ABTEILUNG MIKROBIOLOGIE
ZENTRALINSTITUT FÜR ERNÄHRUNGS- UND LEBENSMITTELFORSCHUNG WEIHENSTEPHAN
TECHNISCHE UNIVERSITÄT MÜNCHEN

Microbial consortia from smear-ripened cheese: Biodiversity, incidence of commercial starter microorganisms and anti-listerial activity of yeasts

STEFANIE GOERGES

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Haushalts- und Ernährungswissenschaften
(Dr. oec. troph.)

genehmigten Dissertation.

Vorsitzender:		Univ.-Prof. Dr. G. Cerny
Prüfer der Dissertation:	1.	Univ.-Prof. Dr. S. Scherer
	2.	Univ.-Prof. Dr. K. Heller
		(Christian-Albrechts-Universität Kiel)

Die Dissertation wurde am 18.08.2005 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 12.10.2005 angenommen.

Acknowledgments

The present work has been performed at the "Abteilung Mikrobiologie, Zentralinstitut für Ernährungs- und Lebensmittelforschung (ZIEL) Weihenstephan, TU München" under supervision of Prof. Dr. Siegfried Scherer. Special thanks go to my supervisor Siegfried Scherer for his excellent support, for giving me the possibility to follow my own ideas and to continue research on anti-listerial properties in yeast although this task was canceled ahead of schedule in the SCM project.

I am very grateful to Prof. Dr. Knut Heller (Christian-Albrechts-Universität, Kiel) for being my second examiner.

Furthermore, I am very thankful to Dr. Barbara Silakowski and Ulrike Aigner for their contribution to the section on anti-listerial yeast. My cordial thanks also go to Dr. Roberto Gelsomino (BCCM/LMG Bacteria Collection, University of Ghent, Belgium), Jérôme Mounier (Dairy Products Research Centre, Teagasc, Fermoy, Co. Cork, Ireland) and Mary Rea (Biotechnology Department, Moorepark Food Research Centre, Teagasc, Fermoy, Co. Cork, Ireland). Their reliability enabled an excellent cooperation.

Sincere thanks are given to all of my colleagues, especially to these with whom I shared office and labs, for providing a pleasant working atmosphere. The freely given technical assistance of Lisa Rieder and Andreas Bischof is also greatly appreciated.

Special thanks are addressed to Dr. Kinga Jakob and Dr. Mareike Wenning for fruitful discussion, helpful advices and critical reading of my manuscript.

Finally, I would like to cordially thank my parents and especially Sebastian Müller for their interest in my Ph.D. work and their constant, infinite encouragement and support throughout this time.

This work was partially supported by the EU, project QLK1-CT-2001-02228: Biodiversity and anti-listerial activity of surface microbial consortia from Limburger, Reblochon, Livarot, Tilsit and Gubbeen cheese; Acronym: SCM (Smear cheese microflora), and by the "Vereinigung zur Förderung der Milchwissenschaftlichen Forschung an der Technischen Universität München in Freising - Weihenstephan e.V.".

Table of contents

List of figures

List of tables

List of abbreviations

Abbreviations for genera

A. ..... *Arthrobacter*
B. ..... *Brevibacterium*
C. ..... *Candida*
Cl. ..... *Clavispora*
D. ..... *Debaryomyces*
G. ..... *Galactomyces*
I. ..... *Issatchenkia*
K. ..... *Kluyveromyces*
L. ..... *Listeria*
Lb. ..... *Lactobacillus*
M. ..... *Microbacterium*
P. ..... *Pichia*
R. ..... *Rhodotorula*
S. ..... *Saccharomyces*
St. ..... *Staphylococcus*
T. ..... *Torulaspora*
Tr. ..... *Trichosporon*
Y. ..... *Yarrowia*

Other abbreviations

1/2/3 (E, M, L) .....Batch 1/2/3 (early, middle, late sampling point)
3 d, 6 d, 9 d, 28 d .....sampling after ~3, ~6, ~9, ~28 days of ripening
AI .....*A. nicotianae*/*A. arilaitensis* isolated from cheese smear
ARDRA.....amplified rDNA restriction analysis
AS.....*A. nicotianae*/*A. arilaitensis* from smear starter culture
ATCC.....American Type Culture Collection, Manassas, Va., USA
BCCM/LMG .....Belgian Co-ordinated Collections of Micro-organisms/Bacteria Collection Laboratorium voor Microbiologie, Ghent University, Ghent, Belgium
BCCM/MUCL.....Belgian Co-ordinated Collections of Micro-organisms/Mycothèque de l'Université catholique de Louvain
BHI .....brain heart infusion
BI .....*B. linens*/*B. aurantiacum* isolated from cheese smear
Bis-Tris .....Bis(2-hydroxyethyl)amino-tris-(hydroxymethyl-)methan
BOX-, $(GTG)_5$-, REP-PCR ...*rep*-PCR using BOX-, $(GTG)_5$-, REP-primers
BS.....*B. linens*/*B. aurantiacum* from smear starter culture
CBS.....Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands
cfu .....colony forming units
DI.....*D. hansenii* isolated from cheese smear
DNA .....deoxyribonucleic acid
DS .....*D. hansenii* from smear starter culture

E, M, L .....early (~day 6), middle (~day 28) and late sampling point (~day 58)
e.g. .....(lat. exempli gratia); for example
EU .....European Union
FTIR spectroscopy.....Fourier transform infrared spectroscopy
G.....glycerol number, Abteilung Mikrobiologie, ZIEL Weihenstephan, Freising, Germany
GB .....Gubbeen
GI.....*G. geotrichum* isolated from cheese smear
GMP .....good manufacturing practice
GS .....*G. geotrichum* from smear starter culture
H_2O .....water
HACCP.....Hazard Analysis Critical Control Point
HGMF .....hydrophobic grid membrane filter
IS1 .....isolation series 1 (surface isolates from Limburger Batches 1 to 3 and one representative of each smear starter organism were available)
IS2 .....isolation series 2 (surface isolates from Limburger Batch 4 and 50 representatives of each smear starter organism were available)
IV .....Livarot
LAB .....lactic acid bacteria
LM.....Limburger
$\log_{(10)}$ .....logarithm (to the basis 10)
Milli-Q-Water.....highly purified water (Millipore)
mt.....mitochondrial
n.d. .....not determined
n.e. .....not evaluable
NaCl.....sodium chloride
no. .....number
OD_{600} .....optical density, wavelength 600 nm
P .....supernatant of *Lactobacillus plantarum* containing pediocin
PCA^{3+}.....plate count agar containing 3 % NaCl
PCR .....polymerase chain reaction
PE1/2/3 .....preliminary experiments 1/2/3
PFGE.....pulsed field gel electrophoresis
pH .....pondus Hydrogenii
RAPD.....random amplified polymorphic DNA
RB .....Reblochon
rDNA .....ribosomal deoxyribonucleic acid
rep-PCR.....repetitive PCR
RFLP .....restriction fragment length polymorphism
rpm.....rounds per meter
rRNA .....ribosomal ribonucleic acid
$_S$ .....isolation from preparations on PCA^{3+} using a spatula technique
SCM.....Smear Cheese Microflora
SDS-PAGE .....Sodium dodecyl sulphate polyacrylamide gel electrophoresis

sp. .....species (singular)
spp.....species (plural)
T.....isolation from preparations on TSA
TS.....Tilsit
TSA.....tryptic soy agar
TSB.....tryptic soy broth
UPGMA .....unweighted pair group method algorithm
w/v.....weight per volume
WSLC.....Weihenstephan *Listeria* Collection, Abteilung Mikrobiologie, ZIEL Weihenstephan, Freising, Germany
WSYC .....Weihenstephan Yeast Collection, Abteilung Mikrobiologie, ZIEL Weihenstephan, Freising, Germany
YG^+A .....yeast extract glucose agar containing 1 % tryptone
YG^+SA .....yeast extract glucose soft agar containing 1 % tryptone
YGB .....yeast extract glucose broth
YGCA.....yeast extract glucose chloramphenicol agar
YGCBA .....yeast extract glucose chloramphenicol bromophenol agar
ZnSe.....zinc selenide

Units of measurement

° C.....degree Celsius
µl .....microliter
µm.....micrometer
cm .....centimeter
cm^{-1}.....1/cm
cm^2 .....square centimeter
d .....day
g.....gram
l .....liter
mg .....milligram
min .....minute
ml.....milliliter
mm .....millimeter
pmol.....picomol
sec .....second

Summary

The present work was performed as part of the EU project QLK1-CT-2001-02228 "Biodiversity and anti-listerial activity of surface microbial consortia from Limburger, Reblochon, Livarot, Tilsit and Gubbeen cheese", Acronym SCM (Smear cheese microflora). It was initiated to analyze the yeast and bacterial floras of these cheeses at different ripening stages using a polyphasic approach including several phenotypic and genotypic methods and to test cheese yeasts for their anti-listerial properties. Main subjects of the present work within the frame of the EU project were to investigate the biodiversity of the yeast floras and the anti-listerial activity of yeasts.

1. The yeast populations of the five European project cheeses varied significantly in their species diversity. Generally, a higher diversity was obtained when cheese batches from different dairies were investigated. Floras were composed of two to 12 different species, however, most of them present in rather low numbers. Most frequently occurring species were *Debaryomyces hansenii* and *Galactomyces geotrichum*. *D. hansenii*, the only yeast present in the smears of all cheeses, dominated the microfloras of Limburger, Tilsit and Gubbeen cheese with 49 to 98 %, whereas *G. geotrichum* was found to be dominant on both French cheeses with 27 and 61 %. A low degree of similarity was detected between representatives of *D. hansenii* and *G. geotrichum* from the five cheeses.

