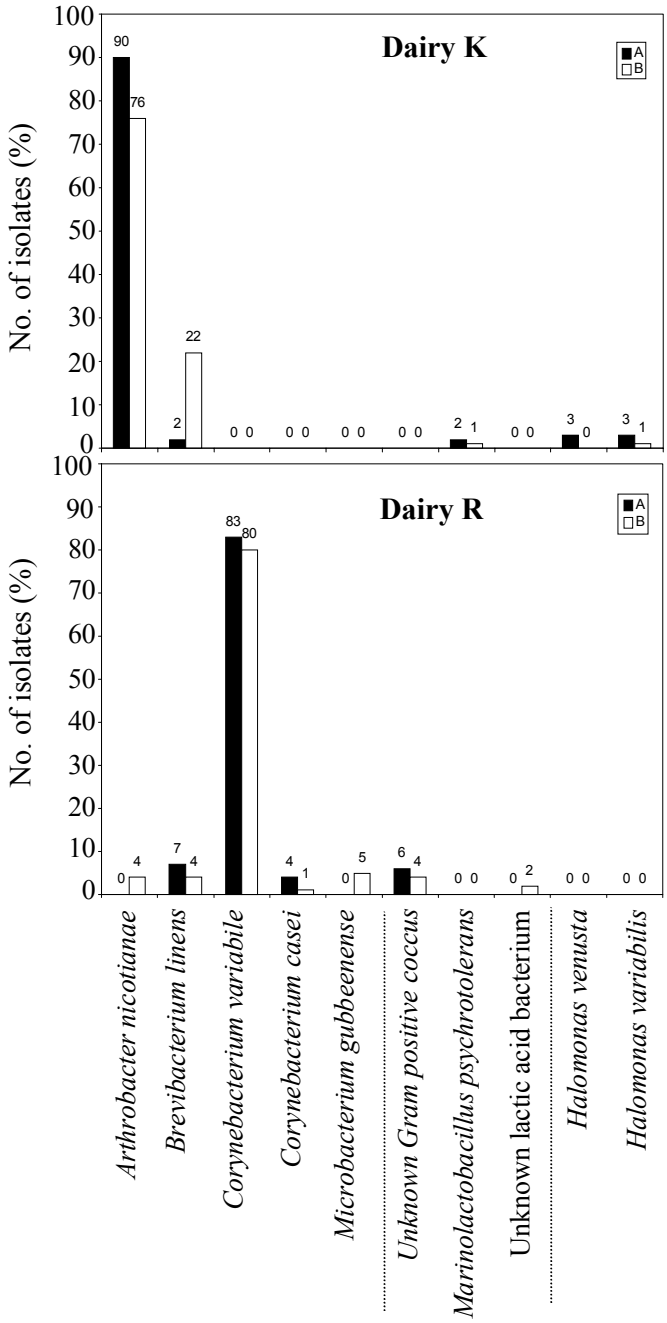

Fig. 3.2 Composition of two undefined surface floras derived from German commercial mature red smear soft cheeses produced in dairy R and K, respectively. Each flora was derived at the beginning (A) and at the end (B) of a six-month period from retail red smear cheeses. The total cell counts of consortium R at sampling (A) and (B) were 4.0 x 10^9 CFU/g smear and 3.2 x 10^9 CFU/g smear, respectively. The total cell counts of consortium K at sampling (A) and (B) were 2.9 x 10^8 CFU/g smear and 3.8 x 10^8 CFU/g smear, respectively. The three groups (⋯⋯) represent the Gram positive and catalase positive isolates (left), Gram positive and catalase negative isolates (center) and the Gram negatives (right).

biochemical tests. However, three isolates could be identified as *Halomonas venusta* and two as *Halomonas variabilis* by 16S rDNA analysis.

The other two appeared to be members of the genus *Halomonas* also, but could not be addressed at the species level.

Tab. 3.1. Acid formation from lactose by Gram positive and catalase negative bacteria isolated from microbial consortia which were derived two times (A and B) from red smear cheeses produced in dairy R and K. (Initial pH of M17 broth: 7.1).

Isolate number	Sampling	Identification	Final pH
R_4	A	Unknown Gram positive coccus	6.61
R_5	A	Unknown Gram positive coccus	6.64
R_8	A	Unknown Gram positive coccus	6.52
R_9	A	Unknown Gram positive coccus	6.61
R_10	A	Unknown Gram positive coccus	6.57
R_11	A	Unknown Gram positive coccus	6.48
R_13	B	Unknown Gram positive coccus	6.87
R_60	B	Unknown Gram positive coccus	6.67
R_62	B	Unknown LAB (uncultured bacterium from feedlot manure)	5.46
R_65	B	Unknown Gram positive coccus	6.95
R_89	B	Unknown LAB (uncultured bacterium from feedlot manure)	5.50
R_100	B	Unknown Gram positive coccus	6.94
K_7	A	*Marinolactobacillus psychrotolerans*	6.50
K_8	A	*Marinolactobacillus psychrotolerans*	5.70
K_25	B	*Marinolactobacillus psychrotolerans*	5.74

3.1.4 *In situ* anti-listerial activity of consortium R and K

Fig. 3.3 displays the anti- listerial activity of consortium R as determined *in situ* on soft cheese. In both experiments A and B, *L. monocytogenes* WSLC 1364 grew to high numbers on the control cheeses (M) with contamination levels of 40 CFU/ml brine and 60 CFU/ml brine, respectively. Applying the same titers, the pathogen could be inhibited completely by consortium R and almost completely with contamination levels of 340 CFU/ml brine and 270 CFU/ml brine. In experiment B the anti-listerial activity of consortium R was tested additionally with a contamination level of 1600 CFU/ml brine and yielded a total inhibition of *L. monocytogenes* (Fig. 3.3 B). The repetitions in both experiments showed similar results (Fig. 3.3).

Figure 3.4 depicts the data on the anti-listerial activity *in situ* of consortium K when derived at the beginning (Fig. 3.4A) and at the end (Fig. 3.4B) of a six month period. In either case it inhibited *L. monocytogenes* at a

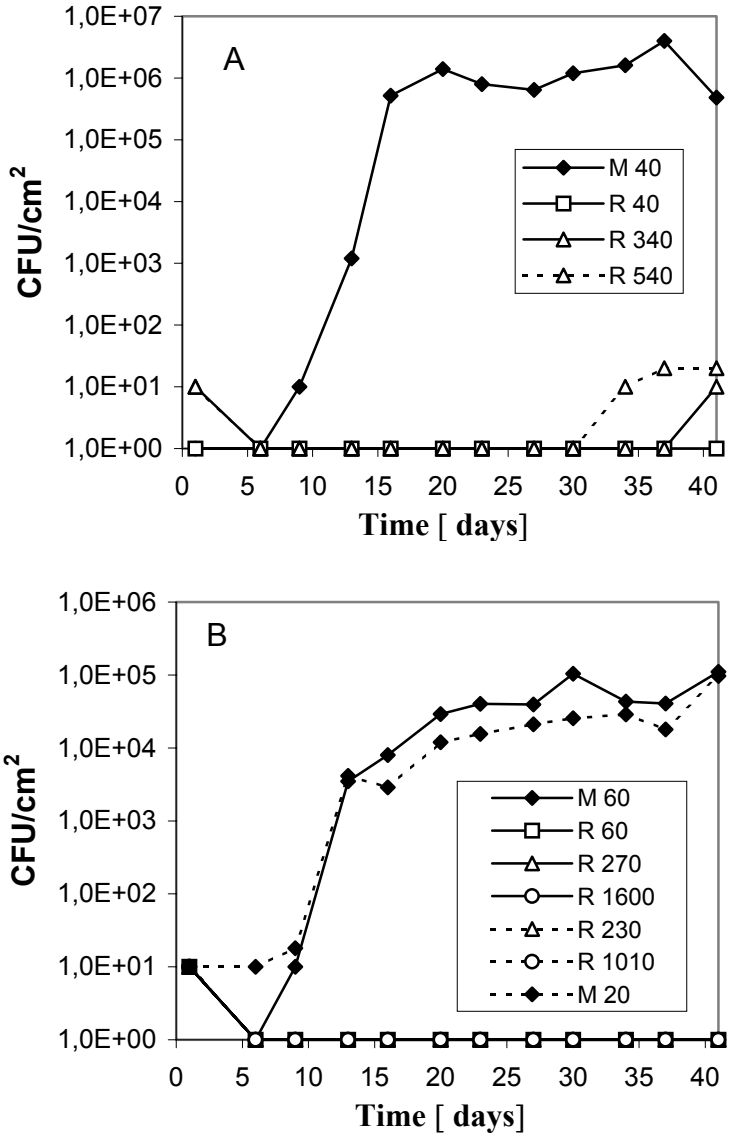

Fig. 3.3 Growth *of Listeria monocytogenes* on soft cheese in the presence of the complex undefined red smear surface flora R. The experimental series (A) and (B) were performed with floras harvested from mature retail red smear soft cheeses of dairy R, with six months apart. *Listeria monocytogenes* WSLC 1364 was used for contamination on day 1. The numbers in the legend indicate the contamination level per ml smear water. Flora M served as a control. It is a defined culture derived from red smear soft cheese of dairy M. The dashed lines represent the repetitions.

contamination level of 10^1 CFU/ml brine. With contamination levels of 10^2 CFU/ml brine, however, the listerial growth could not be inhibited to the same extent. While comparison with the control consortium M still demonstrates a

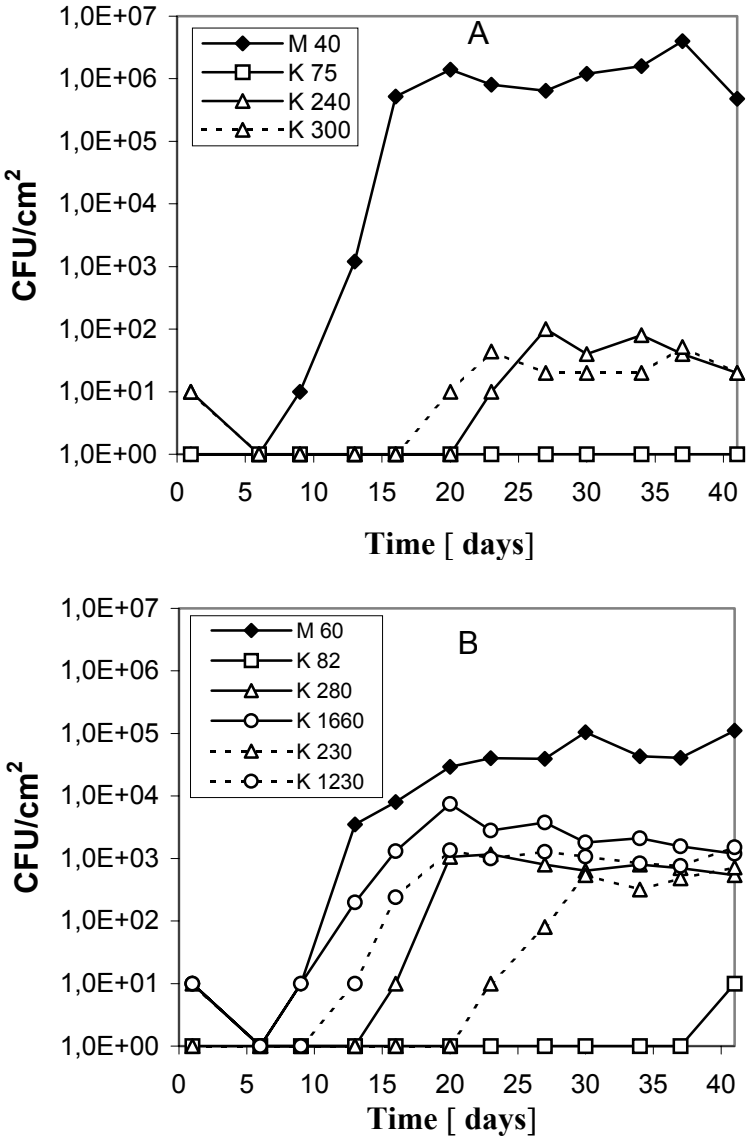

Fig. 3.4 Growth *of Listeria monocytogenes* on soft cheese in the presence of the complex undefined red smear surface flora K. The experimental series (A) and (B) were performed with floras harvested from mature retail red smear soft cheeses of dairy K, with six months apart. *Listeria monocytogenes* WSLC 1364 was used for contamination on day 1. The numbers in the legend indicate the contamination level per ml smear water. Flora M served as control. It is a defined culture derived from red smear soft cheese of dairy M. The dashed lines represent the repetitions.

certain inhibitory effect, *Listeria* analysis yielded positive results after about 3 weeks (Fig. 3.4A) and two weeks (Fig. 3.4B), respectively. Also the repetitions revealed similar results and yielded positive results after three weeks (Fig. 3.4A and Fig. 3.4B). With a contamination level of 10^3 CFU/ml brine *Listeria* analysis yielded a positive result after 9 days but growth of *L. monocytogenes* didn't reach to the same extent like in the control M (Fig. 3.4B). The repetition yielded similar inhibitory effect (Fig. 3.4B).

3.1.5 Anti-listerial activity of individual isolates of consortium R and consortium K.

The antagonistic activity against *L. monocytogenes* has been determined in an agar diffusion-screening assay. While the control strain *St. equorum* WS 2733 yielded clear inhibition zones after 24 h, 48 h and 72 h, none of the 400 isolates tested exhibited an ant-listerial activity as a single culture at any time point of examination. The cumulative anti-listerial effect of each consortium has been examined also. None of the four consortia exhibited inhibition zones.

3.1.6 Development of the pH on the cheese surface

The development of the pH in the presence of undefined surface floras derived from factory R and K with six months apart is shown in Fig. 3.5. Within 10 days, the pH reached to about 7.5 in each experiment (Fig. 3.5A and Fig. 3.5B), followed by a slight increase towards pH 8.0 during the ripening until day 41.

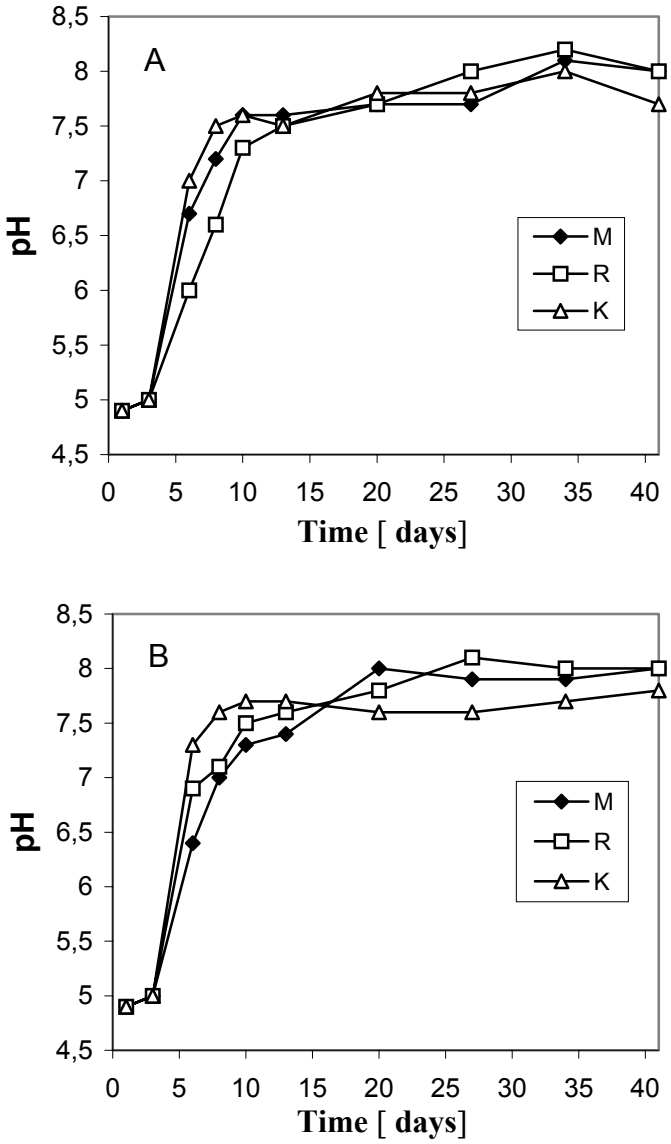

Fig 3.5 Development of the pH on soft cheese surfaces when different red smear surface cultures are applied. The experimental series (A) and (B) were performed six months apart. Flora M served as control. It is a defiend culture derived from red smear soft cheese of dairy M. Floras R and K are complex undefined red smear consortia harvested two times each with six month apart from the surface of commercial red smear soft cheeses from factory R and K, respectively.

3.2 Ripening temperature effect on biodiversity and anti-listerial activity of two complex undefined microbial red smear cheese ripening consortia

In order to evaluate the effect of ripening temperature regimes on the composition of red smear cheese surface floras and their anti-listerial activity, two different, complex and undefined microbial consortia were derived two times each (I and II) from red smear soft cheeses produced in two dairies (R and K). Each of the four consortia was used for inoculation of soft cheeses, which were subsequently ripened at two temperature regimes (13°C and 16/12°C) (see Fig. 2.1). A total of 1200 isolates, representing 4 consortia, determined in the smear water and the corresponding 8 surface floras derived from ripened cheeses at the expiry date, were identified by FT-IR spectroscopy, 16S rDNA sequence analysis and classical methods. In addition, the anti-listerial potential of each consortium was investigated *in situ* in both ripening temperature regimes.

3.2.1 Identification of 1200 representative bacterial isolates

According to the results of the preliminary characterization, the total of 1200 isolates was subdivided into three groups, (i) Gram positive and catalase positive non-sporeformers (968 isolates), (ii) Gram positive and catalase negative non-sporeformers (207 isolates) and (iii) Gram negatives (25 isolates) (see Fig. 3.8 and Fig. 3.11 on pages 39 and 46, respectively). 934 isolates of the first group were identified as coryneforms representing 78% of the total isolates. 34 isolates were S*taphylococcus* spp. As an example, the FT-IR dendrogram stemming from matured soft cheese inoculated with flora R II and ripened at 16/12 °C is displayed in Fig. 3.6, where bold letters indicate additional 16S rDNA sequence analysis. In this case, the identification of 87 coryneform isolates resulted in 13 major FT-IR clusters of *Brevibacterium linens*, *Microbacterium gubbeenense*, *Corynebacterium casei* and *Corynebacterium variabile*. All of the four species formed more than one major cluster. This phenomenon was also observed with flora K. For example, the

56 coryneforms isolated from cheese at the expiry date ripening with flora K II

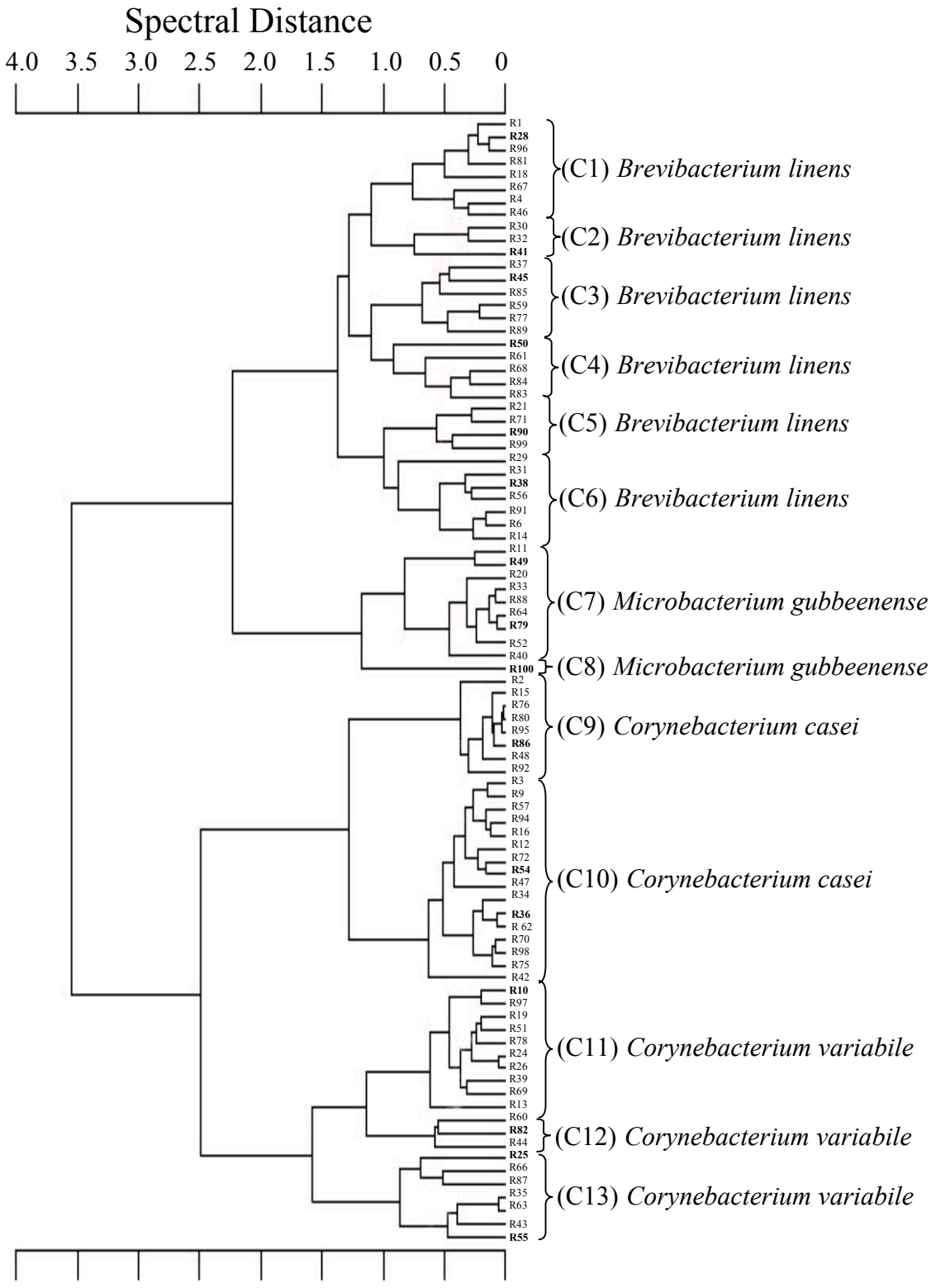

Fig. 3.6 Dendrogram of 87 coryneforms identified by FT-IR spectroscopy. The isolates were derived at the expiry date (day 41) from the red smear surface flora of soft cheese inoculated with consortium R II (see Fig. 3.8-II) and ripened at 16/12°C (see Fig. 2.1). Bold letters indicate additional 16S rDNA sequence analysis.

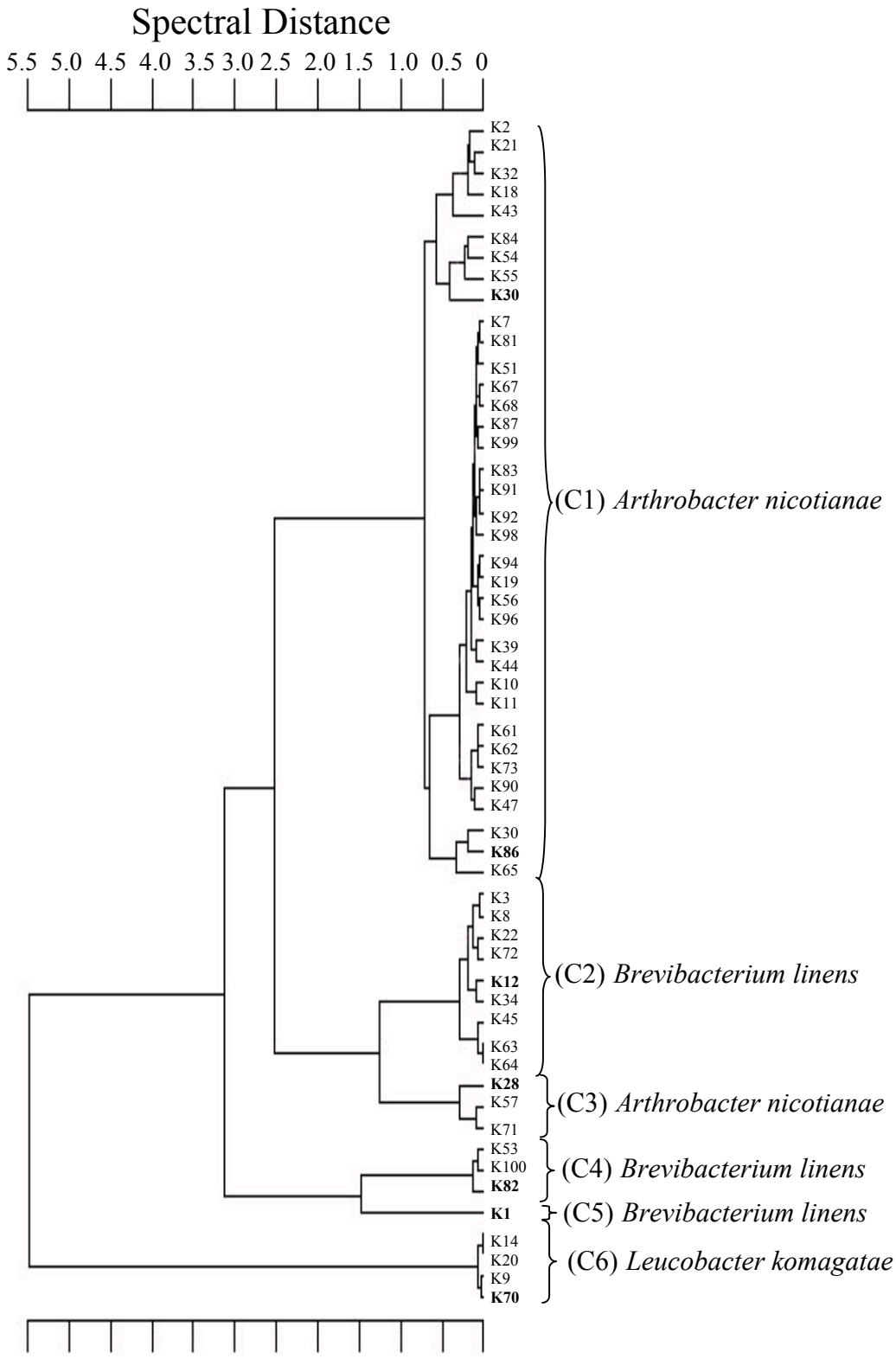

Fig. 3.7 Dendrogram of 56 coryneforms identified by FT-IR spectroscopy. The isolates were derived at the expiry date (day 41) from the red smear surface flora of soft cheese inoculated with consortium K II (see Fig. 3.11-II) and ripened at 13°C (seeFig. 2.1). Bold letters indicate additional 16S rDNA sequence analysis.

at 13°C, represented 3 species and formed 6 major clusters in the FT-IR dendrogram (Fig. 3.7). In this case, *Arthrobacter nicotianae*, *B. linens* and *Leucobacter komagatae* were found.

3.2.2 Bacterial composition of consortium R I.

Every consortium had been analyzed three times to determine its bacterial composition in the smear water (100 isolates), as well as at the expiry date after the two ripening procedures (13°C and 16/12°C, 100 isolates each). The results of consortium R I are displayed in Fig. 3.8. In the smear water, coryneform bacteria represented 82% of the isolates. The predominant species was *C. variabile* (57%), followed by *M. gubbeenense*, *A. nicotianae*, *B. linens*, and *C. casei/ammoniagenes*, representing 11%, 8%, 5% and 1% of the isolates, respectively. Gram positive and catalase negative bacteria were the predominant non-coryneform isolates.14 isolates represented a coccus-shaped unknown lactic acid bacterium (LAB). All the 14 isolates lowered the pH of M17 broth to about 5.6 (Tab. 3.2). They could be referred to an uncultured bacterium from feedlot manure by 16S rDNA sequence analysis (GeneBank, accession numbers AF317370 and AF317352). Both sequences were revealed similarities above 99%, while the third hit was at 94%. In the FT-IR dendrogram all of them were located in one cluster (CL 2, see in Fig 3.9). The other four were identified as *Vagococcus carniphilus* and *Vagococcus* sp., respectively.

All 100 isolates of the bacterial surface flora derived at the expiry date from cheeses incubated at 13°C were coryneform bacteria. The predominant species was *B. linens* representing 38% of the isolates, followed by *C. casei/ammoniagenes* (24%). The latter was marked as *casei/ammoniagenes*, since neither 16S rDNA analysis nor FT-IR spectroscopy nor phenotypic tests yielded decisive results and some *C. casei* isolates might belong to the species *C. ammoniagenes*. *M. gubeenense* and *A. nicotianae* represented 23% and 13% of the isolates, respectively. One *C. variabile* was found, as well as one *Microbacterium* sp. which had not been detected in the smear water.

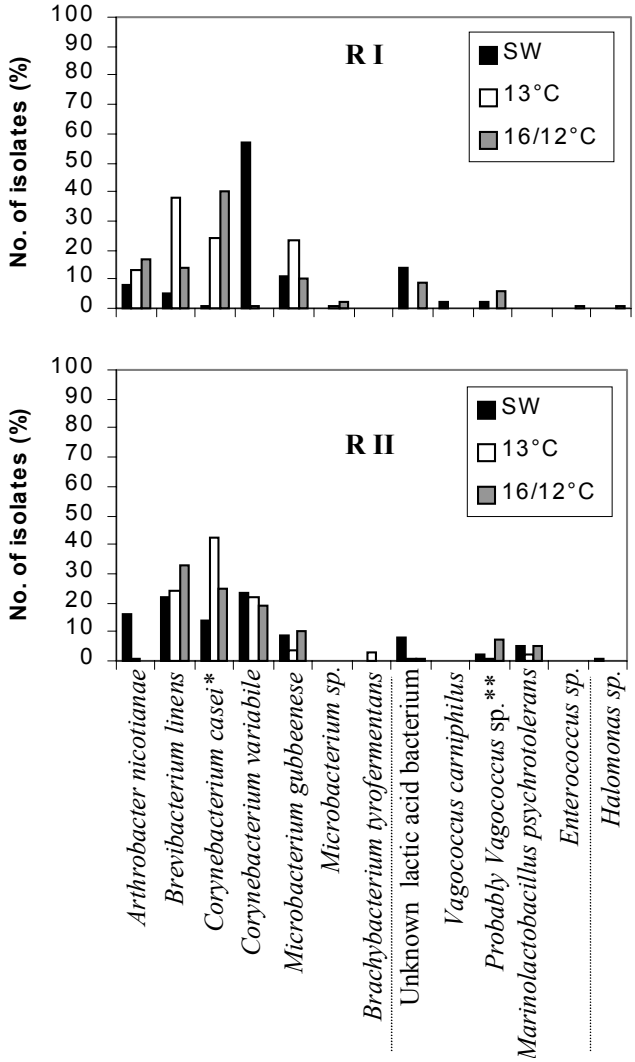

Fig. 3.8 Bacterial composition of the two red smear consortia R I and R II, determined in the smear water (SW) and the corresponding surface floras derived at the expiry date (day 41) from inoculated soft cheeses, matured according to two different ripening regimes (13°C and 16/12°C, respectively; see Fig. 2.1). The respective smear waters (I and II) were prepared using undefined red smear surface flora derived two times (I and II) from mature commercial German soft cheese produced in dairy R. The three groups (⋯⋯) represent the Gram positive and catalase positive isolates (left), Gram positive and catalase negative isolates (center) and the Gram negatives (right).