2. The microflora of three batches of Limburger cheese produced by a single German dairy was investigated in the early, middle and late stages of ripening. A total of 450 yeasts and 450 bacteria were isolated. The yeast flora was composed of *D. hansenii* and *G. geotrichum* strains. Members of the former taxon dominated the yeast flora during different stages of ripening in all of the batches, apart from the early stage of Batch 2. Two *D. hansenii* types were distinguishable, one of which was identified as a strain included in a commercial smear starter culture used for the Limburger cheese production in subsequent analyses. Most of the bacterial isolates were assigned to the newly described species *Arthrobacter arilaitensis* and *Brevibacterium aurantiacum*. Isolates within the same species showed a high degree of homogeneity. It became apparent that the surface microbial flora of Limburger cheese is dominated by a small number of yeast and bacterial species which is in contrast to other European red smear cheeses.

3. The German dairy used a modified old-young smearing technique for producing the Limburger cheese investigated. Furthermore, the cheese milk was supplemented with a commercial smear starter preparation containing *D. hansenii*, *Geotrichum candidum* (anamorphic form of *G. geotrichum*), *Arthrobacter nicotianae* and *B. linens* to support the ripening process. A total of four Limburger batches were examined for the presence of the yeast and bacterial smear starter organisms on the cheese surface during several ripening stages. For this purpose 639 yeast isolates and 725 bacterial isolates were characterized by using phenotypic and genotypic methods. The *D. hansenii* starter strain was detectable on the cheese surfaces of all four batches. It dominated the ripening flora after the first smearing in the fourth batch with 88 %, but it played an inferior role on surfaces of later ripening stages in all batches where its fraction amounted to a maximum of 14 %. The *G. geotrichum* starter strain was not recovered from cheeses of Batches 1 to 3. On cheeses of the fourth batch it occurred in a frequency of 6 to 20 %. Similar to the cheese surface isolates, the bacterial smear starters were also identified as *A. arilaitensis* and *B. aurantiacum*. However, the starter strains were not reisolated from the cheese surface at any time point during ripening in all four batches. The application of the commercial smear starter preparation appeared not to be optimal. The relevance of this culture for the ripening process still has to be investigated.

4. A total of 413 yeasts, mainly isolates from red smear cheeses, were screened for an inhibitory activity against five *Listeria monocytogenes* strains. Using an agar-membrane screening assay, a fraction of approximately 10 % of the isolates inhibited growth of one or more *L. monocytogenes* strains. Fourteen out of the 413 yeasts were co-cultivated together with the most sensitive indicator strain on a solid medium to test the anti-listerial activity of yeast in direct cell contact with *Listeria*. All yeasts inhibited *L. monocytogenes* to a low degree, which is most probably due to competition for nutrients. Some yeasts, however, reduced listerial growth by up to four log units. They are promising candidates for further characterization of the inhibitory principle and a potential use as a protective culture. Inhibition of *L. monocytogenes* was clearly pronounced in the co-cultivation assay which simulates the conditions and contamination rates present on smear cheese surfaces. There was no evidence that the unknown inhibitory molecule(s) is (are) able to diffuse

through soft agar. It is therefore suggested that the inhibitory principle is not of bacteriocin- or killer-toxin-type.

Zusammenfassung

Die vorliegende Arbeit war Teil des EU-Projektes QLK-CT-2001-02228 „Biodiversity and anti-listerial activity of surface microbial consortia from Limburger, Reblochon, Livarot, Tilsit and Gubbeen cheese“, Acronym SCM (Smear cheese microflora). Dieses wurde initiiert, um die Hefen- und Bakterienfloren dieser Käse zu verschiedenen Reifungsstadien mittels eines polyphasischen Ansatzes, der phänotypische und genotypische Methoden umfasst, zu analysieren und um Käsehefen auf ihre anti-listeriellen Eigenschaften zu untersuchen. Der Schwerpunkt der vorliegenden Arbeit im Rahmen des Projektes lag auf der Untersuchung der Biodiversität der Hefenfloren und der anti-listeriellen Aktivität von Hefen.

1. Die Hefenpopulationen der fünf europäischen Projektkäse unterschieden sich signifikant hinsichtlich ihrer Speziesdiversität. Im Allgemeinen ergab sich eine höhere Diversität für einen Käsetyp, wenn Chargen aus verschiedenen Käsereien untersucht wurden. Die Floren setzten sich aus zwei bis 12 verschiedenen Spezies zusammen, wobei die meisten jedoch nur in relativ geringen Anteilen auftraten. Am häufigsten vorkommende Spezies waren *Debaryomyces hansenii* und *Galactomyces geotrichum*. *D. hansenii*, die einzige Hefe, die in den Schmieren aller Käse vorkam, dominierte die Mikrofloren von Limburger, Tilsiter und Gubbeen Käse mit 49 bis 98 %, während *G. geotrichum* sich auf den beiden französischen Käsen mit 27 bis 61 % als dominant erwies. Eine geringe Ähnlichkeit wurde zwischen Vertretern von *D. hansenii* und *G. geotrichum* der fünf Käse festgestellt.

2. Die Mikroflora von drei Limburger-Chargen, produziert in derselben deutschen Käserei, wurde jeweils zu einem frühen, mittleren und späten Reifungsstadium untersucht. Insgesamt wurden je 450 Hefen und Bakterien isoliert. Die Hefenflora bestand aus *D. hansenii* and *G. geotrichum* Stämmen. Vertreter des ersten Taxons dominierten die Hefenflora während der verschiedenen Reifungsstadien in allen Chargen, abgesehen vom frühen Stadium von Charge 2. Zwei *D. hansenii*-Typen waren unterscheidbar. Einer von diesen wurde in anschließenden Untersuchungen als Stamm einer kommerziellen Schmierestarterkultur identifiziert, die zur Produktion des Limburgers eingesetzt wurde. Die meisten Bakterienisolate wurden den neu beschriebenen Spezies *Arthrobacter arilaitensis* und *Brevibacterium aurantiacum* zugeordnet. Innerhalb einer Spezies wiesen die Isolate große Homogenität auf. Es wurde deutlich, dass die Oberflächenflora des Limburger Käses, im

Gegensatz zu anderen europäischen Rotschmierekäsen, von einer geringen Zahl an Hefen- und Bakterienspezies dominiert wird.

3. Die deutsche Käserei wandte eine modifizierte Technik des Alt-Jung-Schmierens zur Produktion des untersuchten Limburger Käses an. Zudem wurde die Kesselmilch mit einem kommerziellen Schmierestarter-Präparat versetzt, welches *D. hansenii*, *Geotrichum candidum* (anamorphe Form von *G. geotrichum*), *A. nicotianae* und *B. linens* enthält, um den Reifungsprozess zu unterstützen. Insgesamt wurden vier Limburger Chargen hinsichtlich des Vorkommens der Hefen- und Bakterienstarterorganismen auf der Käseoberfläche zu verschiedenen Reifungsstadien untersucht. Hierzu wurden 639 Hefen- und 725 Bakterienisolate mittels phänotypischer und genotypischer Methoden charakterisiert. Der *D. hansenii*-Starterstamm wurde auf den Käseoberflächen aller vier Chargen detektiert. Während er die Reifungsflora nach dem ersten Schmiervorgang in der vierten Charge mit 88 % dominierte, spiele dieser in späteren Reifungsstadien aller Chargen mit Anteilen von maximal 14 % eine untergeordnete Rolle. Der *G. geotrichum* Starterstamm wurde auf Käsen der Chargen 1 bis 3 nicht gefunden. Auf Käsen der vierten Charge trat er zu 6 bis 20 % auf. Ebenso wie die Käseoberflächenisolate wurden die Bakterienschmierestarter als *A. arilaitensis* und *B. aurantiacum* identifiziert. Jedoch konnten die Starterstämme bei allen vier Chargen zu keinem Reifungszeitpunkt von der Käseoberfläche reisoliert werden. Der Einsatz des kommerziellen Schmierestarter-Präparates erwies sich als nicht optimal. Die Relevanz dieser Kultur für den Reifungsprozess muss noch untersucht werden.

4. Insgesamt wurden 413 Hefen, überwiegend Isolate von Rotschmierekäsen, auf ihre anti-listerielle Aktivität untersucht. Etwa 10 % der Isolate hemmten das Wachstum eines oder mehrerer *L. monocytogenes* Stämme in einem Agar-Membran-Screening-Assay. Vierzehn der 413 Hefen wurden mit dem sensitivsten Indikatorstamm auf festem Medium co-kultiviert, um die anti-listerielle Aktivität von Hefen in direktem Zellkontakt mit Listerien zu testen. Alle Hefen hemmten *L. monocytogenes* zumindest in geringem Maß, was sich vermutlich durch Nährstoffkonkurrenz erklären lässt. Einige Hefen jedoch reduzierten das Listerienwachstum um bis zu vier Zehnerpotenzen. Diese sind aussichtsreiche Kandidaten zur weiteren Charakterisierung des Hemmprinzips und für einen möglichen Einsatz als Schutzkultur. Die Hemmung von *L. monocytogenes* war in dem Co-Kultivierungsassay, der

die Gegebenheiten und die Kontaminationsraten auf Rotschmierekäseoberflächen simuliert, deutlich ausgeprägt. Es gab keinen Hinweis darauf, dass das (die) unbekannte(n) hemmende(n) Molekül(e) durch Softagar diffundieren kann (können). Es deutet demnach darauf hin, dass das hemmende Prinzip nicht vom Bakteriozin- oder Killertoxintyp ist.

1 Introduction

The present work was involved in the EU project QLK1-CT-2001-02228 "Biodiversity and anti-listerial activity of surface microbial consortia from Limburger, Reblochon, Livarot, Tilsit and Gubbeen cheese", Acronym SCM (Smear cheese microflora). Knowledge about the flora composition of smear-ripened cheeses is limited but essentially needed for developing defined, commercially available ripening cultures that ensure an optimized ripening and make the red smear cheese production more safe by reducing the risk for contamination with undesirable, even pathogenic organisms such as *Listeria*. The application of ripening organisms with inhibitory properties against *Listeria* in red smear cheese production is another promising method to increase food safety.

1.1 Smear-ripened cheese

Smear-ripened cheeses, such as Limburger, Reblochon, Livarot, Tilsit and Gubbeen cheese (Hohenegger et al.; Larpin et al.; Rea et al.; Thomas et al., manuscripts submitted), are also called red smear cheeses due to a characteristic red-brown to red-orange colored smear (Bockelmann and Hoppe-Seyler 2001; Reps 1987). The smear may also appear light yellow (Kammerlehner 2003).

Old-young smearing as a traditional production method and its hygienic risks

Traditionally, smear-ripened cheeses are produced using the old-young smearing process in which younger cheeses are inoculated with the microorganisms taken from mature cheeses (Carnio et al. 1999; Valdés-Stauber et al. 1997). All microorganisms that are necessary in the ripening process are transferred to the smear brine and thus, to the surface of the young cheeses. The surface microbial organisms are adjusted to the ripening conditions causing a fast development of the smear and, therefore, an optimal ripening (Bockelmann 2002a; Bockelmann and Hoppe-Seyler 2001; Bockelmann et al. 1997b; Kammerlehner 1986). To enhance or support the ripening process, cheese manufacturers often additionally use smear adjuncts composed of a few species, such as *Debaryomyces hansenii* and *Brevibacterium linens*. However, this does not reflect the complexity of the surface microbial consortia found on red smear cheeses which increases the importance of the house microflora (Bockelmann 2002a).

However, the old-young smearing allows potential contamination with spoiling or even pathogenic organisms, such as *L. monocytogenes* or *Staphylococcus aureus* (Carnio et al. 1999; Valdés-Stauber et al. 1997). If the surface of the mature cheeses is contaminated with undesirable organisms, they are brought into the smear water, spread over the surfaces of the young cheeses and may persist in the dairy by becoming a part of the house flora (Bockelmann 2002a). The risk for contaminations is further increased when the cheeses are lying on unhygienic shelfs since cheeses that are wet from smearing offer an ideal growth medium for contaminants. Also floor drains of food processing plants are often colonized by undesirable organisms (Kammerlehner 2003).

Due to these hygienic, but also economic aspects, efforts in developing alternative production methods are necessary. A promising approach would be to use complex, defined ripening cultures. However, limited knowledge about the microbial ecology of these cheeses makes the development of commercially available, defined smear cultures difficult since optimal ripening conditions and cheese quality have to be guaranteed (Bockelmann 2002a). Bockelmann and co-workers are intensely and successfully working on this topic (Bockelmann 2002a, 2002b; Bockelmann et al. 2000; Bockelmann et al. 1997a; Bockelmann et al. 2005).

Composition of surface microbial consortia

The typical smear on the surface of red smear cheeses results from microbial growth and substances produced from microbial metabolic activity (Kammerlehner 2003). The composition of the microbial consortia and interactions which occur between the different ripening organisms are not known in detail (Reps 1987). A complex consortium of yeasts, coryneform and other Gram-positive bacteria, such as staphylococci, have been considered to be involved in ripening (Brennan et al. 2004; Busse 1989; Carnio et al. 1999; Irlinger et al. 1997; Valdés-Stauber et al. 1997). The individual house microflora exerts a large influence on the composition of the surface microbial consortia (Bockelmann 2002a).

D. hansenii is considered to be the most frequently found yeast on red smear cheeses (Bockelmann 2002a; Bockelmann and Hoppe-Seyler 2001; Eliskases-Lechner and Ginzinger

1995b; Valdés-Stauber et al. 1997). Other yeasts isolated from smear-ripened cheeses are, e.g., members of the genera *Candida* and *Pichia*, as well as strains assigned to the species *Geotrichum candidum* and its perfect form *Galactomyces geotrichum*, *Kluyveromyces lactis*, *K. marxianus*, *Trichosporon beigelii*, *Torulaspora delbrueckii* and *Yarrowia lipolytica* (Corsetti et al. 2001; Eliskases-Lechner and Ginzinger 1995b; Valdés-Stauber et al. 1997).

Major organisms involved in the ripening of red smear cheeses are coryneform bacteria (Bockelmann and Hoppe-Seyler 2001; Brennan et al. 2004). Among them, species of the genus *Corynebacterium* have been reported to be the dominant organisms isolated from various red smear cheeses (Bockelmann and Hoppe-Seyler 2001; Brennan et al. 2002; Maoz et al. 2003; Mayr et al. 2004; Oberreuter et al. 2003). Furthermore, representatives of the genus *Arthrobacter* also play a major role (Bockelmann and Hoppe-Seyler 2001; Eliskases-Lechner and Ginzinger 1995a; Feurer et al. 2004a; Irlinger et al. 2005; Maoz et al. 2003; Valdés-Stauber et al. 1997). Species assigned to the genera *Brevibacterium*, *Brachybacterium* and *Microbacterium* are also often mentioned as flora components (Brennan et al. 2002; Eliskases-Lechner and Ginzinger 1995a; Schubert et al. 1996; Valdés-Stauber et al. 1997). *B. linens* was long considered to be the only organism in the cheese smear - due to its orange pigmentation – to be responsible for the color development of the cheese surface (Brennan et al. 2004; Busse 1989). More recent studies show that *B. linens* represents only a maximum proportion of approximately 20 % of the bacterial population (Bockelmann and Hoppe-Seyler 2001; Brennan et al. 2002; Maoz et al. 2003). Bockelmann and Hoppe-Seyler (2001) demonstrated that the typical red-brown color of Tilsit cheese develops from mixed cultures of *Arthrobacter* strains with the proteolytic *B. linens* but also with the proteolytic and non-pigmented *Staphylococcus sciuri*. *Arthrobacter nicotianae* is also able to produce red-brown colored pigments in pure culture when hydrolyzed casein or sulphur-containing amino-acids are present in the medium.

Beside the coryneform bacteria also staphylococci are found on red smear cheeses. Staphylococci are typically isolated from the cheese surface in 0.1 to 5 % (e.g. Bockelmann 2002a; Brennan et al. 2002), but also in higher fractions (Hohenegger et al., submitted; Oberreuter et al. 2003; Rea et al., submitted). The most predominant species found on red

smear cheeses is *Staphylococcus equorum* (Bockelmann 2002a; Bockelmann and Hoppe-Seyler 2001; Brennan et al. 2004; Hoppe-Seyler et al. 2004). *St. saprophyticus*, a typical undesirable contaminant, also often occurs on smear cheese surfaces (Hoppe-Seyler et al. 2004; Rea et al., submitted). Species such as *St. epidermidis*, *St. hominis*, *St. sciuri*, *St. vitulinus*, *St. warneri* and *St. xylosus* are less frequently isolated (Bockelmann 2002a; Bockelmann et al. 1997b; Carnio et al. 1999; Irlinger et al. 1997; Rea et al., submitted). Due to their tolerance to low pH values staphylococci are also found on cheeses during the early stages of ripening (Bockelmann 2002a; Rea et al., submitted). In studies of Bockelmann et al. (1997a) staphylococci were numerously found in the brine bath which also showed their tolerance to high salt concentrations. Oberreuter et al. (2003) reported on staphylococci dominating the smear liquid flora of a Tilsit cheese.

Role of surface microbial consortia in the ripening of red smear cheeses

Due to the growth and metabolic activity of the microorganisms on the cheese surface, the ripening process is reduced from months to several weeks which is clearly accelerated compared to other types of cheeses (Bockelmann and Hoppe-Seyler 2001). Compared to semi-hard and hard varieties the greatest effect of the surface microorganisms on the ripening process is observed in soft cheeses (Reps 1987). Generally, the surface microflora influences appearance, flavor and texture of the smeared cheeses (Bockelmann and Hoppe-Seyler 2001; Reps 1987). The pigmentation of the microorganisms determines the color of red smear cheeses (Bockelmann and Hoppe-Seyler 2001; Brennan et al. 2004). Enzymes produced by the smear organisms as well as the resulting metabolites that penetrate the cheese body effect the flavor and the cheese quality in general (Kammerlehner 1986, 2003). The formation of an intact smear further protects against mould contamination (Kammerlehner 2003).

Since yeasts are able to grow at low pH they are the dominant organisms found on the cheese surface at the beginning of ripening (Eliskases-Lechner and Ginzinger 1995b; Fleet 1990; Reps 1987). Growth of acid-sensitive but salt-tolerant coryneform bacteria depends on a pH increase on the cheese surface from around 5.0 to around 6.0 which is the result of aerobic lactic acid assimilation and deamination of amino acids by yeast. Yeasts also produce compounds such as vitamins or amino acids stimulating the growth of ripening bacteria

(Brennan et al. 2004; Eliskases-Lechner and Ginzinger 1995b; Prillinger et al. 1999). Bacteria dominate the surface microbial consortia in the later stages of ripening when the pH has risen (Beresford and Williams 2004).