*Since in case of some isolates of experiment R I neither 16S rDNA analysis nor FT-IR spectroscopy nor phenotypic tests yielded decisive results, some *C. casei* isolates might belong to the species *C. ammoniagenes*.

** Since the 16S rDNA sequence similarities were found to be 93% to 96% with *Vagococcus spp*., it might be that these isolates are *Vagococcus sp*.

The final bacterial surface flora of cheeses matured at 16/12°C was mainly formed by coryneform bacteria, representing 83% of the isolates. The predominant species was *C. casei/ammoniagenes* (40%). Additionally, *A. nicotianae* (17%), *B. linens* (14%) and *M. gubbeenense* (10%) were isolated. *Microbacterium* sp. appeared with 2% of the isolates, while *C. variabile* was not detected. 9 isolates could be referred to the same uncultured bacterium from feedlot manure (GeneBank, accession numbers AF317370 and AF317352) than the respective 14 isolates from the smear water of

Tab. 3.2 Acid formation from lactose by Gram positive and catalase negative bacteria isolated from consortium R I. They were isolated from the smear water (SW) and from the corresponding surface flora derived at the expiry date from inoculated soft cheeses matured at 16/12°C. (Initial pH of M17 broth: 7.1).

Isolate number	Identification	Final pH
SW_13	Unknown LAB (uncultured bacterium from feedlot manure)	5.57
SW_25	Unknown LAB (uncultured bacterium from feedlot manure)	5.51
SW_30	Unknown LAB (uncultured bacterium from feedlot manure)	5.65
SW_53	Unknown LAB (uncultured bacterium from feedlot manure)	5.61
SW_55	Unknown LAB (uncultured bacterium from feedlot manure)	5.61
SW_59	Unknown LAB (uncultured bacterium from feedlot manure)	5.63
SW_61	Probably *Vagococcus* sp.	6.55
SW_62	Unknown LAB (uncultured bacterium from feedlot manure)	5.61
SW_64	Unknown LAB (uncultured bacterium from feedlot manure)	5.61
SW_67	Unknown LAB (uncultured bacterium from feedlot manure)	5.57
SW_70	Unknown LAB (uncultured bacterium from feedlot manure)	5.60
SW_71	Unknown LAB (uncultured bacterium from feedlot manure)	5.57
SW_74	*Vagococcus carniphilus*	6.93
SW_79	*Vagococcus carniphilus*	6.99
SW_80	Probably *Vagococcus* sp.	6.74
SW_82	Unknown LAB (uncultured bacterium from feedlot manure)	5.60
SW_85	Unknown LAB (uncultured bacterium from feedlot manure)	5.58
SW_92	Unknown LAB (uncultured bacterium from feedlot manure)	5.59
16/12_1	Unknown LAB (uncultured bacterium from feedlot manure)	5.57
16/12_2	Unknown LAB (uncultured bacterium from feedlot manure)	5.56
16/12_3	Unknown LAB (uncultured bacterium from feedlot manure)	5.53
16/12_13	Unknown LAB (uncultured bacterium from feedlot manure)	5.68
16/12_29	Unknown LAB (uncultured bacterium from feedlot manure)	5.58
16/12_33	Unknown LAB (uncultured bacterium from feedlot manure)	5.68
16/12_37	Unknown LAB (uncultured bacterium from feedlot manure)	5.73
16/12_39	Probably *Vagococcus* sp.	6.56
16/12_43	Unknown LAB (uncultured bacterium from feedlot manure)	5.65
16/12_47	Unknown LAB (uncultured bacterium from feedlot manure)	5.60
16/12_60	Probably *Vagococcus* sp.	6.61
16/12_63	Probably *Vagococcus* sp.	6.59
16/12_73	Probably *Vagococcus* sp.	6.59
16/12_74	Probably *Vagococcus* sp.	6.73
16/12_75	Probably *Vagococcus* sp.	6.60

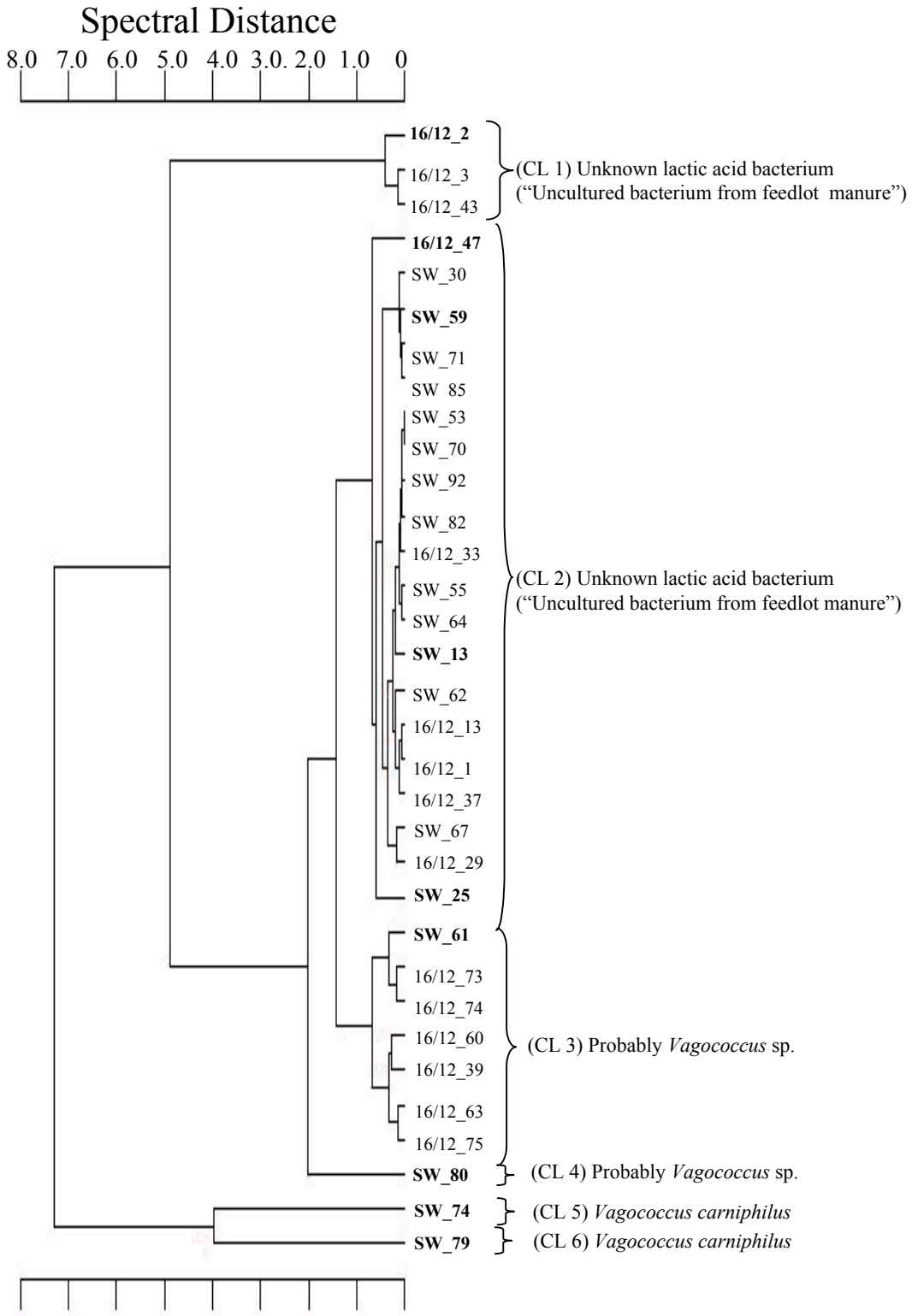

Fig. 3.9 FT-IR dendrogram of 33 Gram positive and catalase negative bacteria isolated from consortium R I**.** They were isolated from the smear water (SW) and from the corresponding surface flora derived at the expiry date from inoculated soft cheeses matured at 16/12°C. Representative strains of similarity clusters which were subjected to 16S rDNA sequence analysis are designated by bold letters.

consortium R I. Again all of them were coccus-shaped and fermented lactose. However, in the FT-IR dendrogram they were located in two different clusters (Fig. 3.9). Furthermore, one *Enterococcus* sp. and one *Halomonas* sp. were found (Fig. 3.8).

3.2.3 Bacterial composition of consortium R II

Coryneform bacteria formed the majority (84%) of consortium R II in the smear water. In this case, there was no predominant species in the coryneform subflora, which was composed of *C. variabile* (23%), *B. linens* (22%), *A. nicotianae* (16%), *C. casei* (14%) and *M. gubbeenense* (9%) (Fig. 3.8). Gram positive and catalase negative bacteria were the most frequent non-coryneform isolates (15 isolates). 8 of them could be referred to an uncultured bacterium from feedlot manure. While all of them were coccus-shaped only one isolate fermented lactose (Tab. 3.3) to the same extent like the respective 23 isolates which were found in consortium R I. The others lowered the pH to about 6.5 (Tab. 3.3). The strong fermenting isolate formed an independent cluster (CL11) by FT-IR spectroscopy (Fig. 3.10). 5 of the isolates were identified as *Marinolactobacillus psychrotolerans* by 16S rDNA sequence analysis. Within the species also some of the isolates fermented lactose weakly (final pH about 6.5) and some fermented lactose strongly (final pH about 5.7) (Tab. 3.3). The members of this species were located in different FT-IR clusters, independently from the intensity of lactose fermentation (Fig. 3.10). The remaining two Gram positive catalase negative isolates were probably *Vagococcus* sp., since the highest 16S rDNA sequence similarities were found to be 93% to 96% with *Vagococcus spp*. (Fig. 3.8). In addition, one Gram negative bacterium (*Halomonas* sp.) was detected.

The core part of the final bacterial surface flora on cheeses incubated at 13°C was formed by coryneform bacteria (96%) (Fig. 3.8). The predominant species was *C. casei*, representing 42% of the isolates. *B. linens*, and *C. variabile*, represented 24% and 22% of the isolates, respectively. Additionally, *M. gubbeenense* (4%), *A. nicotianae* (1%) and *Brachybacterium tyrofermentans* (3%) were isolated. The latter was not found in the smear

water. The non-coryneform bacteria (4%) were *Marinolactobacillus psychrotolerans* (2%), unknown lactic acid bacterium which could be referred to an uncultured bacterium from feedlot manure (1%) and *Vagococcus* sp. (1%).

Tab. 3.3 Acid formation from lactose by Gram positive and catalase negative bacteria isolated from consortium R II. They were isolated from the smear water (SW) and from the corresponding surface floras derived at the expiry date from inoculated soft cheeses, matured at 13°C and 16/12°C, respectively. (Initial pH of M17 broth: 7.1).

Isolate number	Identification	Final pH
SW_5	Unknown LAB (uncultured bacterium from feedlot manure)	5.70
SW_13	Probably *Vagococcus* sp.	6.17
SW_17	Marinolactobacillus psychrotolerans	5.69
SW_21	*Marinolactobacillus psychrotolerans*	6.42
SW_26	*Marinolactobacillus psychrotolerans*	6.55
SW_29	*Marinolactobacillus psychrotolerans*	5.81
SW_30	Unknown LAB (uncultured bacterium from feedlot manure)	6.49
SW_52	Unknown LAB (uncultured bacterium from feedlot manure)	6.53
SW_53	Unknown LAB (uncultured bacterium from feedlot manure)	6.56
SW_62	Probably *Vagococcus* sp.	6.66
SW_63	Unknown LAB (uncultured bacterium from feedlot manure)	6.57
SW_72	Unknown LAB (uncultured bacterium from feedlot manure)	6.53
SW_79	*Marinolactobacillus psychrotolerans*	6.47
SW_94	Unknown LAB (uncultured bacterium from feedlot manure)	6.45
SW_97	Unknown LAB (uncultured bacterium from feedlot manure)	6.56
13_16	Probably *Vagococcus* sp.	6.77
13_20	*Marinolactobacillus psychrotolerans*	6.81
13_55	Unknown LAB (uncultured bacterium from feedlot manure)	6.53
13_67	*Marinolactobacillus psychrotolerans*	5.74
16/12_5	*Marinolactobacillus psychrotolerans*	5.56
16/12_7	Probably *Vagococcus* sp.	6.38
16/12_8	Probably *Vagococcus* sp.	6.44
16/12_17	Probably *Vagococcus* sp.	6.51
16/12_22	*Marinolactobacillus psychrotolerans*	6.46
16/12_23	Probably *Vagococcus* sp.	6.44
16/12_27	Probably *Vagococcus* sp.	6.41
16/12_53	Unknown LAB (uncultured bacterium from feedlot manure)	6.56
16/12_58	*Marinolactobacillus psychrotolerans*	5.61
16/12_65	Probably *Vagococcus* sp.	6.58
16/12_73	*Marinolactobacillus psychrotolerans*	6.42
16/12_74	*Marinolactobacillus psychrotolerans*	5.64
16/12_93	Probably *Vagococcus* sp.	6.42

At 16/12°C, coryneforms represented 87% of the final bacterial surface flora. This subflora was composed of *B. linens* (33%), *C. casei* (25%), *C. variabile* (19%) and *M. gubbeenense* (10%). *A. nicotianae* was not found in this surface flora. All non-coryneforms were Gram positive catalase negative bacteria.

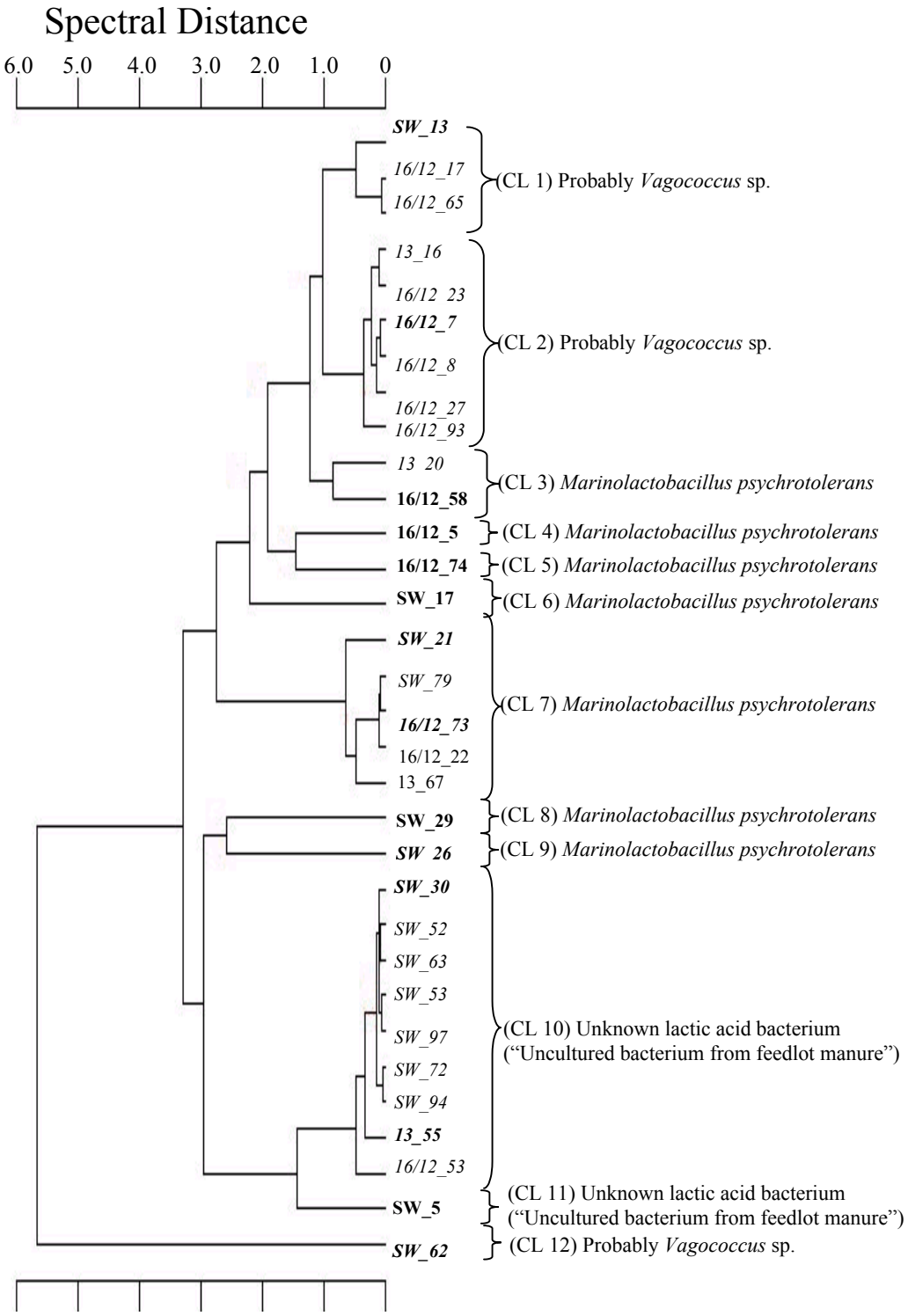

Fig. 3.10 FT-IR dendrogram of 32 Gram positive and catalase negative bacteria isolated from consortium R II**.** They were isolated from the smear water (SW) and from the corresponding surface flora derived at the expiry date from inoculated soft cheeses, matured at 13°C and 16/12°C, respectively. Representative strains of similarity clusters which were subjected to 16S rDNA sequence analysis are designated by bold letters. Italic letters indicate isolates with only weak acid formation from lactose.