1.2 Taxonomy and identification of ripening organisms

Yeast

Yeasts are mainly unicellularly growing fungi with a diameter of five to ten µm (Barnett 1992; Krämer 1992). Generally, they prefer aerobic conditions for growth but - with only few exceptions - they are able to change their metabolism to fermentation under anaerobic conditions. In general, growth is observed within a wide range of pH (pH 3 to 11) and temperature (0 to 45° C). Some yeasts can still multiply at -10° C or at pH values of 1.5 (Krämer 1992). Most yeasts reproduce vegetatively by budding (anamorphic or imperfect forms), but there are also several yeasts for which a sexual reproduction has been reported (teleomorphic or perfect form) (Barnett 1992; Barnett et al. 2000). Depending on the environmental conditions, many yeasts are able to appear either as anamorphic or teleomorphic form which are genetically identical but differ in morphological criterions. Thus, the anamorphic and teleomorphic form have often been described as separate species.

Within the fungi, yeast belong to two phyla, the *Ascomycota* and the *Basidiomycota*, which are characterized by forming ascospores or basidiospores, respectively (Barnett et al. 2000). In the past, classification and identification of yeasts was mostly based on the microscopic appearance of the cells, mode of sexual reproduction, physiological activities and biochemical features (Barnett et al. 2000; Kreger-van Rij 1984). However, gene sequence analyses that are more and more used, show a conflict between the classical taxonomic structure and the placement of species on gene trees (Kurtzman and Robnett 2003). Thus, a reorganization of the yeast taxonomy is required. Kurtzman and Robnett (2003) already evaluated the taxonomy of the *Saccharomycetaceae* using multigene analyses resulting in several reassignments and the proposal of five new genera.

Since the classical phenotypic tests described by Barnett et al. (2000) and Kreger-van Rij (1984) are extremely laborious and time consuming, physiological patterns can be

determined in a simplified way by using commercially available kits such as API 20C and ID 32C, RapID Yeast Plus or the MicroScan system. They are working well for the identification of clinical isolates (Moghaddas et al. 1999; Ramani et al. 1998; St.-Germain and Beauchesne 1991), however they are less or not suitable for food relevant yeast (Arias et al. 2002; Deák and Beuchat 1995; Rohm et al. 1990; Török and King (Jr.) 1991). Fourier transform infrared (FTIR) spectroscopy, a further phenotypic method, is used to identify yeast by means of fingerprint-like patterns showing the total chemical composition of the individual organisms (Kümmerle et al. 1998; Wenning et al. 2002). Random amplified polymorphic DNA (RAPD) analysis and restriction fragment length polymorphism (RFLP) analysis were not only described as tools used to identify to species level but also to strain level since specific fragment patterns are generated (Andrighetto et al. 2000; Baleiras Couto et al. 1994; Dlauchy et al. 1999; Esteve-Zarzoso et al. 1999; Marcellino et al. 2001; Petersen et al. 2001; Petersen et al. 2002; Romano et al. 1996; Vasdinyei and Deak 2003). Further, analysis of the D1/D2 domain of the 26S rDNA allows a reliable identification at species level (Kurtzman and Robnett 1998).

Coryneform bacteria

Initially, all Gram-positive bacteria showing irregular cell morphology and a G+C content higher than 50 mol % were called coryneform bacteria (Buchanan et al. 1974; Funke et al. 1997). The name is derived from the Greek word coryne (club) since most of the coryneform bacteria are characterized by a club shaped cell morphology. Although the term "coryneform bacteria" is beyond the up-dated classification system (Stackebrandt et al. 1997) it is still commonly used (e.g. Bockelmann 2002a; Brennan et al. 2002; Brown et al. 2005; Daneshvar et al. 2004; Funke et al. 1998; Oberreuter et al. 2003). The so called snapping-divisions are also typical for this bacterial group. An irregular splitting of the cell walls during the cell division results in a V- or Y-shaped appearance (Holt et al. 1994; Schlegel 1992). Furthermore, many coryneform bacteria undergo a rod to cocci transformation during their cell cycle which is influenced by the cultural conditions, especially the composition of the medium (Cure and Keddie 1973). Generally, the classical coryneform bacteria grow aerobically, they are catalase-positive, non-spore-forming and non-partially acid-fast (Funke et al. 1997; Holt et al. 1994).

Stackebrandt et al. (1997) proposed a new hierarchic classification system for Gram-positive bacteria with high G+C content, in which phylogenetically neighboring taxa at the genus level are clustered into families, suborders, orders, subclasses, and a class based on 16S rDNA/rRNA sequence data. The hierarchical structure of the new class called *Actinobacteria* comprises five subclasses including several orders, suborders and families with the classical coryneform bacteria grouping into the suborders *Corynebacterineae* and *Micrococcineae* (Stackebrandt et al. 1997). With the addition of novel taxa to the suborder *Micrococcineae* this hierarchical system was restructured (Stackebrandt and Schumann 2000).

Identification and classification of coryneform bacteria proves to be rather difficult and labor-intensive. Classical methods include chemotaxonomic studies, e.g., analyses of polyamines (Altenburger et al. 1997; Busse and Schumann 1999), fatty acids (Bernard et al. 1994; Kämpfer and Kroppenstedt 1996) as well as mycolic acids (de Briel et al. 1992), and numerical taxonomic analysis based on physiological characteristics (Kämpfer et al. 1993; Seiler 1983). Commercial identification kits such as API (RAPID) Coryne (BioMerieux) and RapID CB Plus System (Remel Inc.) or MicroSeq 500 (Perkin Elmer) are particularly suitable for the identification of clinical isolates. Methods such as comparative 16S rRNA analyses (Bockelmann et al. 1997b) or the application of genus- and species-specific oligonucleotide probes are used for identifying coryneform bacteria on molecular level (Hou et al. 1997; Kollöffel et al. 1997). Amplified rDNA restriction analysis (ARDRA) is not only considered to be a suitable tool for differentiation but also for identification of these organisms (Hoppe-Seyler et al. 2003; Vaneechoutte et al. 1995). Strain typing of coryneform bacteria was successfully performed by pulsed field gel electrophoresis (PFGE) (Brennan et al. 2002). FTIR spectroscopy allows an identification and differentiation of coryneform bacteria with high accuracy. Due to its easy handling and cost efficiency it has clear advantages compared to other methods routinely used for identifying and differentiating coryneforms (Oberreuter et al. 2002b).

1.3 *Listeria*

Taxonomy, morphology and cultural characteristics

Six species currently belong to the genus *Listeria*: *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. ivanovii* and *L. grayi* (Vazquez-Boland et al. 2001) of which *L. monocytogenes* is the only human pathogenic species (Pschyrembel 1994). *L. monocytogenes* is a gram-positive, nonsporing, peritrichous flagellated, rod-shaped bacterium (0.4 – 0.5 x 0.5 - 2 µm) which is oxidase-negative and catalase-positive (Holt et al. 1994; Krämer 1992). It is ubiquitously found and grows under facultative anaerobic conditions (Holt et al. 1994). Growth occurs from 1 to 45° C, with a growth optimum between 30 and 37° C (Ryser and Marth 1991), and within a wide pH ranging from pH 5.0 to 9.5 (Krämer 1992). Further, *L. monocytogenes* is highly salt tolerant and able to grow at 10 % NaCl concentrations, some strains even at higher concentrations (Krämer 1992).

Intracellular infectious cycle

L. monocytogenes is able to invade host cells such as macrophages or nonprofessional phagocytes such as epithelial cells, fibroblasts, hepatocytes, endothelial cells and various types of nerve cells by induced phagocytosis. Due to the production of a hemolysin, designated listeriolysin O, and phospholipases they can leave the phagocytic vacuole and reach the cytoplasm where they multiply protected from any immune response. By polymerizing actin filaments *Listeria* are able to move around in the host cells until they get in contact with the surface of the host cell which induces the host cell membrane to produce extrusions that penetrate an uninfected neighboring cell. Phagocytosis of these ramifications including *L. monocytogenes* results in the formation of a two-membrane vacuole from which the *Listeria* invade the cytoplasm of the second host cell by lysing the membranes. This mechanism enables *L. monocytogenes* to spread from cell to cell and also to cross the intestinal, blood-brain and placental barrier without being detected by the cell′s defense mechanism (Dussurget et al. 2004; Hof 2003; Vazquez-Boland et al. 2001).

Human listeriosis

Human listeriosis is a food-borne disease that can be caused by *L. monocytogenes* of all 13 serovars which are 1/2 (a, b, c), 3 (a, b, c), 4 (a, ab, b, c, d, e) and 7 (Farber and Peterkin 1991; Hof 2003). However, serovars 1/2a, 1/2b and 4b account for most of the cases (Farber and Peterkin 1991; Vazquez-Boland et al. 2001). Although listeriosis is a very rare disease with an incidence of 6 cases / 10^6 inhabitants / year (Kayser et al. 1998), severe listeriosis are lethal in 20 to 50 % of the cases (Dussurget et al. 2004; Low and Donachie 1997; Vazquez-Boland et al. 2001). The minimum infectious dose is not known, but is considered to vary in a wide range depending on the *Listeria* strain, the host susceptibility and other factors (Farber and Peterkin 1991). Elderly, immunocompromised or debilitated adults, pregnant women and neonates are specifically susceptible for listeriosis (Vazquez-Boland et al. 2001). Compared to the normal population, pregnant women have a 12-fold increased risk to get listeriosis after consumption of contaminated food (Hof 2003). *Listeria* are able to cross the placental barrier in infected pregnant women which may cause an intrauterine infection of the fetus leading to abortion, stillbirth, premature birth or birth of a baby with a generalized infection. In the mother, however, the infection is often asymptomatic or it takes a flu-like course (Hof 2003; Krämer 1992; Vazquez-Boland et al. 2001). Listeriosis in nonpregnant people often manifests itself as sepsis, meningitis and/or encephalitis but also many other clinical manifestations have been observed. In contrast to this, most immunocompetent people overcome an initial attack and shedding of *Listeria* through the feces stops after a few days (Hof 2003).

Incidence of *L. monocytogenes* in food, especially in smear-ripened cheese

L. monocytogenes is widely distributed in the environment. Therefore it is also often found on food, such as meat, poultry, egg products, vegetables, seafood and dairy products (Farber and Peterkin 1991; Krämer 1992). A lot of worldwide listeriosis outbreaks have been associated with contaminated food (for overview to outbreaks of human food-borne listeriosis from 1976 – 2001 see McLauchlin et al. (2004)). Generally, not only raw food but also processed food is concerned in listerial contaminations (Bereksi et al. 2002). The ability of *L. monocytogenes* to grow at refrigeration temperature (Walker et al. 1990), in low pH environments (Bereksi et al. 2002; Cotter and Hill 2003), in presence of high sodium chloride con-

centrations (Bereksi et al. 2002) and on dry surfaces (Lee Wong 1998) makes food preservation difficult (Barker and Park 2001).

Among dairy products cheeses are often contaminated with *Listeria* (Farber and Peterkin 1991; McLauchlin et al. 2004; Ryser and Marth 1991). Studies of Terplan et al. (1986) showed that a higher contamination frequency occurs in red smear cheeses compared to other cheeses. Rudolf and Scherer (2001) also reported about a particularly high incidence of *L. monocytogenes* in red smear cheeses, even when cheeses were made from pasteurized milk. *Listeria* may colonize food processing factories by chronic carriers, carry-over infections or due to lacking hygienic steps in the dairy; by faulty or insufficiently sanitized equipment they are brought in the food products (Hof 2003; Loessner and Scherer 2002). Floors, floor drains or sites that are difficult to clean or to sanitize are often found to be a reservoir for *Listeria* strains which are able to become a member of the resident microbial flora of food processing facilities over several months or years (Loessner and Scherer 2002; Tompkin 2002; Zhao et al. 2004). The growth of smear-ripening cultures on the cheese surface rises the pH in the cheese rind very fast which causes ideal conditions for listerial multiplication (Krämer 1992). *Listeria* are found to be especially localized on the cheese surface because of the pH gradient which develops in these cheeses (Farber and Peterkin 1991). The traditional old-young smearing including frequent handlings and washings still used in red smear cheese production often leads to contaminations with *L. monocytogenes* (Loessner et al. 2003). If the brine solution is contaminated not only the desired smear flora but also the pathogens are transferred from one cheese batch to another and therefore *Listeria* spread over the whole cheese production (Bockelmann and Hoppe-Seyler 2001; Eppert et al. 1997). High-moisture cheeses - soft or semi-soft cheeses - are more exposed to listerial contamination (Rudolf and Scherer 2001; Ryser and Marth 1991).

1.4 Biological preservation of food

Outbreaks of food-borne diseases such as listeriosis are repeatedly noticed all around the world (McLauchlin et al. 2004) which shows that food safety is of worldwide interest and points out the necessity of adequate methods for food preservation. However, consumers increasingly prefer more natural and minimally processed food as well as light and mild

products which are characterized by low sugar, fat and acid contents (Cleveland et al. 2001; Holzapfel et al. 1995; Leistner and Gorris 1995). This poses several food safety risks since these products are especially susceptible for contaminations with pathogens or spoilage organisms (Holzapfel et al. 1995). On the other hand, consumers set more and more store by producing food without any chemical preservatives which increases the interest in biological preservation (Cleveland et al. 2001; Holzapfel et al. 1995; Leistner and Gorris 1995). Biopreservation by protective cultures is considered to be a promising concept to improve food safety without changing the sensory quality of a product (Bredholt et al. 2001; Holzapfel et al. 1995; Vermeiren et al. 2004). The anti-microbial effect of protective cultures is caused by a pH reduction due to the production of organic acids such as lactic acid, by hydrogen peroxide, by enzymes such as lysozyme, by low-molecular metabolites such as fatty acids, by bacteriocins such as nisin or by competition for nutrients. For improving food safety and assuring food quality it is not enough to rely solely on protective cultures, but their implementation should rather support safety concepts such as good manufacturing practice (GMP) or HACCP to reduce risks of growth and survival of pathogens and spoilage microorganisms (Holzapfel et al. 1995). Protective cultures or their metabolic products such as bacteriocins can be applied as additional hurdles in food preservation concepts by hurdle technology in which several preservative factors are combined to guarantee a gentle but effective preservation (Cleveland et al. 2001; Leistner 2000; Leistner and Gorris 1995).

Most often lactic acid bacteria (LAB) are used as protective cultures since they are traditionally involved in many food fermentations (e.g., Abee et al. 1995; Bredholt et al. 2001; Holzapfel et al. 1995; Loessner et al. 2003; Ryan et al. 1996; Vermeiren et al. 2004). However, also ripening organisms found on the surface of red smear cheeses have characteristics required for protective cultures: food-grade safety, adaptation and suitability for a specific food system and the occurrence of antagonistic effects, especially in ripening bacteria, against pathogens such as *Listeria* (Carnio et al. 1999; Eppert et al. 1997; Holzapfel et al. 1995; Ryser et al. 1994; Valdés-Stauber et al. 1991). Details about anti-bacterial and especially anti-listerial properties in ripening organisms are presented in the following chapter.

1.5 Anti-bacterial, in particular, anti-listerial activity of smear-ripening organisms

Bacteria

Lactic acid bacteria (LAB) are known for the production of inhibitory substances, especially bacteriocins (e.g. Barefoot and Klaenhammer 1983; Eijsink et al. 1998; Ennahar et al. 2000; Klaenhammer 1993; Ryan et al. 1996), some of which inhibit *Listeria* (e.g. Ennahar et al. 1996; Loessner et al. 2003; Maisnier-Patin et al. 1996; Ryan et al. 1996; Torri Tarelli et al. 1994). Compared to this, the knowledge about smear-ripening bacteria that have anti-bacterial or particularly anti-listerial properties is somewhat limited. In order to develop potential counter measures against *Listeria* in red smear cheese production, anti-listerial actions exerted by ripening bacteria have been studied in some detail (Carnio et al. 1999; Carnio et al. 2000; Eppert et al. 1997; Maisnier-Patin and Richard 1995; Ryser et al. 1994; Valdés-Stauber et al. 1991; Valdés-Stauber and Scherer 1994). In some cases, the anti-listerial effect was assigned to bacteriocins (Motta and Brandelli 2002; Valdés-Stauber and Scherer 1994) or bacteriocin-like substances (Boucabeille et al. 1997; Ryser et al. 1994). So far, linocin M18 is the only bacteriocin produced by a *B. linens* strain that has been characterized at molecular level (Valdés-Stauber and Scherer 1994). Micrococcin P_1, a macrocyclic peptide antibiotic, was found to be secreted by a *St. equorum* strain isolated from Raclette cheese (Carnio et al. 2000). In other cases, the character of the anti-listerial substances produced by different ripening bacteria, or even details about the molecular basis of the inhibitory action remain unresolved (Carnio et al. 1999; Valdés-Stauber et al. 1991). Unknown factors inhibiting *Listeria*, such as competitive or symbiotic interactions within the complex cheese smear ecosystem, may also exist (Eppert et al. 1997).

Different methods have been used to screen for anti-listerial activity in ripening bacteria: An agar disc diffusion assay was performed by Motta and Brandelli (2002) in which a disc soaked with a cell free culture supernatant fluid was applied to an agar surface previously inoculated with a *Listeria* indicator strain. After incubation inhibition zones were evaluated. An agar diffusion assay was also performed by Valdés-Stauber et al. (1991). However, in this case, sterile filtered supernatants of the organisms to be tested for their anti-listerial activity were filled into wells punched in solidified agar which was previously inoculated with a *Listeria*

strain. Ryser et al. (1994) detected anti-listerial effects of ripening bacteria by a hydrophobic grid membrane filter (HGMF) technique. Cheese smear suspensions were filtered through HGMF membranes on which the ripening bacteria were grown by applying the membrane onto an appropriate solid medium. The membrane with the bacteria grown on its surface was removed after the incubation and the agar overlayed with soft agar containing a *Listeria* indicator strain. Inhibition zones, caused by inhibitory substances released to the agar, were examined visually after further incubation. Carnio et al. (1999) modified this method as they transferred the membrane, on which the smear organisms were grown, to a soft agar inoculated with a *Listeria* indicator strain. Due to the grid structure on the membrane the inhibition can easily be attributed to the respective colony which allows the screening of a high number of isolates at the same time. To test for inhibitory activity in individual strains or for anti-listerial substances released to the growth medium, suspensions or sterile-filtered samples of the test organisms can be spotted onto soft agar inoculated with an indicator strain or onto a lawn of indicator cells before incubation (Carnio et al. 1999; Valdés-Stauber and Scherer 1994). The anti-listerial potential of ripening organisms was also evaluated *in situ* on model cheeses inoculated with *Listeria* in different studies. Eppert et al. (1997) established cheese-ripening experiments under laboratory conditions using a single-strain culture of *B. linens* M18, a producer of bacteriocin linocin M18. A significant reduction of *Listeria* cells on these cheeses was detected compared to the control. Studies of Carnio et al. (2000) with a single-strain culture of a *St. equorum* strain that secreted Micrococcin P_1 revealed similar results. Cheese-ripening experiments were also done with different undefined, complex industrial red smear cheese floras (Eppert et al. 1997; Maoz et al. 2003). Depending on the ripening culture the effect on the multiplication of the *Listeria* indicator strain varied extremely. The *in situ* experiments clearly show the suitability of ripening bacteria as protective cultures on smear-ripened cheeses.