7 isolates most likely belong to the genus *Vagococcus*. 5 of the isolates were identified as *Marinolactobacillus psychrotolerans*. Again, they differed in the intensity of lactose fermentation (Tab. 3.3) and were located in different FT-IR clusters, independent from this feature (Fig. 3.10). Additionally one unknown lactic acid bacterium was found which could be referred to an uncultured bacterium from feedlot manure.

3.2.4 Bacterial composition of consortium K I

The results of the bacterial composition analysis of consortium K I in the smear water (100 isolates), as well as at the expiry date after the two ripening procedures (13°C and 16/12°C, 100 isolates each) are displayed in Fig. 3.11. 98% of the isolates were coryneform bacteria. The predominant species was *A. nicotianae* (85%). The second coryneform was *B. linens*, representing (13%). The non-coryneform bacteria were Gram negative bacteria, namely *Halomonas* sp. and one isolate which could be referred to an uncultured proteobacterium.

The final bacterial surface flora on cheeses incubated at 13°C was mainly formed by coryneform bacteria, representing 76% of the isolates (Fig. 3.11). 51% of these isolates were *A. nicotianae,* while 25% were identified as *B. linens*. 22% of the isolates belonged to the group of Gram positive catalase negative bacteria. While 4 isolates could be identified as *Marinolactobacillus psychrotolerans*, the other 18 isolates could not be referred to a specific genus since they revealed high 16S rDNA sequence similarities to both *Carnobacterium* sp. and *Lactobacillus* sp. All of them were rod-shaped bacteria and fermented lactose (Tab. 3.4). Therefore they were considered as rod-shaped lactic acid bacteria. None of these had been found in the smear water of consortium K I, as well as the two Gram negatives that were identified as *Providencia* sp.

At 16/12°C, coryneforms represented 74% of the isolates. The predominant species was *B. linens* (46%) followed by *A. nicotianae* (28%). Gram positive catalase negative bacteria (14%) and Gram negative bacteria (12%) composed the non-coryneform subflora. The former group was composed of *Marinolactobacillus psychrotolerans* (4%) and rod-shaped lactic

acid bacteria (10%). The latter group was dominated by *Psychrobacter* sp. (11%), while also one *Marinobacter* sp. was detected.

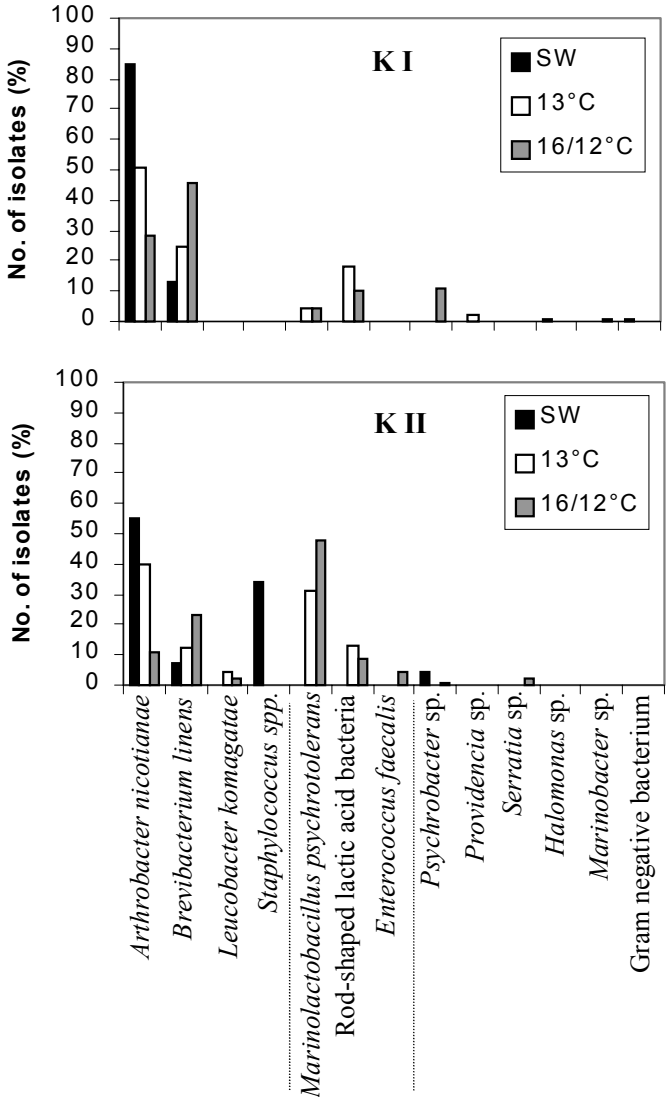

Fig. 3.11 Bacterial composition of the two red smear consortia K I and K II, determined in the smear water (SW) and the corresponding surface floras derived at the expiry date (day 41) from inoculated soft cheeses, matured according to two different ripening regimes (13°C and 16/12°C, respectively; see Fig. 2.1). The respective smear waters (I and II) were prepared using undefined red smear surface flora derived two times (I and II) from mature commercial German soft cheese produced in dairy K. The three groups (·····) represent the Gram positive and catalase negative isolates (left), Gram positive and catalase negative isolates (center) and the Gram negatives (right).

Tab. 3.4 Acid formation from lactose by Gram positive and catalase negative bacteria isolated from consortium K I. They were isolated from the corresponding

surface floras derived at the expiry date from inoculated soft cheeses, matured at 13°C and 16/12°C, respectively. (Initial pH of M17 broth: 7.1).

Isolate number	Identification	Final pH
13_9	*Carnobacterium* sp./*Lactobacillus* sp.	5.64
13_12	*Carnobacterium* sp./*Lactobacillus* sp.	5.59
13_13	*Marinolactobacillus psychrotolerans*	5.69
13_14	*Carnobacterium* sp./*Lactobacillus* sp.	5.57
13_20	*Carnobacterium* sp./*Lactobacillus* sp.	5.59
13_21	*Marinolactobacillus psychrotolerans*	6.19
13_26	*Carnobacterium* sp./*Lactobacillus* sp.	5.64
13_27	*Carnobacterium* sp./*Lactobacillus* sp.	5.63
13_31	*Carnobacterium* sp./*Lactobacillus* sp.	5.68
13_33	*Marinolactobacillus psychrotolerans*	6.24
13_46	*Carnobacterium* sp./*Lactobacillus* sp.	5.62
13_47	*Carnobacterium* sp./*Lactobacillus* sp.	5.65
13_58	*Carnobacterium* sp./*Lactobacillus* sp.	5.61
13_66	*Carnobacterium* sp./*Lactobacillus* sp.	5.63
13_70	*Marinolactobacillus psychrotolerans*	5.65
13_75	*Carnobacterium* sp./*Lactobacillus* sp.	5.62
13_77	*Carnobacterium* sp./*Lactobacillus* sp.	5.70
13_83	*Carnobacterium* sp./*Lactobacillus* sp.	6.53
13_88	*Carnobacterium* sp./*Lactobacillus* sp.	5.69
13_89	*Carnobacterium* sp./*Lactobacillus* sp.	5.56
13_90	*Carnobacterium* sp./*Lactobacillus* sp.	5.55
13_96	*Carnobacterium* sp./*Lactobacillus* sp.	5.63
16/12_4	*Carnobacterium* sp./*Lactobacillus* sp.	5.62
16/12_8	*Carnobacterium* sp./*Lactobacillus* sp.	5.68
16/12_15	*Carnobacterium* sp./*Lactobacillus* sp.	5.63
16/12_21	*Carnobacterium* sp./*Lactobacillus* sp.	5.62
16/12_22	*Marinolactobacillus psychrotolerans*	6.39
16/12_34	*Marinolactobacillus psychrotolerans*	6.38
16/12_50	*Carnobacterium* sp./*Lactobacillus* sp.	5.61
16/12_54	*Carnobacterium* sp./*Lactobacillus* sp.	5.59
16/12_59	*Carnobacterium* sp./*Lactobacillus* sp.	5.62
16/12_70	*Carnobacterium* sp./*Lactobacillus* sp.	5.64
16/12_79	*Carnobacterium* sp./*Lactobacillus* sp.	5.71
16/12_80	*Marinolactobacillus psychrotolerans*	6.37
16/12_87	*Carnobacterium* sp./*Lactobacillus* sp.	5.58
16/12_98	*Marinolactobacillus psychrotolerans*	6.41

3.2.5 Bacterial composition of consortium K II

The bacterial flora of consortium K II in the smear water was mainly composed by coryneform bacteria (62%) and coagulase negative *Staphylococcus* spp (34%). The latter could not be identified at the species level with the methods described in this study since several species of this genus revealed 16S rDNA sequence similarities above 97%. The predominant coryneform species was *A. nicotianae* (55%) while *B. linens* represented 7%

of the isolates. The Gram negative bacteria (4%) were identified as *Psychrobacter* sp.

After cheese maturation at 13°C, only 56% of the isolates were coryneforms, namely, *A. nicotianae* (40%), *B. linens* (12%) and *Leucobacter komagatae* (4%). The latter had not been detected in the smear water of consortium K II. On the other side the *Staphylococcus* spp. found in the smear water of consortium K II were not detected on this surface flora. The group of Gram positive catalase negative isolates (44%) included *Marinolactobacillus psychrotolerans* (31%) and other rod-shaped lactic acid bacteria (13%) which could not be identified with the applied methods. Both of them had not been detected in the smear water of consortium K II. While the latter formed acid from lactose, none of the former isolates formed acid from lactose (Tab. 3.5).

Gram positive catalase negative bacteria were the main bacterial group in the final bacterial surface flora on cheeses incubated at 16/12°C with *Marinolactobacillus psychrotolerans* (48%) and not identifiable rod-shaped lactic acid bacteria (9%), as well as *Enterococcus faecalis* (4%) were found. The coryneforms formed only 34% of the bacterial flora and were represented by *B. linens* (23%) and *A. nicotianae* (11%). The three Gram negative isolates were identified as *Serratia* sp. (2%) and *Psychrobacter* sp.

Tab. 3.5 Acid formation from lactose by Gram positive and catalase negative bacteria isolated from consortium K II. They were isolated from the corresponding surface floras derived at the expiry date from inoculated soft cheeses, matured at 13°C and 16/12°C, respectively. (Initial pH of M17 broth: 7.1).

Isolate number	Identification	Final pH
13_4	*Marinolactobacillus psychrotolerans*	6.32
13_5	*Marinolactobacillus psychrotolerans*	6.33
13_6	*Carnobacterium* sp./*Lactobacillus* sp.	5.67
13_13	*Carnobacterium* sp./*Lactobacillus* sp.	5.66
13_15	*Marinolactobacillus psychrotolerans*	6.33
13_16	*Carnobacterium* sp./*Lactobacillus* sp.	5.81
13_17	*Marinolactobacillus psychrotolerans*	6.36
13_23	*Marinolactobacillus psychrotolerans*	6.36
13_24	*Marinolactobacillus psychrotolerans*	6.36
13_25	*Marinolactobacillus psychrotolerans*	6.36
13_26	*Carnobacterium* sp./*Lactobacillus* sp.	5.85
13_27	*Marinolactobacillus psychrotolerans*	6.45
13_29	*Carnobacterium* sp./*Lactobacillus* sp.	5.77
13_31	*Marinolactobacillus psychrotolerans*	6.39
13_33	*Carnobacterium* sp./*Lactobacillus* sp.	5.76
13_35	*Marinolactobacillus psychrotolerans*	6.38
13_36	*Marinolactobacillus psychrotolerans*	6.40
13_37	*Carnobacterium* sp./*Lactobacillus* sp.	5.73
13_38	*Marinolactobacillus psychrotolerans*	6.36
13_40	*Marinolactobacillus psychrotolerans*	6.37
13_41	*Carnobacterium* sp./*Lactobacillus* sp.	5.71
13_42	*Marinolactobacillus psychrotolerans*	6.35
13_46	*Marinolactobacillus psychrotolerans*	6.34
13_48	*Marinolactobacillus psychrotolerans*	6.35
13_49	*Carnobacterium* sp./*Lactobacillus* sp.	5.82
13_50	*Carnobacterium* sp./*Lactobacillus* sp.	5.73
13_52	*Marinolactobacillus psychrotolerans*	6.31
13_58	*Marinolactobacillus psychrotolerans*	6.36
13_59	*Marinolactobacillus psychrotolerans*	6.40
13_60	*Marinolactobacillus psychrotolerans*	6.40
13_66	*Marinolactobacillus psychrotolerans*	6.37
13_69	*Marinolactobacillus psychrotolerans*	6.40
13_74	*Marinolactobacillus psychrotolerans*	6.38
13_75	*Carnobacterium* sp./*Lactobacillus* sp.	5.86
13_76	*Carnobacterium* sp./*Lactobacillus* sp.	5.80
13_77	*Marinolactobacillus psychrotolerans*	6.35
13_78	*Marinolactobacillus psychrotolerans*	6.38
13_79	*Marinolactobacillus psychrotolerans*	6.41
13_80	*Carnobacterium* sp./*Lactobacillus* sp.	5.84
13_85	*Marinolactobacillus psychrotolerans*	6.38
13_88	*Marinolactobacillus psychrotolerans*	6.36
13_89	*Carnobacterium* sp./*Lactobacillus* sp.	5.82
13_93	*Marinolactobacillus psychrotolerans*	6.39
13_95	*Marinolactobacillus psychrotolerans*	6.39
16/12_2	*Marinolactobacillus psychrotolerans*	6.28
16/12_10	*Enterococcus faecalis*	5.23
16/12_13	*Marinolactobacillus psychrotolerans*	6.41
16/12_14	*Enterococcus faecalis*	5.21
16/12_16	*Marinolactobacillus psychrotolerans*	6.41
16/12_17	*Marinolactobacillus psychrotolerans*	6.45

Tab. 3.5 continued

Isolate number	Identification	Final pH
16/12_20	*Carnobacterium* sp./*Lactobacillus* sp.	5.97
16/12_21	*Marinolactobacillus psychrotolerans*	6.47
16/12_22	*Carnobacterium* sp./*Lactobacillus* sp.	5.86
16/12_23	*Carnobacterium* sp./*Lactobacillus* sp.	5.88
16/12_25	*Marinolactobacillus psychrotolerans*	6.38
16/12_27	*Marinolactobacillus psychrotolerans*	6.28
16/12_28	*Marinolactobacillus psychrotolerans*	6.48
16/12_30	*Marinolactobacillus psychrotolerans*	6.68
16/12_31	*Marinolactobacillus psychrotolerans*	6.36
16/12_33	*Marinolactobacillus psychrotolerans*	6.39
16/12_35	*Marinolactobacillus psychrotolerans*	6.45
16/12_36	*Marinolactobacillus psychrotolerans*	6.39
16/12_38	*Marinolactobacillus psychrotolerans*	6.47
16/12_39	*Marinolactobacillus psychrotolerans*	6.36
16/12_41	*Marinolactobacillus psychrotolerans*	6.43
16/12_44	*Carnobacterium sp./Lactobacillus sp.*	5.89
16/12_45	*Marinolactobacillus psychrotolerans*	6.46
16/12_48	*Carnobacterium sp./Lactobacillus sp.*	5.88
16/12_49	*Carnobacterium sp./Lactobacillus sp.*	5.96
16/12_50	*Carnobacterium sp./Lactobacillus sp.*	5.70
16/12_52	*Marinolactobacillus psychrotolerans*	6.66
16/12_54	*Marinolactobacillus psychrotolerans*	6.49
16/12_55	*Enterococcus faecalis*	5.32
16/12_56	*Marinolactobacillus psychrotolerans*	6.53
16/12_57	*Marinolactobacillus psychrotolerans*	6.41
16/12_58	*Marinolactobacillus psychrotolerans*	6.44
16/12_59	*Marinolactobacillus psychrotolerans*	6.40
16/12_60	*Marinolactobacillus psychrotolerans*	6.39
16/12_63	*Marinolactobacillus psychrotolerans*	6.44
16/12_64	*Carnobacterium sp./Lactobacillus sp.*	5.95
16/12_66	*Marinolactobacillus psychrotolerans*	6.45
16/12_67	*Marinolactobacillus psychrotolerans*	6.84
16/12_68	*Marinolactobacillus psychrotolerans*	6.43
16/12_70	*Marinolactobacillus psychrotolerans*	6.40
16/12_71	*Marinolactobacillus psychrotolerans*	6.73
16/12_73	*Marinolactobacillus psychrotolerans*	6.69
16/12_74	*Marinolactobacillus psychrotolerans*	6.65
16/12_75	*Marinolactobacillus psychrotolerans*	6.67
16/12_76	*Marinolactobacillus psychrotolerans*	6.69
16/12_77	*Marinolactobacillus psychrotolerans*	6.50
16/12_78	*Marinolactobacillus psychrotolerans*	6.41
16/12_81	*Marinolactobacillus psychrotolerans*	6.46
16/12_82	*Enterococcus faecalis*	5.25
16/12_85	*Marinolactobacillus psychrotolerans*	6.43
16/12_86	*Marinolactobacillus psychrotolerans*	6.46
16/12_87	*Carnobacterium sp./Lactobacillus sp.*	5.92
16/12_89	*Marinolactobacillus psychrotolerans*	6.39
16/12_90	*Marinolactobacillus psychrotolerans*	6.42
16/12_92	*Marinolactobacillus psychrotolerans*	6.43
16/12_93	*Marinolactobacillus psychrotolerans*	6.36
16/12_95	*Marinolactobacillus psychrotolerans*	6.47
16/12_96	*Marinolactobacillus psychrotolerans*	6.42
16/12_97	*Marinolactobacillus psychrotolerans*	6.46
16/12_98	*Marinolactobacillus psychrotolerans*	6.47
16/12_99	*Marinolactobacillus psychrotolerans*	6.40

3.2.6 *In situ* anti-listerial activity of the consortia R I and R II the consortia K I and K II in two ripening procedures

Fig. 3.12 displays the anti-listerial activity of consortium R I, R II, K I and K II. With a contamination level of 10^1 CFU/ml brine, *L. monocytogenes* WSCL 1364 grew to high numbers on the control cheeses (M) in all experiments with both ripening temperature regimes (Fig. 3.12). Applying the same *Listeria* titer in the smear water of consortia K I and K II, at 16/12°C the pathogen reached similar or even higher numbers as on the control cheeses. At 13°C, however, the results were different. While with consortium K I it was not clear whether there is inhibition or not (Fig 3.12), in case of flora K II, *Listeria* analysis yielded first positive results after about 23 days and cell counts remained below the detection limit until the end of the experiment (Fig. 3.12). This observation has been confirmed with another repetition that is marked as dashed line (Fig. 3.12). In case of consortium R the contamination level of *L. monocytogenes* was 10^2 CFU/ml brine. With both consortia R I and R II, the pathogen could be inhibited completely with both ripening procedures (Fig. 3.12).

3.2.7 Development of the pH on the cheese surface

Development of the pH on the cheese surface in presence of M, K I and R I is shown in Fig. 3.13. Within 10 days, the pH increased to about 7.5, followed by a slight increase towards pH 8.0 until the expiry date (day 41) with both ripening procedures. Similar results were obtained in the experiments with consortia K II and R II (Fig 3.13).

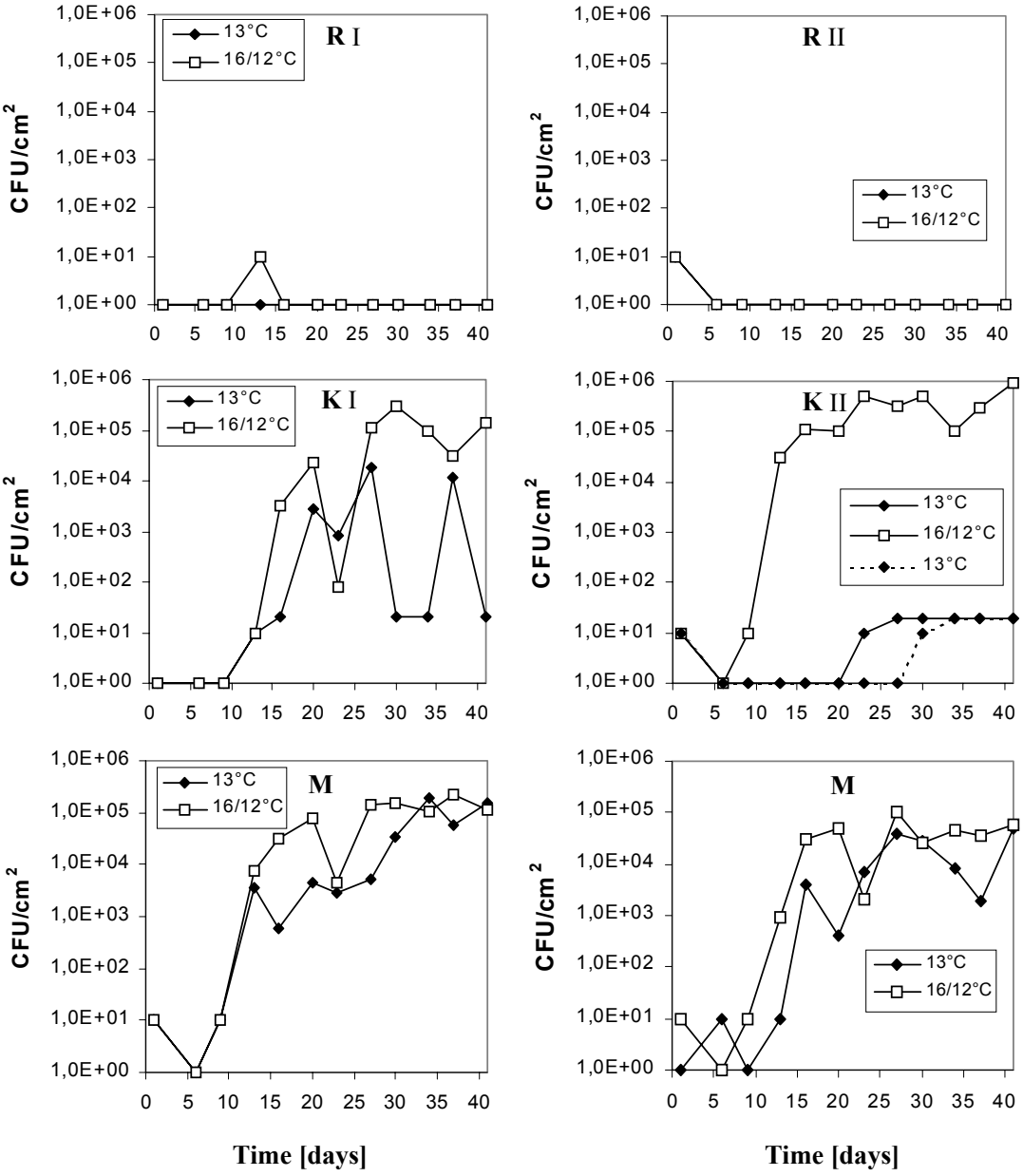

Fig. 3.12 Growth of *Listeria monocytogenes* on soft cheese in the presence of complex undefined red smear surface floras during two ripening regimes (13°C and 16/12°C). Floras R I, R II, K I, K II are complex undefined red smear consortia harvested twice (I and II) from the surfaces of commercial red smear soft cheeses produced in factory R and K, respectively. The consortia R I, R II, K I, K II were used for the subsequent cheese ripening experiments (I and II), respectively. Flora M served as control. It is a defined culture derived from red smear soft cheese of factory M. *L. monocytogenes* WSLC 1364 was used for contamination on day 1. The contamination levels were 10^1 CFU/ml brine in case of flora K and M, and 10^2 CFU/ml brine in case of flora R. The dashed line in the K II subfigure is a repetition.

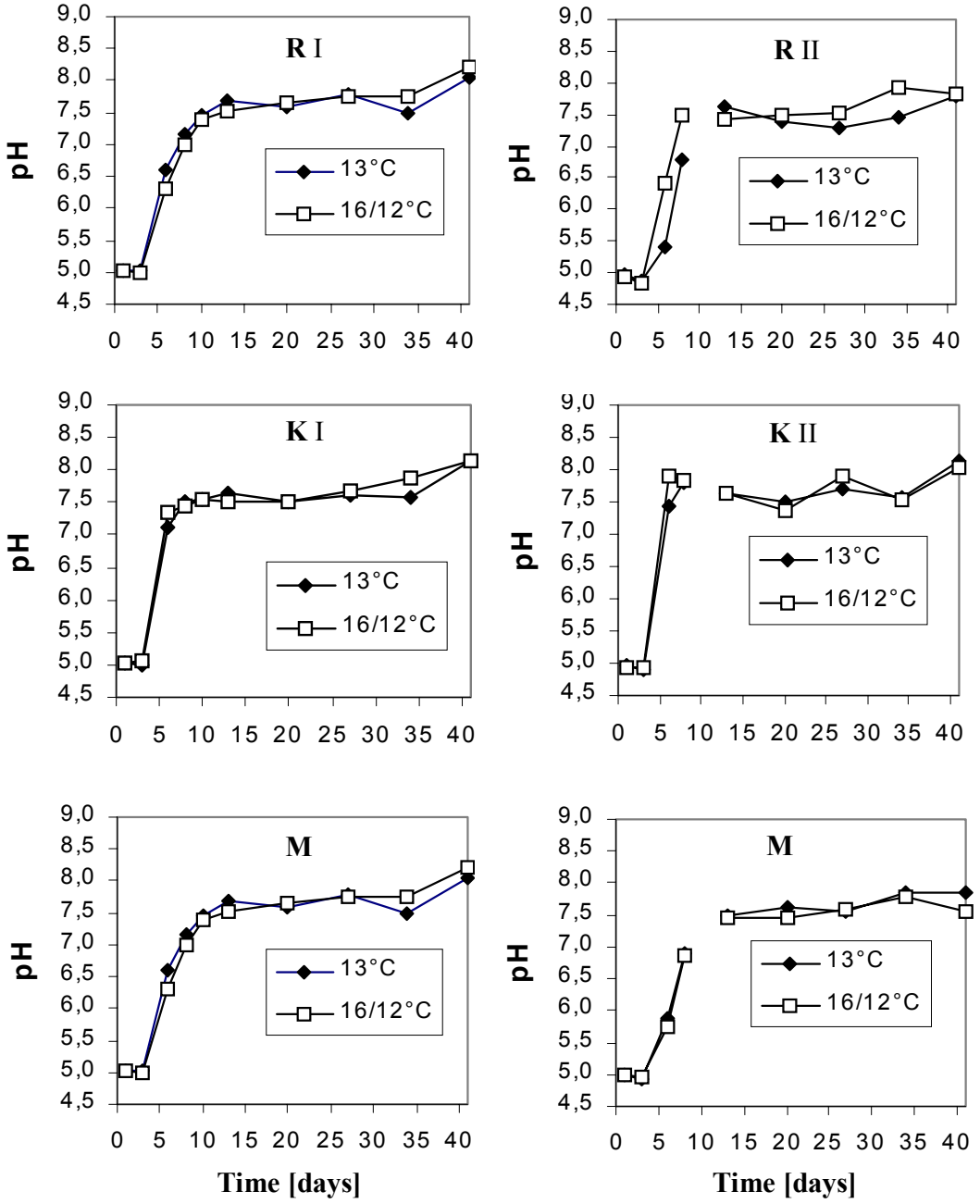

Fig. 3.13 Development of the pH on soft cheese surfaces ripened at two different temperature regimes (13°C and 16/12°C) when different red smear surface cultures are applied. Floras R I, R II, K I, K II are complex undefined red smear consortia harvested twice (I and II) from the surfaces of commercial red smear soft cheeses produced in factory R and K, respectively. The consortia R I, K I R II, K II were used for the subsequent cheese ripening experiments (I and II), respectively. Flora M served as control. It is a defined culture derived from red smear soft cheese of factory M. On day 10 in the experimental series II the pH electrode was broken.

3.3 Sensitive *in situ* monitoring of a recombinant bioluminescent *Yersinia enterocolitica* reporter mutant in *real time* on Camembert cheese

In order to avoid sacrificing of samples during long term *in situ* monitoring of a pathogen in food without addition of antibiotics or aldehyde, bioluminescent mutants of *Yersinia enterocolitica* were generated by transposon mutagenesis using a promoterless complete *lux* operon (*lux*CDABE).

3.3.1 Selection of a suitable reporter strain

Since the Tn*5* *lux* transposon integrates randomly into the *Yersinia* chromosome, transposants were screened for strongly expressed bioluminescence in the cheese environment. To screen for a suitable mutant, cheeses were covered with a filter membrane with a pore size of 0.22 µm and a variety of strains picked from the initial conjugation plates were spread on the filter surface. After overnight incubation at 30°C, six of the brightest mutants designated A1, B12, B94, C95, D16 and D22 were chosen for further experiments. The light emission of these six mutants was measured on Camembert cheese during 7 days at 10°C (Fig. 3.14A). The mutant B94 exhibited the highest light emission during the first 3 days without any lag time and from day 4 to day 7 the light emission remained at about 1 x 10^6 RLU/cheese. Mutant D16 had a lag time of one day and then the light emission increased continuously until day 5. Between day 5 and day 7 it remained at about 2 x 10^6 RLU/cheese. In order to choose the mutant that shows the highest photon output per CFU, bioluminescence of these six mutants was calculated on a CFU basis by enumerating *Y. enterocolitica* on the cheese surface. The results are displayed in Fig. 3.14B. Strain B94 showed the highest photon output per CFU. For example, 1 x 10^5 CFU/cm^2 of B94 emitted light of 1 x 10^6 RLU/cheese while the same number of cells of D16 emitted only 4.5 x 10^5 RLU/cheese. Therefore, B94 was chosen for further experiments.