Yeast

Growth and, therefore, bacteriocin production of cheese ripening bacteria depends on a pH increase on the cheese surface from around 5.0 to around 6.0 which is the result of aerobic lactic acid assimilation by yeast, which form an important part of the surface microbial consortia of red smear cheeses (Eliskases-Lechner and Ginzinger 1995b; Reps 1987). Thus,

anti-listerial substances produced by ripening bacteria become only effective after the bacterial members of the consortia develop. This is a potential problem since *L. monocytogenes* is more pH tolerant than the ripening bacteria and grows even at pH values of around 5.0 (Farber and Peterkin 1991). During the early stages of ripening, yeasts dominate the microbial flora of red smear cheeses due to their pH tolerance (Eliskases-Lechner and Ginzinger 1995b). Therefore, it would be reasonable to search for yeast demonstrating anti-listerial activity to combat *Listeria* in its initial stages of multiplication on cheese.

Generally, antagonistic effects of yeasts against other yeasts are well known. The production of killer toxins was already recognized in the 1960s (Magliani et al. 1997). Killer toxins were described as exotoxins lethal against susceptible strains of the same or congeneric species whereas the toxin-producing yeast itself is immune against its own killer toxin (Magliani et al. 1997; Polonelli and Morace 1986). Polonelli and Morace (1986) also reported on a killer phenomenon directed against unrelated microorganisms, among others bacteria. However, in their study a screening medium supplemented with methylene blue was used and Bilinski et al. (1985) demonstrated a correlation between the anti-bacterial activity in yeast and the use of methylene blue in the medium. There exist some older clinical studies which describe an anti-bacterial activity of yeasts (Budak et al. 1982; Hipp et al. 1974; Provost et al. 1995; Wallin and Gnarpe 1975). However, until now little effort has been put into investigating yeasts isolated from food or food environments for an inhibitory potential against pathogenic bacteria, such as *L. monocytogenes*. To our information there are only two studies reporting on this topic. One deals with biofilms from floor drains at different food processing plants (Zhao et al. 2004). Three out of 156 non-identified yeast isolates showed a very low anti-listerial activity. In liquid medium listerial growth was repressed by 0.7 $\log_{10}$ cfu/ml and in a biofilm on stainless steel by 0.2 and 0.5 $\log_{10}$ cfu/cm^2, but no further details were reported. Dieuleveux et al. (1997) described an anti-listerial *Geotrichum candidum* strain which was isolated from a French red smear cheese.

1.6 Objective

The EU project "Biodiversity and anti-listerial activity of surface microbial consortia from Limburger, Reblochon, Livarot, Tilsit and Gubbeen cheese" was initiated to characterize and identify yeasts and bacteria present in the smears of the five cheeses at different stages of ripening using a polyphasic approach and to screen for anti-listerial activity of yeast originating from these cheese surfaces. The main emphasis of the present work within this project was to study the biodiversity of yeast populations and the anti-listerial properties of yeast.

The composition and similarity of yeast microfloras from all five cheeses were determined using FTIR spectroscopy. Based on FTIR data as well as physiological tests the interspecies and intraspecies diversity of the Limburger yeast flora of different batches at several ripening stages was evaluated in more detail. FTIR measurements were also performed on a selection of Limburger bacteria but further data on bacterial isolates from Limburger cheese were supplied by different project partners. All results, however, were put together and interpreted in the present work to evaluate the overall biodiversity.

For the production of the same Limburger cheese a commercial smear starter culture was used. The incidence of the commercial smear starter microorganisms was examined during the ripening of four Limburger batches. These analyses were done in cooperation with two research groups that have also been involved in the mentioned EU project.

At the beginning of the EU project no screening method was available to detect yeast with anti-listerial properties. Therefore, a suitable assay was developed for screening a high number of isolates. Since *Listeria* and yeast were separated from each other by a membrane in the resulting assay, a co-cultivation assay was additionally developed to test the anti-listerial activity of yeast in direct cell contact with *Listeria* as it is found on cheese surfaces.

2 Materials and methods

2.1 Determination of yeast populations from different European smear-ripened cheeses

Yeasts from five cheeses produced in four different countries were investigated within the SCM EU project "Biodiversity and anti-listerial activity of surface microbial consortia from Limburger, Reblochon, Livarot, Tilsit and Gubbeen cheese". Yeast isolates were collected from three cheese batches, as in Limburger cheese, or from cheeses sampled in three different dairies, as in Reblochon and Livarot cheese. Gubbeen cheese yeasts were obtained from a total of six batches where the second three batches were produced around six years after the first three batches. Tilsit cheese yeasts were isolated from cheeses obtained from 15 dairies. For details see Table 1.

Table 1: European red smear cheeses investigated

Cheese	Country	Source of cheese samples	No. of isolates
Limburger	Germany	Batches 1 - 3	469*
Reblochon	France	Dairies 1 - 3	404
Livarot	France	Dairies 1 - 3	457
Tilsit	Austria	Dairies 1 - 15	325
Gubbeen I	Ireland	Batches 1 - 3	386
Gubbeen II	Ireland	Batches 4 - 6	450
Total			2491

*Subcultures of the initial 450 yeasts that showed different colony morphologies were investigated separately

Strains were isolated by the individual partners (for Limburger see chapter 2.3) and sent either as glycerol stocks or stab cultures for identification and differentiation by Fourier transform infrared (FTIR) spectroscopy. Some isolates of Livarot, Tilsit and Gubbeen cheese were also used in screenings for anti-listerial activity (see chapter 2.9).

2.2 Production of Limburger cheese

The Limburger cheese investigated in this study was produced by a German dairy that uses pasteurized milk and a thermophilic lactic acid starter culture. A commercial smear starter culture containing *D. hansenii*, *Geotrichum candidum,* the anamorphic form of *G. geotrichum*, *A. nicotianae* and *B. linens* was added to the cheese milk. Within the production period of about 14 days (Figure 1), cheeses were smeared four times. Traditional old-young smearing was modified as the first smearing of a batch followed the second smearing of the previous produced one. Once packaged, cheeses were ready for sale. The expiry date was set about 60 days after the production of the green cheeses.

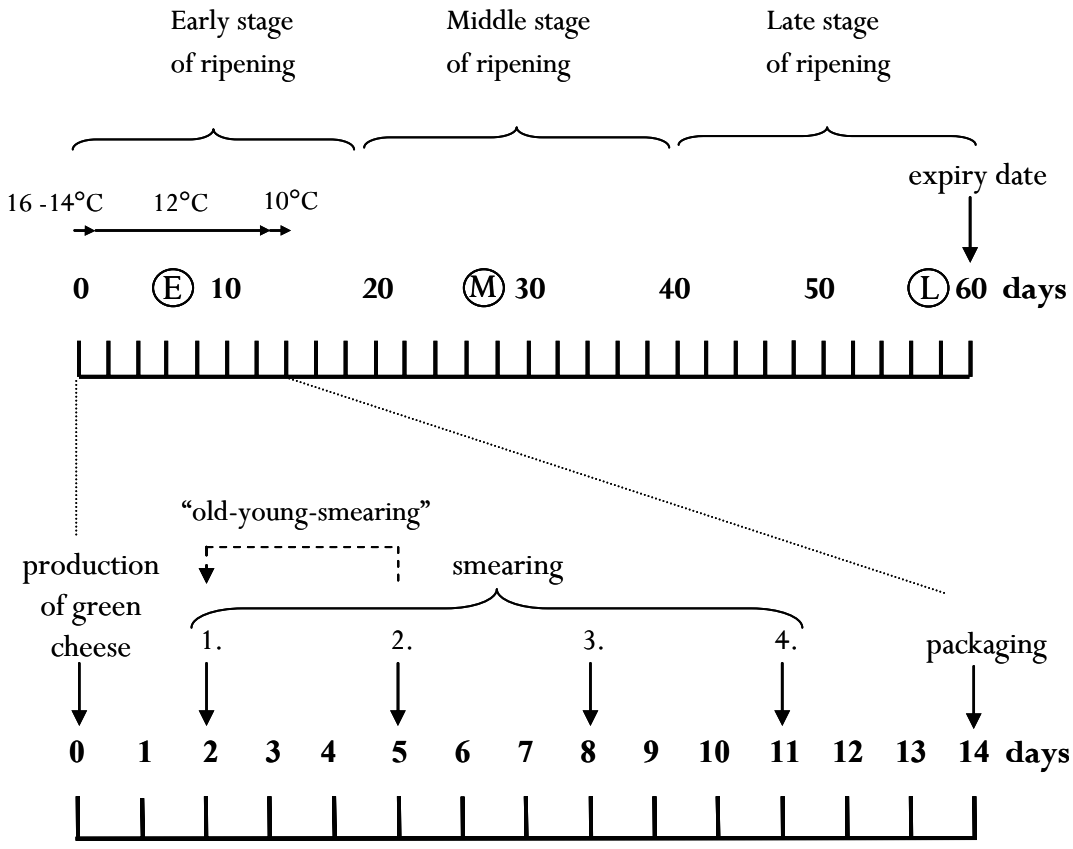

Figure 1: Production and ripening scheme for Limburger cheese. Cheeses are smeared four times and packaged between day 11 and 14. Sampling points: (E) after second smearing; (M) around day 28; (L) about two days before the expiry date.

2.3 Isolating microorganisms from the surface of Limburger cheeses

The microflora of three Limburger batches produced at five to six weeks intervals were investigated for their biodiversity. To examine the incidence of the commercial smear starter organisms in the microbial smear composition, a fourth Limburger batch was used that was produced approximately three years later than the other three batches. All cheeses were frozen at -70° C to preserve their status of ripening for different investigations. Fifty cm^2 of cheese surface was cut with a sterile knife, tenfold diluted with tri-sodium citrate buffer (2 g/100 ml), pH 7.5, and homogenized. Liquid samples were serially diluted and plated out. Yeasts were grown on yeast extract glucose chloramphenicol agar (YGCA; Merck, Darmstadt, Germany) supplemented with 10 mg/l bromophenol blue (YGCBA; Seiler 1991). Bacteria of batches 1 to 3 were cultivated on plate count agar containing 3 % NaCl (PCA^{3+}):

tryptone	5.0 g
glucose	1.0 g
yeast extract	2.5 g
NaCl	30.0 g
agar	15.0 g
distilled water	1.0 l

adjusted to pH 7.0
autoclaved at 121° C for 15 min

Since experience showed that several coryneform bacteria prefer to be cultivated on tryptic soy agar (TSA, Roth) rather than on PCA^{3+}, both media were used for isolating coryneforms from cheese smears of the fourth batch. Development of yeast and fungi was suppressed by spreading 100 µl 2 % pimaricin (approx. 2.5 % aqueous suspension, Sigma) on the surface of each PCA^{3+} or TSA plate before use. Viable yeast and bacteria counts were determined after a 5 d incubation at 20° C under aerobic conditions. The isolates were subcultured on YGCBA and PCA^{3+} lacking pimaricin.

Yeasts of the first three Limburger batches were isolated from cheeses at early (6 d), middle (28 d) and late (58 d) stages of ripening. Fifty yeasts were collected from appropriate dilutions of each cheese sample yielding a total of 450 yeasts. In the fourth batch yeasts were obtained from cheese smears after the first (3 d; 50 isolates), second (6 d; 50 isolates) and

third smearing (9 d; 20 isolates) as well as from a cheese after 28 d of ripening (50 isolates) yielding a total of 170 strains. Isolation of bacteria was generally focused on coryneforms and Gram-positive, catalase-positive cocci. In Batches 1 to 3 the same cheese samples were used as selected for the yeast to isolate fifty bacteria from appropriate dilutions of each cheese sample resulting in a total of 450 isolates. Isolating bacteria from cheese surfaces of the fourth batch started with a cheese after the second smearing of which 50 isolates were picked from TSA plates. After the third smearing 11 coryneforms were isolated from countable dilutions on PCA^{3+}. An additional isolation of coryneform bacteria from a plate on which the lowest dilution of the cheese rind homogenate was plated should help to record strains that were present in low numbers onto the cheese surface. Since it is difficult to pick colonies from these dilutions a spatula was touched onto the nearly overgrown agar plate and the cell material was spread onto a fresh PCA^{3+} plate. Using a fresh spatula, cell material from this agar surface was transferred onto another PCA^{3+} plate just as described before. This procedure was repeated further two times to achieve a dilution series. Plates were then incubated at 30° C for 3 d. After the incubation, 50 coryneform bacteria were isolated from an appropriate dilution. Additionally, 50 isolates were collected from a cheese smear of the same ripening stage prepared in the conventional way on TSA. From a cheese smear after 28 d of ripening, 50 coryneform bacteria were isolated. Dilutions of this homogenized cheese rind have been streaked on PCA^{3+}. A total of 211 coryneforms resulted, but the sampling was extended for further investigations by isolating additional 25 coryneform bacteria from each of the above mentioned cheese preparations apart from the preparation made from the cheese sample after the third smearing on PCA^{3+}, hence yielding a total of 311 isolates.

2.4 Isolating microorganisms from a commercial smear starter culture

The freeze-dried commercial smear starter mixed culture used to produce the Limburger cheese was investigated in this study. For the isolation of the individual organisms one spatula of powder was added to 1 ml of yeast extract glucose broth (YGB):

yeast extract (Oxoid)	5.0 g
glucose (Fluka)	20.0 g
distilled water	1.0 l
adjusted to pH 6.6 with HCl	
autoclaved at 121° C for 15 min	

The suspension was held at refrigeration temperature for around two hours to give the organisms time for regeneration. After that time the suspension was tenfold diluted and aliquots of appropriate dilutions were plated in duplicates on YGCBA for yeast isolation or on PCA^{3+} with 100 µl pimaricin spread onto the agar surface for collecting bacteria. From each of the four organisms – *D. hansenii*, *Geotrichum candidum, A. nicotianae* and *B. linens* - included in the commercial smear starter preparation, 50 colonies were picked.

2.5 Cultivation and maintenance

Isolates were stored in glycerol stocks. Cell material of a fresh yeast or bacterial culture grown on YGCBA or PCA^{3+} was suspended in 5 ml of the following suspension medium:

sodium glutamate	10,0 g
lactose x H_2O	16,0 g
agar	1,0 g
ascorbic acid	0,1 g
glycerol	120,0 g
tap water	ad 1.0 l
autoclaved at 121° C for 15 min.	

Aliquots of the inoculated suspension medium were filled in cryotubes. Before freezing at -80° C cultures were incubated overnight at refrigeration temperature to improve the uptake of glycerol and other substances in the cells for better protection.

Additionally, representative bacterial strains have been deposited in the public BCCM/LMG Bacteria Collection (http://www.belspo.be/bccm/index.htm). For this purpose, strains were grown on tryptic soy broth (TSB, BBL, Sparks, USA) supplemented with 1.5 % agar at 30° C for 2-3 days and stored at -80° C. Representative yeast strains were freeze-dried and deposited in the Weihenstephan Yeast Collection.

2.6 Fourier transform infrared (FTIR) spectroscopy

Sample preparation

Sample preparation for yeast was performed according to Kümmerle et al. (1998). Several colonies of a yeast culture grown on YGCBA were streaked as a lawn onto one third of a YGCA (Merck) plate using a Drigalski spatula. After incubation at 27° C for 24 h +/- 0.5 h one loop of cell material (1 mm in diameter) was suspended in 100 µl distilled water. 35 µl of this suspension were applied onto one of the 15 sample positions of a ZnSe-sample wheel and dried for around 50 min at 42° C.

Cultivation of coryneform bacteria as cell lawns was done on TSA (Oxoid) for 24 h +/- 0.5 h at 30° C as described by Oberreuter et al. (2002b). One loop of material (2 mm in diameter) was suspended in 100 µl distilled water and then an aliquot of 35 µl was applied onto a ZeSe-sample wheel and dried.

Measurement and evaluation

Yeast spectra were recorded and evaluated according to Kümmerle et al. (1998) using an IFS-28B FTIR spectrometer and the software OPUS 3.17 for Windows (both Bruker, Karlsruhe, Germany). Identification of the isolates was achieved by comparing the second derivative of the original spectra - smoothed by a nine-point- polynome - against around 2500 yeast spectra held in reference databases. Three spectral windows, all with the weight of one and a reproducibility of 30, were selected (Table 2). According to the weights and reproducibility levels a yeast isolate was considered to be identified if the spectral distance (hit quality) was below 1.0.

Table 2: Spectral windows, weights and reproducibility levels for the identification of yeasts

Spectral window		Weight	Reproducibility level
3030 - 2830	Fatty acid region	1	30
1350 - 1200	"Mixed" region	1	30
900 - 700	Fingerprint region	1	30

The second derivative of the spectra as well as the relevant frequency ranges (spectral windows) were also used for cluster analyses.

Coryneform bacteria spectra were recorded and evaluated according to Oberreuter et al. (2002b). For identification and cluster analysis the first derivative was calculated with nine smooting points and the following spectral windows, weights and reproducibility levels were used:

Table 3: Spectral windows, weights and reproducibility levels for the identification of coryneform bacteria

Spectral window		Weight	Reproducibility level
3.000 – 2.800	Fatty acid region	0.8	3.3
1.800 – 1.500	Amide region	0.8	5
1.500 – 1.200	"Mixed" region	0.9	20
1.200 - 900	Polysaccharide region	0.9	33
900 - 700	"Fingerprint" region	0.9	116

The reference database applied for the identification of coryneform bacteria comprised around 1200 spectra of coryneform bacteria and staphylococci. A valid identification required a spectral distance of below 1.5 between the measured and the reference spectrum.

2.7 Physiological tests on yeasts

Additionally to FTIR spectroscopy classical physiological tests were used for identification. Tests were performed according to the methods of Barnett et al. (2000), as modified by Seiler and Busse (1988). Growth on several carbon and nitrogen substrates as well as growth without vitamins or thiamine were tested in microplates. In the same way, urea hydrolysis and the resistance to cycloheximide was investigated.