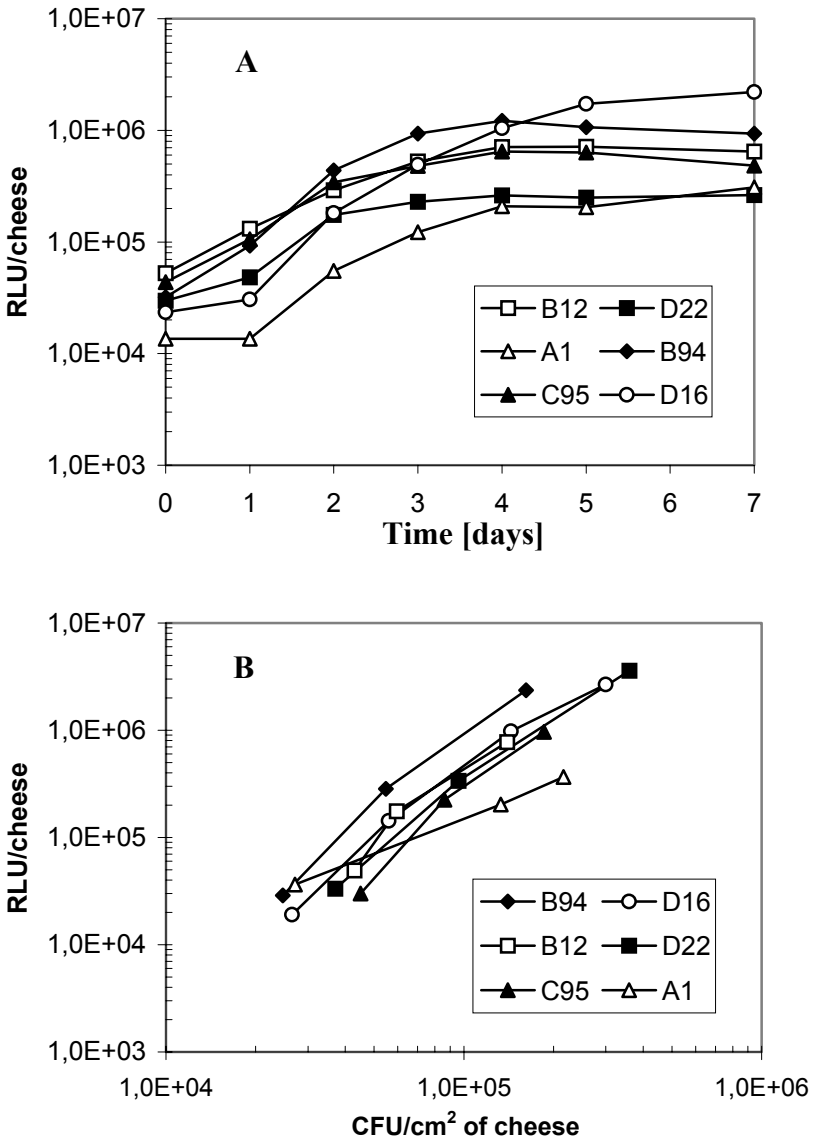

Fig. 3.14 Light emission of six *lux*-transposon mutants of *Yersinia enterocolitica* on Camembert cheese at 10°C after initial inoculation of about 10^4 CFU/cm^2. The total photon count of one cheese surface is given. (**A**) Time dependence of light emission during storage of the contaminated cheese. Panel (**B**) shows the correlation between light emission and colony forming units per cm^2. Immediately after measuring light emission, the measured cheeses were homogenized and the *Yersinia* cell number was determined on CIN agar (see methods section).

In order to see whether a recombinant strain would exhibit a loss of fitness compared to the wild type strain, the growth rate of mutant B94 and the wild type *Y. enterocolitica* WS 3371 strain were examined aerobically in LB

broth at 30°C with shaking of 175 rpm (Fig. 3.15). The growth rates of both strains were comparable.

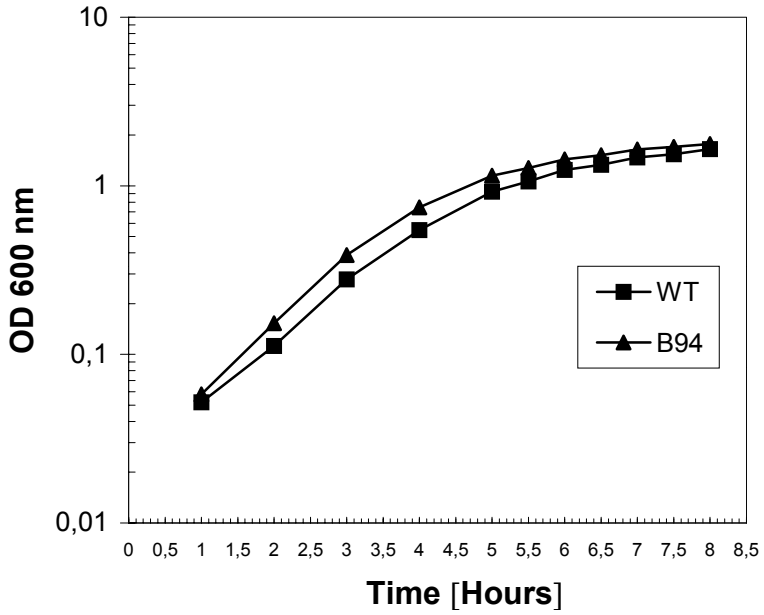

Fig 3.15 Growth curve of B94 and wild type WS 3371(WT) *Y. enterocolitica* strains at 30°C .

3.3.2 Microheterogeneity of *Yersinia* on cheese

Images of contaminated Camembert cheese stored at 10°C are displayed in Fig. 3.16. The entire cheese surface was initially contaminated with *Yersinia*. During the storage time certain areas appeared to become more highly colonized than others. Alongside with increasing of the light intensity from day 1 to day 3, the photon counts increased also.

3.3.3 Detection limit of strain B94

In order to estimate the sensitivity of the method, bioluminescence of the entire cheese surface was measured and correlated with viable cell count on the cheese surface. The results in Fig. 3.17 demonstrate a high correlation between culture derived viable cell count and bioluminescence (r^2= 0.98). The equation for calculating the derived viable cell count and bioluminescence on cheese surface is described in Appendix B. We found an *in situ* detection limit of about 200 CFU/cm^2 for *Y. enterocolitica* B94 on cheese.

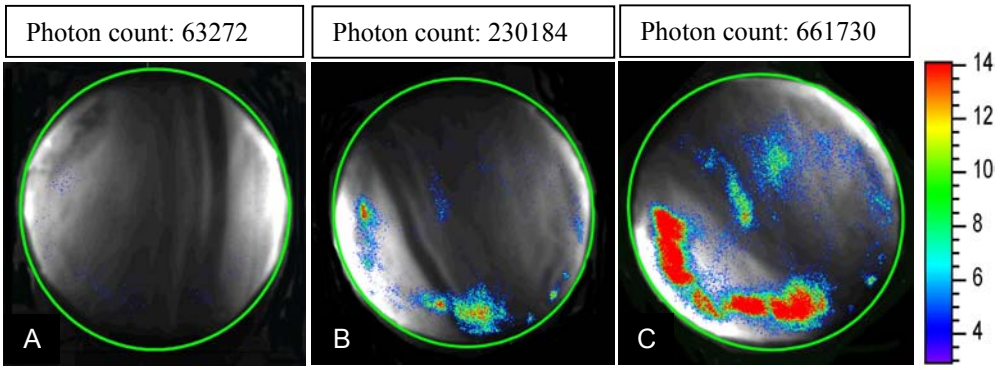

Fig. 3.16 Localization of bioluminescent *Yersinia enterocolitica* B94 on Camembert cheese. (**A**) Directly after contamination, (**B**) after two days and (**C**) after 3 days. Initial contamination level was 5 x 10^3 CFU/cm^2. Measurement and storage were at 10°C. Above each image, in the total photon count of the cheese surface with a diameter of 6.5 cm is shown. The scale at the right indicates light intensity (red is high and blue is low).

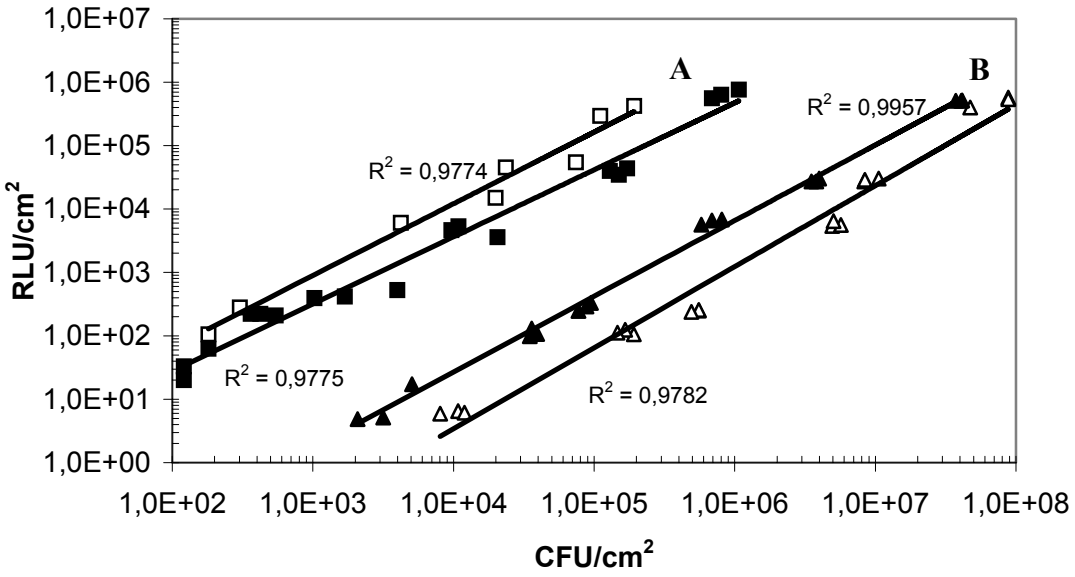

Fig. 3.17 Correlation of bioluminescence (RLU) and culture regime dependent viable cell count (CFU/cm^2) measured at 10°C. (A) The correlation is shown for cheese after 14 days of storage (open squares) and during the first 3 days after inoculation (closed squares). (B) Correlation for *Y. enterocolitica* on BHI agar with 2.5% salt (closed triangles) and without salt (open triangles) at the first 5 days. The background luminescence of cheese and BHI agar has been subtracted. Immediately after measuring light intensity, the plates or the cheeses measured to determine the viable counts (see sections 2.3.4 and 2.3.5).

In addition, on Camembert cheese after 14 days at 10°C, we observed a higher RLU rate per cell than during day 1-3. Furthermore, the correlation between bioluminescence and viable cells was examined on BHI agar with 2.5% NaCl and without NaCl. The equation for calculating the derived viable cell count and bioluminescence on BHI agar is described in Appendix B. The bioluminescence of mutant B94 on BHI agar was much lower compared to the cheese environment, even in the presence of sodium chloride (Fig. 3.17).

3.3.4 Temperature dependence of luciferase activity of *Y. enterocolitica* B94

The light emission of mutant B94 was detected in temperature measurements between 4°C and 37°C and the results are shown in Fig. 3.18. Between 4°C and 30°C, with increasing measurement temperatures the luciferase activity appears higher. At 37°C, the luciferase activity was about the same level observed at 30°C. At 10°C, luciferase activity is about 15% of the one at 30°C.

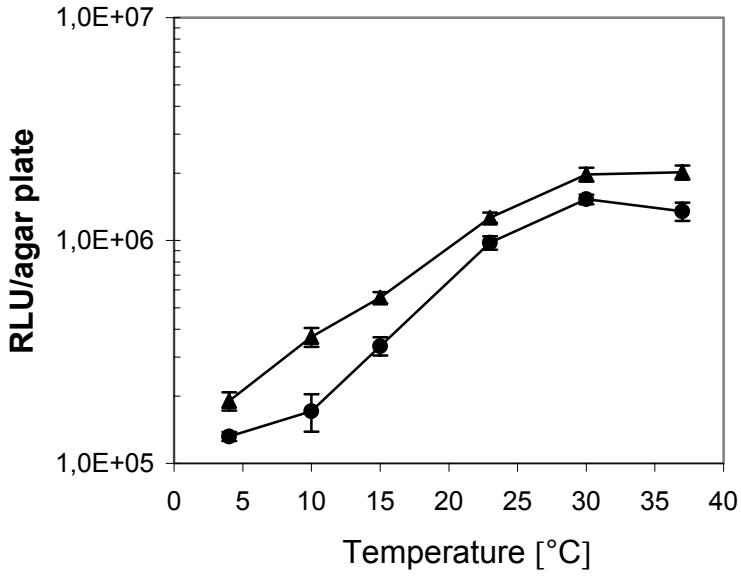

Fig. 3.18 Temperature dependence of luciferase activity of *Y. enterocolitica* B94. The mutant was grown in BHI broth with 2.5% NaCl (triangels) and without NaCl (circles) at 10°C to 1.5 x 10^8 CFU/ml. 100 µl of this culture was spread on BHI plates pre-adjusted to, and after inoculation held at, the appropriate temperature for 5 minutes. Subsequently, bioluminescence was measured at the same temperature.

3.3.5 Light emission of *Y. enterocolitica* B94 is partially growth phase dependent

Fig. 3.17 showed that the bioluminescence of strain B94 increases (per cell) with its duration on the cheese surface. Therefore, we compared the bioluminescence of strain B94 when this bacterium is grown at various temperatures between 4°C and 30°C to an OD_{600} of 0.15 (log phase) and an OD_{600} of 1.1 (stationary phase), respectively (Fig. 3.19). To exclude the temperature dependence of luciferase activity mentioned above (see Fig. 3.18), cultures of all growth temperatures were measured at the same temperature (10°C). In the logarithmic growth phase, the light emission didn't differ significantly and ranged from 5 x 10^3 to 9 x 10^3 light per cell in the whole growth temperature range. In the stationary phase, the expression of light per cell remained about 4 x10^3 at 4°C and at 10°C. From 15°C forwards, it decreased continuously, and resulted a 5 x 10^2 light per cells at 30°C.

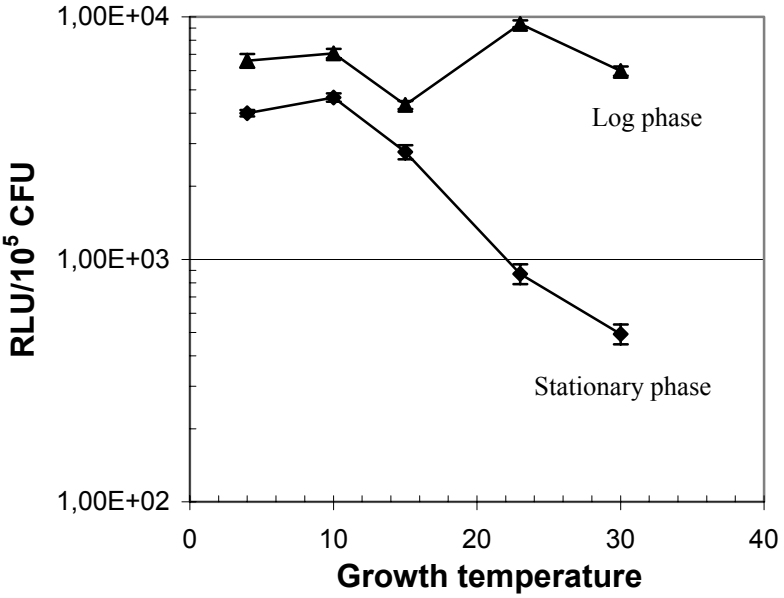

Fig. 3.19 Influence of growth temperature and growth phase on bioluminescence of *Y.enterocolitica* B94. The mutant was grown in BHI broth with 2.5% salt at different temperatures to OD_{600nm} = 0.15 (5 x 10^7 CFU/ml) (triangles) and to OD_{600nm} = 1.1 (4.5 x 10^8 CFU/ml) (diamonds). 100 µl of the culture was spread on BHI plates pre-adjusted to 10°C. After 5 minutes bioluminescence was measured at 10 °C.

3.3.6 Light emission of *Y. enterocolitica* B94 is salt dependent.

As shown in Fig. 3.17, the ratio of bioluminescence to viable cells was higher on BHI agar with salt compared to the ratio obtained on BHI agar without salt. Thus it was interesting to know if the promoter that regulates the *lux* cassette in strain B94 also involved in a salt stress response of B94. Therefore, we checked if salt is a stress factor for B94 growth. The growth rate of B94 was compared in BHI broth with and without 2.5% NaCl at 30°C with shaking of 175 rpm. The results are displayed in Fig. 3.20. The lag phase in BHI medium with salt was much longer that obtained in BHI broth without salt (7 h and 2 h, respectively). In addition, the growth rate in BHI medium with salt was lower than without salt (Fig. 3.20). Furthermore, as shown in Fig. 3.18, in the whole range of measurement temperatures tested, presence of salt enhanced light emission.

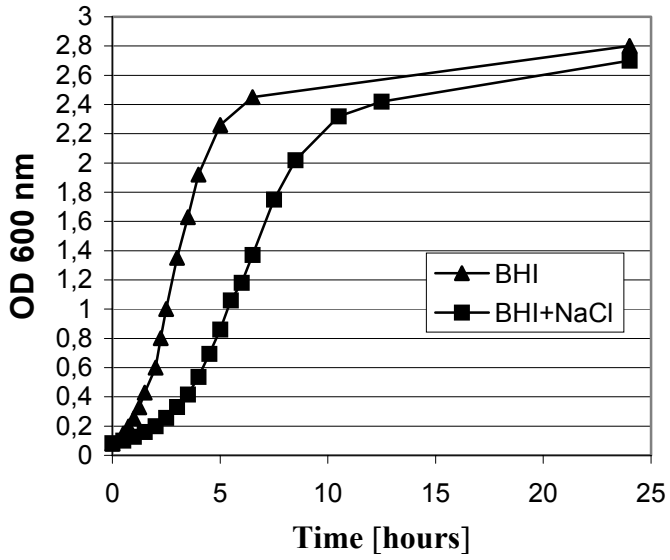

Fig. 3.20 Growth of *Y. enterecolitica* B94 in BHI broth with and without NaCl at 30°C. The lag phase of B94 in BHI broth with 2.5% and without salt was 7 h and 2 h, respectively. The time point zero in this figure is related to the beginning of the log phase.

4 Discussion

4.1 Temporal stability and biodiversity of two complex, anti-listerial cheese ripening microbial consortia

4.1.1 Composition and temporal stability of consortium R

Coryneform bacteria formed the core part of consortium R, representing 94% of the isolates. The predominant coryneform at both sampling points was *C. variabile* (Fig 3.2). This species has been isolated from the surface of German red smear cheeses previously, but in much lower numbers (Ryser *et al*., 1994; Valdés-Stauber *et al*., 1997). The proportions of all other species were very low (≤ 7% of the isolates). However, some of them were formed more than one independent FT-IR cluster (Fig 3.1). This phenomenon has been described by Kümmerle et al. (1998) as an indicator of intraspecific heterogeneities with regard to the cellular composition. Interestingly the position of the *C. variabile* and *C. casei* clusters (Fig. 3.1) corresponds with the results of Brennan et al. (2001), who reported on a close phylogenetic relationship of these two species. While the general structure of the consortium appeared to be stable over six months, the diversity at the species level was found higher at the end (B) of this period, exhibiting *Arthrobacter nicotianae* and *Microbacterium gubbeenense* as additional coryneform species and two lactic acid bacteria which could be related to an uncultured bacterium from feedlot manure (Ouwerkerk and Klieve, 2001). The latter is reported for the first time in such a consortium. Although the overall predominance of coryneform bacteria is comparable, the composition as well as the structure of consortium R is completely different to the recent findings with Irish Gubbeen cheese. The latter consortium was dominated by *C. casei*, which yielded, however, a proportion of 50% only, while no *C. variabile* has been isolated at all (Brennan *et al*., 2002). Furthermore, these authors report on *M. gubbeenense* to represent a high proportion of the total isolates. While the low incidence of microbacteria in consortium R is in contrast to these findings, it is in agreement with other studies where *Microbacterium spp*. also

have been found in low numbers only (Seiler, 1986; Eliskases-Lechner and Ginzinger, 1995; Valdés-Stauber *et al*., 1997).

4.1.2 Composition and temporal stability of consortium K

With regard to its composition and stability, consortium K differed significantly from consortium R (Fig. 3.2). The dominant species of the latter has not been isolated at all in consortium K. Instead, in both analyses consortium K was clearly dominated by *A. nicotianae*, which had been only a minor constituent of flora R in the second analysis. This species has been isolated from red smear surface floras previously (Seiler, 1986; Eliskases-Lechner and Ginzinger, 1995; Valdés-Stauber *et al*., 1997), but none of these authors reported on a comparable predominance. After six months, *A. nicotianae* dropped from 90% to 76% and *B. linens*, the only other coryneform isolated from consortium K, yielded an eleven fold higher proportion (22%) (Fig. 3.2). These numbers mark a significant structural change of consortium K within six months. Nevertheless, the development of the pH as indicator for successful maturation appeared to be the same (Fig. 3.5). It showed a typical pH development for ripening of red smear cheese (Reps, 1987; Eppert *et al*., 1997; Carminati *et al*., 1999).

The non-coryneform subflora was composed by Gram positive catalase negative and Gram negative bacteria. The Gram positive catalase negative bacteria were identified as *Marinolactobacillus psychrotolerans.* This species has never been related to red smear cheese or any other dairy product, but has only been isolated from marine organisms near Japan (Ishikiwa *et al*., 2002, in press). Due to its halophilic character, however, it obviously found a niche in the salty smear of a German milk product, representing an interesting case of Beijerinck's law (everything is everywhere, but the environment selects). The Gram negatives isolated from red smear surface flora were *Halomonas* spp. According to our knowledge, this is the first time that *Halomonas* spp. are reported to be present in red smear cheese ripening consortia. Since they are Gram negatives while the cheese had been produced from pasteurized milk, their presence may indicate hygienic problems in factory K. As these species compensate for the low numbers of

coryneforme species of consortium K, the overall biodiversity on the species level of the two consortia R and K was comparable.

4.1.3 *In situ* anti-listerial activity of consortium R and K

The *in situ* evaluation of anti-listerial activity revealed a stable anti-listerial activity of consortium R over six months. The high incidence of *Listeria* on red smear cheeses (Rudolf and Scherer, 2001) indicates that many of the respective industrial surface consortia exhibit no significant anti-listerial activity or, alternatively, were contaminated by resistant *Listeria* strains (Carnio *et al*., 1999). In our study, the contaminating *L. monocytogenes* strain has been propagated under optimal conditions and the contamination was performed with the first smearing. Hence, the ripening consortium had no possibility to establish itself on the cheese surface in advance. Taking in account these facts, as well as the high contamination levels, the anti-listerial potential of consortium R is extraordinarily high. Its inhibitory effect is also much stronger than the one observed by Eppert et al. (1997), who did not analyze the composition of the respective ripening cultures. In contrast, consortium K showed lower anti-listerial activity, which decreased with time. Interestingly, this change of anti-listerial activity is accompanied by a significant change of the species composition (Fig. 3.2). It is possible that the increasing numbers of *B. linens* resulted in a loss of anti-listerial activity. This observation is of importance for the food industry, since the contribution of red smear ripening consortia to food safety appears to be not necessarily a stable factor over time.

The interaction of microorganisms on red smear cheese surfaces is unknown and, most probably, very complex. It was therefore of considerable interest to test whether the extraordinary high inhibitory potential of consortium R and the unstable one of consortium K may be due to the secretion of bacteriocins, as has been demonstrated in a couple of studies (Sulzer and Busse, 1991; Eppert *et al*., 1997; Giraffa and Carminati, 1997; Valdés-Stauber *et al*., 1997; Ennahar *et al*., 1998 Carnio *et al*., 2000). Since none of the 400 isolates tested exhibited an anti-listerial activity as a single culture, the cumulative effect of each of the four consortia was examined, too. None of them exhibited inhibition zones. According to these results, it is

unlikely that the anti-listerial effects observed *in situ* (Fig. 3.3 and Fig 3.4) are due to inhibitory substances of singles strains or total consortia secreted into the growth medium. Instead, hitherto unknown factors appear to be responsible for the inhibition of *Listeria* in this food ecosystem.

4.2 Ripening temperature effect on biodiversity and anti-listerial activity of two complex undefined microbial red smear cheese ripening consortia

4.2.1 Bacterial composition of consortium R I

The bacterial composition of the consortium R I was analyzed in the smear water (SW) and on the cheese surface at the expiry date after two different ripening procedures (13°C and 16/12°C). In the smear water coryneforms represented 82% of the isolates (Fig. 3.8). The predominant bacterium *C. variabile* (57%) had been isolated from the surface of German red smear cheeses previously, but in lower numbers (Seiler, 1986; Valdés-Stauber et al., 1997). The other coryneforms contributing to this consortium were *C. casei*, *B. linens*, *A. nicotianae* and *M. gubbeenense*. Theses species had been found also in a previous study on red smear surface flora of dairy R (see Fig. 3.2 sampling point B in section 3.1.2). While all 100 isolates from the bacterial surface flora derived at the expiry date from cheeses incubated at 13°C were coryneforms, at 16/12°C they remained at the same level as found in the smear water.

During maturation at 16/12°C the proportion of *B. linens* raised 3 fold and at 13°C even 7.5 fold up to 38% (Fig. 3.8). This number is a little bit higher than the maximum numbers reported by several studies in red smear surfaces, 30% (Eliskases-Lechner and Ginzinger, 1995; Valdés-Stauber *et al*., 1997) and 32% (Seiler, 1986; Kollöffel *et al*., 1999) of the total isolates. In parallel the number of *C. casei/ammoniagenes* increased from 1% in the smear water to 24% (13°C) and even 40% (16/12°C). Alongside the increase of these two species, former predominant *C. variabile* dropped from 57% to 1% at 13°C (Fig. 3.8). Since this dramatic decrease occurred also at 16/12°C,

where it could not be detected at all at the expiry date, this phenomenon may not just be due to the temperature regime. The other changes of the flora composition were of minor importance. Some species were found in low numbers at the expiry date that had not been isolated from the smear water. They may have been present in the smear water at lower numbers or might originate from the green cheeses. The most frequent non-coryneform was an hitherto unknown species which could be related to an uncultured bacterium from feedlot manure (Ouwerkerk and Klieve, 2001). All isolates of this bacterium fermented lactose in M17 broth, revealing a final pH of about 5.6 (Tab 3.2). In the FT-IR dendrogram, they were found in more than one cluster (Fig. 3.9). This phenomenon has been described by Kümmerle et al. (1998) as an indicator of intraspecific heterogeneities with regard to the cellular composition. Additionally, two *Vagococcus carniphilus* isolates were found in consortium R I. This species had been isolated from ground beef (Shewmaker *et al*., unpublished, Accession number in gene bank is AY179329). According to our knowledge, this is the first time that this bacterium was detected in a dairy product.

4.2.2 Bacterial composition of consortium R II

In the smear water of consortium R II the same coryneform species could be isolated as described in R I. Although the overall predominance of coryneform bacteria was almost identical in both consortia of R, the proportions of the 4 coryneform species were completely different, indicating a significant structural change. Since the interval time between the sampling of consortium R I and consortium R II was one and half year, these results show that cheese-ripening consortia may not be stable for this period of time. This is interesting since consortium R showed temporal stability for six months (Fig. 3.2 in section 3.1.2). The fifth coryneform in both consortia of R was *M. gubbeenense*. Its occurrence was similar R I and R II (Fig. 3.8) and its proportion was similar to the one found in Irish Gubbeen cheese (Brennan *et al*., 2002). Again some Gram positive cocciod isolates appeared that could be related to an uncultured bacterium from feedlot manure (Ouwerkerk and Klieve, 2001). Furthermore *Marinolactobacillus psychrotolerans* was found in

consortium R II, which has been isolated from marine organisms near Japan (Ishikiwa *et al*., 2002, in Press) and found recently in consortium K (see section 3.1.3). Within these two Gram positive catalase negative species, some isolates fermented lactose strongly and some fermented lactose weakly (Tab. 3.3), indicating the phenotypic heterogeneity of those species. However, this phenotypic heterogeneity is not always expressed by different independent FT-IR clusters (see Fig. 3.10). The reason for the different intensity of lactose fermentation remained unknown. It might originate from the mutation of one or more genes coding for enzymes involved in this fermentation process, leading to a reduced formation of enzyme-substrate complexes due to changes in the conformation of the enzyme's binding site.

Cheeses, which were inoculated with consortium R II and ripened at 13°C revealde a predominance of *C. casei* (42% of the isolates). This number is close to the level found in Irish Gubbeen cheese (Brennan *et al*., 2002). *B. linens* (24%) and *C. variabile* (22%) contributed also significantly to the flora. While *A. nicotianae* almost disappeared at 13°C, an additional coryneform was found and identified as *Brachybacterium tyrofermentans*. This bacterium is known from French smear cheeses (Schubert *et al*., 1996; Burri, 1999), but according to our knowledge this is the first time that it is isolated from German red smear cheeses.

After ripening at 16/12°C, the bacterial composition differed somewhat from that observed at 13°C with less *C. casei* and more *B. linens* after the two-steps-ripening. The latter species formed six independent clusters in the FT-IR dendrogram (Fig. 3.6). Actually, all four coryneforms present in this flora (R II, 16/12°C), formed more than one independent cluster (Fig. 3.6). This phenomenon has been described by Kümmerle et al. (1998) as an indicator of intraspecific heterogeneities with regard to the cellular composition. *A. nicotianae* which had been present in the smear water was not detected after the two-steps-ripening.

4.2.3 Bacterial composition of consortium K I

Regarding its composition and structure in the smear water, consortium K I differed significantly from consortia R I and R II (Fig. 3.8 and Fig. 3.11). Only two coryneforms, were found, namely, *B. linens* and *A. nicotianae*. The latter was extraordinary predominant (85%). While, this species had been isolated from red smear surface floras previously (Seiler, 1986, Elsikases-Lechner and Ginzinger, 1995; Valdés-Stauber *et al*., 1997), none of these authors reported on a comparable predominance. The only non-coryneform bacteria found in this consortium were two Gram negative bacteria. The presence of these Gram negatives may indicate hygienic problems in dairy K.

The corresponding surface flora of cheeses matured at 13°C exhibited a significant decrease of *A. nicotianae* alongside a doubling of *B. linens*. However, the former was still the predominant species (51%). Additionally, rod-shaped lactic acid bacteria, which had not been detected in smear water, appeared in this flora (18%, see Fig. 3.11). Due to their high proportion, they probably originated from the green cheeses, rather than they might have been present in the smear water in low numbers. Since most lactic acid bacteria are microaerophilic (Leclercq-Perlat *et al*., 2000), it is interesting that we found it as a member of the smear surface flora. However, some lactic acid bacteria have also been detected in red smear surface floras previously (Seiler, 1983; Gobbetti *et al*., 1997). Two-Gram negative bacteria were identified as *Providencia* sp. Members of this genus had been isolated from French and Swiss smear cheeses recently (Gianotti, 1999). But, according to our knowledge, this is the first time that it was isolated from German red smear also.

At 16/12°C, *A. nicotianae* lost its predominance to *B. linens* that finally represented 46% of the isolates, which it is quite a big proportion. While in former times this bacterium was supposed to be an extremely important member of red smear surface floras, nowadays its importance is under discussion (Bockelmann *et al*., 1997a; Bockelamann and Hopper-Seyler, 2001 Brennan, 2002). Additionally, some Gram positive catalase negative and some Gram negative bacteria were isolated. Since the genera *Marinobacter*, *Psychrobacter* and *Marinolactobacillus* comprise halotolerant or even

halophilic bacteria (Cavanagh *et al*., 1996 , Sørensen *et al*., 2002; Ishikiwa *et al*., 2001, in press), the salty surface of smear cheeses obviously is a suitable habitat for these organisms.

4.2.4 Bacterial composition of consortium K II

In the smear water, the main difference between consortium K II and consortium K I was the presence of coagulase negative *Staphylococcus* spp in the former where they represented even 34% of the isolates. While, *Staphlococcus* spp. have been isolated from other red smear cheeses (Ryser *et al*., 1994; Bockelmann *et al*., 1997a; Gobbetti *et al*., 1997; Burri, 1999), they neither were found in consortium K I which was harvested only one week before nor in previous analysis of consortium K (see section 3.1.3). Hence *Staphylococcus* spp. do not appear to be typical major constituents of red smear surface flora of dairy K. Instead, most likely their presence is due to a contamination of the mature cheeses which were used for the preparation of consortium K II. As with consortium K I, again *A. nicotianae* (55%) and *B. linens* (7%), were the only coryneforms isolated from the smear water. And again, the only none-coryneform bacteria found in K II was *Psychrobacter* sp. (4%). The latter had also been found in K I at the expiry date cheese (16/12°C) (Fig. 3.11). They were identified as *Psychrobacter* sp. According to our knowledge this is the first time that this genus is reported to be present in red smear cheeses.

Cheeses ripened at 13°C revealed an additional coryneform bacterium identified as *Leucobacter komagatae* (Takeuchi *et al*., 1996). According to our knowledge, this is the first time that this bacterium was isolated from a dairy product. Despite reduced numbers *A. nicotianae* was still the predominant species (40%). The major change after ripening at 13°C was the total disappearance of Staphylococci accompanied by a very strong development of *Marinolactobacillus psychrotolerans* to a proportion of 31% (Fig. 3.11). The latter species had not been detected at all in the smear water. Due to its strong presence at the expiry date, the *Marinolactobacilli* may have originated

from the green cheeses, rather than from low initial numbers in the smear water.

At 16/12°C, the coryneforms (36%) lost their typical predominance to Gram positive catalase negative bacteria that represented 61% the isolates. This is an amazing result, since the pH development during this temperature regime (Fig. 3.11) as an indicator for successful maturation of red smear surface floras, appeared to be typical for ripening of red smear cheeses (Reps, 1987; Eppert *et al*., 1997; Carminatti *et al*., 1999). The trend of reduction of *A. nicotianae* alongside with an increase of *B. linens* was similar to consortium K I. Additional bacteria such as *Enterococcus faecalis* and *Serratia* sp. were found after this temperature regime. Both bacteria have been previously isolated from red smear cheeses (Ryser *et al*., 1994; Burri, 1999; Gianotti, 1999), but indicate hygienic problem in dairy K and/or the dairy where the green cheeses had been produced.

4.2.5 *In situ* anti-listerial activity of consortia R I, R II, K I and K II in two ripening temperature regimes

Despite the significant difference of the bacterial composition between R I and R II, both consortia exhibited remarkable and comparable anti-listerial activity at both ripening temperature regimes (Fig. 3.12). These results demonstrate the fact that a stable bacterial composition may not always be the necessary key for a stable anti–listerial activity of consortium R. Instead, these results showed the flexibility of the surface flora of dairy R for different ripening temperature regimes without loosing its functionality as a protective culture (Fig. 3.12). In contrast, the anti-listerial activity of consortia K I and K II appeared to be dependent on the ripening temperature regime (Fig.3.12).

4.3 Sensitive *in situ* monitoring of a recombinant bioluminescent *Yersinia enterocolitica* reporter mutant in *real time* on Camembert cheese

4.3.1 Selection of a suitable reporter strain

Mutants of *Yersinia enterocolitica*, which had the complete lux cassette inserted into an open reading frame of unknown function were screened for light production on Camembert cheese under natural conditions. Mutant B94 was selected for further experiments since it showed the highest photon output per CFU (Fig.14A and Fig.14B). The reporter mutant did not exhibit a loss of fitness compared to the wild type (Fig. 3.15).

4.3.2 Microheterogeneity of *Yersina* on cheese

Using the intensified charge-coupled device camera (ICCD), it is possible to determine the localization of the bioluminescent *Yersinia* on the cheese surface. The distribution of the bioluminescent cells was quite irregular (Fig. 3.16). Although the entire cheese surface was initially contaminated with *Yersinia*, certain areas appeared to become more highly colonized than others (Fig. 3.16). This was also observed for *E. coli* on beef carcasses (Siragusa *et al*., 1999) and reflects the potential of this approach to characterize the local microdiversity of pathogen distribution in food.

4.3.3 Detection limit of strain B94

The introduction of plasmids containing *luxCDABE* and *luxABCDE* constructs derived from *P. luminescens* into *E. coli* and *S. aureus* (Francis *et al*., 2000) has demonstrated that the yield of light per cell strongly depends on the measurement temperature, *lux* construct, and host strain used. As shown with *Y. enterocolitica* (Fig. 3.18), the significant dependence of light emission on temperature can also be observed when the *luxCDABE* construct is acquired by transposition. At 10°C, luciferase activity is about 15% of the one at 30°C (Fig. 3.18). Strain B94, nevertheless, is a highly sensitive reporter. To estimate the sensitivity of the method, bioluminescence of the entire cheese surface was measured. The results (Fig. 3.17) demonstrate a high correlation

between culture-derived viable cell count and bioluminecence (r^2 = 0.98) and the detection limit found on cheese was about 200 CFU/cm^2 (Fig. 3.17) which corresponds to 33 CFU/gr. This limit is similar to the value reported by Siragusa et al. (1999), who could detect 50 *E. coli* O157:H7 cells per well in an artificial microtiter plate system containing broth suspensions, and lower than those found by Chen and Griffiths (1996) measured in various foodstuff. The latter authors contaminated dilutions (1:10) of homogenized meat, fluid milk and liquid whole egg with non-cured bioluminescent *Salmonella*, reporting detection limits of 180 CFU/ml, 1900 CFU/ml and 6760 CFU/ml, respectively. However, since these and other authors (Ahmad *et al*., 1991; Francis *et al*., 2000; Francis *et al*., 2001) measured light emission at high temperatures, their results are not directly comparable to those reported in this study.

4.3.4 Light emission of *Y. enterocolitica* B94 is partly dependent on the growth phase.

Figure 3.17 shows that bioluminescence per cell of strain B94 increases on the cheese surface over time. We therefore compared the bioluminescence of strain B94 when this bacterium was grown at various temperatures to the log phase and to the stationary phase. The results showed (Fig. 3.19) that in the logarithmic growth phase, the expression of light per cell appeared to be little affected by the growth temperature, indicating a temperature-independent regulation of the promoter in exponentially growing cultures. At higher temperatures, stationary phase resulted in a significant reduction of light emission. This latter phenomenon corresponds with the observations of other authors at temperatures between 28°C and 40°C using different *lux* systems derived from marine and terrestrial bacteria in various Gram negative as well as Gram positive transformants. Unge et al. (1999) used the *luxAB* of *Vibrio harveyi* to build a *psbA-gfp-luxAB* cassette, which they introduced into the chromosome of *Escherichia coli* and *Pseudomonas fluorescens*. Upon entrance into stationary phase and during starvation, in both cultures they observed a decrease in luciferase activity that corresponded to a decrease in metabolic activity of the population while the number of culturable cells remained relatively stable at 28°C and 32°C,

respectively. Marincs (2000) reports on similar results between 28°C and 40°C for *E. coli* harboring the whole *luxCDABE* operon from *P. luminescens* linked to the *lac* promoter on pLITE27 (Marincs *et al*., 1994). The same was true with *Streptococcus pneumoniae* chromosomally tagged with a promoterless *luxABCDE* operon derived from *P. luminescens* when grown at 37°C (Frackman *et al*., 1990). Investigating several transformants, the latter authors could show that this phenomenon was not caused by reduced transcription from a specific promoter. Waterfield et al. (1995) fused the *luxAB* of *Vibrio fisheri* with various lactococcal promoters on plasmids. Transforming *Lactococcus lactis* with these constructs, all clones exhibited a decline of light emission after entering the stationary phase. Since the catalytic activity of bacterial luciferase requires reducing equivalents ($FMNH_2$), this growth phase dependent decay of light emission is generally discussed to reflect a decrease in the metabolic activity of the cell. Despite the general phenomenon however, important differences can be found when different studies are compared in detail. In our study the stationary phase cultures where not measured at the very beginning of this growth phase (Fig. 3.19). Nevertheless, at 23°C and 30°C they still yield a photon count of about 10 % compared to the logarithmic cultures. These findings are in contrast to Francis and coworkers (2001) who found a very steep decline of bioluminescence to next to zero within one to three hours after entering the stationary phase. They are, however, consistent with those of Unge et al. (1999) and Marincs (2000) who report on a gradual growth phase dependent light reduction. Observing similar differences with a number of lactococcal promoters, Waterfield et al. (1995) demonstrated that the rate of decrease of bioluminescence depends on both, the growth phase of the culture and the strength of the promoter, with low activity promoters displaying a more rapid decay. Hence, our data may indicate that the *lux* cassette of *Y. enterocolitica* B94 is regulated by a rather strong promoter.

The growth phase-dependent decrease of bioluminescence in *Y. enterocolitica* B94 is temperature dependent. Light emission of stationary – phase cultures of *Y. enterocolitica* grown at 4, 10, and 15°C is strikingly different from that of cultures propagated at higher temperatures (Fig. 3.19). Although the photon counts of stationary-phase cultures at low temperatures are lower than those of logarithmic cultures, they do not exhibit the steep

growth phase-dependent decline observed at 23 and 30°C. Instead, they are of the same magnitude as the ones detected in logarithmic cultures. We could not find any corresponding data on low-temperature measurements in the literature. Obviously, the regulation of the promoter that controls the *lux* operon in *Y. enterocolitica* B94 is temperature dependent during stationary phase, yielding stronger expression at low temperatures. This observation indicates an important function of the promoter and its originally corresponding gene(s) in cold stress response of *Yersinia enterocolitica* in the stationary phase. Furthermore, we observed an even higher relative light unit rate per cell on Camembert cheese after 14 days at 10°C than during day 1 to 3, with a detection limit around 200 CFU/cm^2 (Fig. 3.17). These data suggest an additional upregulation of the promoter in advanced stationary-phase cultures. According to these results, the promoter and the gene(s) originally linked to it may also play a role in starvation metabolism of *Y. enterocolitica*. These results demonstrate the suitability of mutant B94 as a sensitive reporter in long term experiments at low temperature, which is a setting common to food storage at refrigeration temperature.

4.3.5 Light emission of *Y. enterocolitica* B94 is salt dependent.

The correlation between bioluminescence and viable cell count for cells grown on BHI agar with and without 2.5% salt showed clearly that the expression of luciferase is stimulated in the presence of sodium chloride (Fig. 3.17). This is significant since the concentration of sodium chloride in soft cheese may well reach this concentration (Shaw, 1986). The elevated light emission in the presence of 2.5% NaCl is detectable in the whole range of temperatures tested from 4°C to 30°C (Fig. 3.18). While the presence of 2.5% salt stimulates the light emission of *Y. enterocolitica* B94 in BHI broth, it reduces its growth rate in this medium (Fig. 3.20), indicating stress conditions. Hence the promoter that regulates the inserted *lux* cassatte in strain B 94 may also be involved in the salt stress response of *Yersinia enterocolitica*.

It was quite unexpected that bioluminescence of mutant B94 on BHI agar was much lower compared to the cheese environment, even in the presence of sodium chloride (Fig. 3.17). This could have been due to an underestimation of the viable cell count on cheese. CIN medium, however, is

the most efficient medium for enumeration of *Y. enterocolitica* (Schiemann, 1979; Schiemann, 1982; Hamama *et al*., 1992) and it appears unlikely that this approach should underestimate the cell count by a factor of 20. The difference could also be due to a repression of the promoter by BHI. Also stimulation by some cheese constituents, the influence of the competitive flora, or the influence of more than one factor may play a role.

5 Concluding remarks

The temporal stability of the bacterial composition and the anti-listerial activity appear to be individual characteristics of undefined industrial cheese ripening consortia. The continuous carry-over of microorganisms between subsequent productions due to the old young smearing procedure does not necessarily lead to a significant shift of the species composition within six months. A predominant species may represent up to 90% of the isolates, possibly indicating a certain “backbone function” within the respective consortium, but the functionality of accordingly structured ripening consortia is independent of the genus affiliation of the dominating species. Although the anti-listerial effect of such cheese ripening consortia may not constitute a completely stable factor over time, our data clearly indicate a considerable potential of such cultures to be used for a safe food production without artificial preservatives.

It has been shown that the successions within the bacterial subflora of red smear consortia and their final biodiversity strongly dependent on the temperature regime during cheese ripening. Additionally the flora composition of a respective consortium appears to be a determinative factor whether a certain species increases or decreases at a certain ripening temperature. Furthermore it was shown that coryneform bacteria may loose their typical predominance on the cheese during ripening. However, it also has been proven that substantial changes in the flora composition do not necessarily lead to a loss of anti listerial activity of protective cultures. Finally the results show that in depth flora analysis may still reveal hitherto unknown bacteria or species that had not been attributed to this kind of ecosystem so far. While the study sheds some more light on the poorly understood ecology of complex red smear cheese surface floras, it also provides interesting insights of practical relevance that can contribute to an increase of food safety.

We have shown in this study that insertion of a transposon carrying a promoter-less modified *lux* operon downstream of a suitable host promoter allows sensitive *real time* monitoring of *Yersinia enterocolitica in situ*, even at

low temperature. Since strongly expressed promoters, however, are often regulated by a number of environmental factors, it is mandatory to select appropriate reporter strains carefully with respect to the conditions prevalent in the food stuff which will be under investigation. The cheese samples were not artificially influenced by any chemical, antibiotic or physical treatment for light measurement in order for bioluminescence measurements to be made. Our study shows that it is possible to bridge the gap towards a real-product status assessment of pathogens in real time and *in situ*, even at low temperature.

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Appendices

Appendix A

Calculation of *Listeria* cell counts per cm^2 of cheese surface

$$[CFU / cm^2] = \frac{[CFU / g] \bullet 200\ g}{50\ cm^2}$$

200 g	Factor for the homogenized sample
50 cm^2	The area of two sides cheese surfaces which equal to 20 g

Appendix B

Calculation of *Yersinia* cell counts per cm^2 of cheese surface

$$[CFU / cm^2] = \frac{[CFU / g] \bullet 200\ g}{33.18\ cm^2}$$

200 g Factor for the homogenized sample

33.18 cm^2 The area of one side of Camembert cheese surface which equals to 20 g

Calculation of *Yersinia* cell counts per cm^2 of BHI agar

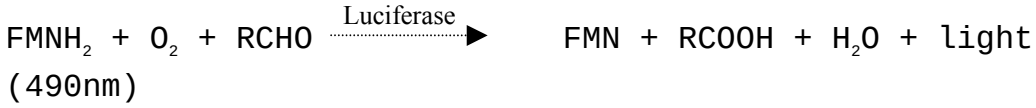
$$[CFU / cm^2] = \frac{[CFU / g] \bullet 200\ g}{56.75\ cm^2}$$

200 g Factor for the homogenized sample

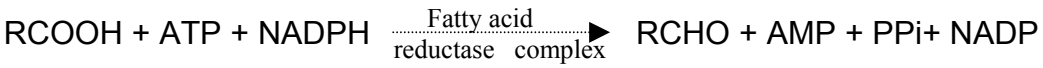
56.75 cm^2 The area of one side of BHI agar surface which equals to 20 g

Appendix C

Bioluminescent reaction



Aldehyde biosynthesis



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2. **Ariel Maoz, Ralf Mayr and Siegfried Scherer** (2003) Temporal stability and biodiversity of two complex, anti-listerial cheese ripening microbial consortia. Appl. Environ. Microbiol. **(In press).**

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