Suspension medium

purified agar (Oxoid)	0.10 g
Na_2HPO_4	0.09 g
H_2PO_4	0.32 g
distilled water	100 ml

adjusted to pH 6.2
aliquots of 10 ml, filled in tubes
autoclaved at 121° C for 15 min

Culture medium: Test for growth on carbon substrates

yeast nitrogen base (Difco)	2.0 g
carbon substrate (see below)	2.5 g
chloramphenicol, 0.1 % (w/v)	10 ml
distilled water	90 ml

adjusted to pH 6.0 – 6.4
filter-sterilized

Carbon substrates

D-glucose, D-galactose, L-sorbose, D-glucosamine, D-ribose, D-xylose, L-arabinose, D-arabinose, L-rhamnose, sucrose, maltose, α-α-trehalose, α-D-methyl-D-glucoside, cellobiose, salicin, arbutin, melibiose, lactose, raffinose, melizitose, glycerol, erythritol, ribitol, xylitol, L-arabinitol, D-glucitol, D-mannitol, galactitol, myo-inositol, D-glucono-1,5-lactone, 2-keto-D-gluconate, 5-keto-D-gluconate, D-gluconate, D-glucuronate, DL-lactate, succinate, citrate

Culture medium: Test for growth on nitrogen substrates

yeast carbon base (Difco)	2.0 g
nitrogen substrate (see below)	0.4 g
chloramphenicol, 0.1 % (w/v)	10 ml
distilled water	90 ml

adjusted to pH 6.0 – 6.4
filter-sterilized

Nitrogen substrates

nitrite, ethylamine HCl, L-lysine, cadaverine, D-glucosamine

Culture medium: Test for growth without vitamins

vitamin-free yeast base (Difco)	4 ml
chloramphenicol, 0.1 % (w/v)	10 ml
distilled water	90 ml
filter-sterilized	

Culture medium: Test for growth without thiamine

vitamin-free yeast base (Difco)	4 ml
chloramphenicol, 0.1 % (w/v)	10 ml
vitamin solution (see below)	0.1 ml
distilled water	90 ml
filter-sterilized	

Vitamin solution

p-aminobenzoic acid	1.0 g
biotin	0.1 g
folic acid	0.01 g
m-inositol	0.05 g
nicotine acid	2.0 g
panthotenate (Ca)	0.01 g
pyridoxine HCl	2.0 g
riboflavin	1.0 g
distilled water	1.0 l
filter-sterilized	

Culture medium: Test for urea hydrolysis

yeast extract	0.1 g
tryptone	0.5 g
glucose	0.5 g
KH_2PO_4	1.0 g
urea	2.5 g
aqueous phenol red solution, 0.1 % (w/v)	10 ml
chloramphenicol, 0.1 % (w/v)	10 ml
distilled water	80 ml
adjusted to pH 5.5	
filter-sterilized	

Culture medium: Test for 0.01 or 0.1 % cycloheximide resistance

yeast nitrogen base (Difco)	2.0 g
glucose	2.5 g
cycloheximide	0.5 or 0.05 g
distilled water	100 ml
filter-sterilized	

Twenty five microliters of the appropriate culture medium was added to the wells of a 96 well microtiter plate. One colony from a fresh culture was suspended in 10 ml of suspension medium. Aliquots (100 µl) of the resultant suspensions were dispensed into each well and the microplates covered with a transparent, oxygen impermeable tape and then with lids. The microplates were then shaken for 1-2 minutes and incubated at 27° C. Growth responses were evaluated after 1, 2, 3 and 4 weeks by determining the turbidity within the wells.

The ability to ferment D-glucose was tested in tubes containing an inverted Durham tube. Each test tube was filled with 5 ml of the following medium:

yeast extract (Oxoid)	0.5 g
D-glucose (Fluka)	2.0 g
distilled water	100 ml
adjusted to pH 5.5	
autoclaved at 121° C for 15 min.	

The resultant sterile preparations were each inoculated with 200 µl of the yeast suspension. The tubes were incubated for 5 days at 27° C then examined for the presence of gas in the Durham tube.

2.8 Sequence analysis of the D1/D2 domain of the 26S rDNA

Yeast strains of questionable identity were subjected to a sequence analysis of the 26S rDNA D1/D2 domain. One loop (1 mm in diameter) of cell material of yeast cultures grown on YGCBA for 48 h at 27° C was suspended in 200 ml sterile Milli-Q-Water (Millipore). For this purpose special micro tubes with screw cap (Sarstedt) were used, filled with several zirconium-silica beads (Roth; 0,5 mm diameter). Cell lysis was performed by a RiboLyser (Hybaid). Two runs after another were done, each for 45 sec with 6.5 m/sec. After that

samples were centrifuged at full speed for 3 min. Supernatant was transferred into a new tube and cooled on ice water until use.

The PCR was performed using a 50 µl reaction mix consisting of 8 µl lysate, 0.5 µl of each primer (50 pmol/µl) called NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG) and NL-4 (5′-GGTCCGTGTTTCAAGACGG) (Kurtzman and Robnett 1998), 25 µl of "ABgene Reddy Mix PCR Master Mix" (ABgene®) and 16 µl sterile Milli-Q-Water (Millipore). The PCR conditions were chosen as follows (Oberreuter et al. 2002b): 1 cycle at 95° C for 5 min, 30 cycles consisting of 95° C for 20 sec, 55° C for 40 sec and 72° C for 2 min, and finally 1 cycle at 72° C for further 2 min. The presence of PCR product was checked in a 1 % agarose gel at 90 V for about 40 min. The amplified fragments were stained with ethidium bromide and visualized by a Pharmacia Image Master (Amersham Pharmacia Biotech, Freiburg). After purifying the DNA by using the QIAquick PCR purification kit (Qiagen) following the manufacturer′s instructions the cycle-sequencing PCR was performed by an external sequencing service. A comparative sequence analysis was done using the database GenBank (http://www.ncbi.nlm.nih.gov).

2.9 Screening for anti-listerial activity in food-borne yeast

L. monocytogenes indicator strains and cultivation

L. monocytogenes strains WSLC 1001, 1039, 1211, 1364, 1416 and 1685 were selected from the Weihenstephan strain collection to serve as indicator strains in the agar-membrane screening assay (Table 4). For the co-cultivation experiment *L. monocytogenes* WSLC 1364 was used.

Table 4: *L. monocytogenes* indicator strains. WSLC: Weihenstephan *Listeria* collection, Abteilung Mikrobiologie, ZIEL Weihenstephan, Freising, Germany; ATCC: American Type Culture Collection, Manassas, Va. **L. monocytogenes* Scott A.

Strain	ATCC number	Serovar	Source
WSLC 1001	ATCC 19112	1/2c	spinal fluid
WSLC 1039	ATCC 13932	4b	spinal fluid
WSLC 1211		3a	cheese
WSLC 1364		4b	cheese
WSLC 1416		1/2a	cheese
WSLC 1685*		4b	human isolate

Five milliliters of brain heart infusion (BHI; Merck) was inoculated with *L. monocytogenes* cells from an overnight culture grown on a BHI agar plate (BHI containing 13 g agar (Oxoid) per liter) and incubated at 30° C for 17 - 18 h using a shaker at 180 rpm.

Yeast strains and cultivation

A total of 413 yeasts were screened for their anti-listerial potential using an agar-membrane screening assay (Table 5). One hundred out of these, isolated from different sources but mainly from dairy products, were selected from the Weihenstephan yeast collection (appendix, Table 1), 100 were collected from a German Almkäse, 87 from a German Limburger cheese, three isolates each from a Gubbeen and Livarot cheese, one isolate each from a Tilsit cheese, a Graukäse and another Austrian smear ripened cheese and 117 from 19 different European smear-ripened cheeses (appendix, Tables 2, 3). The yeast isolates were identified using FTIR spectroscopy (Kümmerle et al. 1998; see chapter 2.6), and, where necessary, by physiological tests according to Barnett et al. (2000), modified by Seiler and Busse (1988) (see chapter 2.7), as well as by sequence analysis of the 26S rDNA D1/D2 domain (Kurtzman and Robnett 1998) using a PCR protocol described by Oberreuter et al. (2002b) (see chapter 2.8). Fourteen yeasts out of the latter 117 set were co-cultivated with *L. monocytogenes* WSLC 1364 and, additionally, were tested for inhibitory activity against the same *L. monocytogenes* strain using an assay which was developed by Provost et al. (1995) to type species of the *Nocardia asteroides* complex by a yeast killer system.

Table 5: Yeast species investigated for their anti-listerial activity by the agar-membrane screening assay

Species	No. of isolates
Candida anglica	3
Candida catenulata	5
Candida ethanolica	1
Candida glabrata	1
Candida intermedia	21
Candida magnoliae	1
Candida maltosa	3
Candida parapsilosis	4
Candida tropicalis	1
Candida zeylanoides	6
Clavispora lusitaniae	44
Debaryomyces hansenii	207
Dekkera anomala	1
Galactomyces geotrichum	24
Hanseniaspora uvarum	1
Issatchenkia occidentalis	3
Issatchenkia orientalis	3
Kluyveromyces lactis	8
Kluyveromyces marxianus	8
Pichia anomala	5
Pichia cactophila	1
Pichia guilliermondii	1
Pichia jadinii	5
Pichia membranifaciens	5
Pichia triangularis	6
Saccharomyces castellii	1
Saccharomyces cerevisiae	1
Saccharomyces dairenensis	1
Saccharomyces unisporus	2
Torulaspora delbrueckii	5
Trichosporon beigelii	4
Trichosporon ovoides	7
Williopsis californica	1
Yarrowia lipolytica	13
Not identified cheese yeasts	10
Total	413

For the preculture one loop (2 mm in diameter) of cells of a fresh culture grown on YGCBA (see chapter 2.3) was inoculated in 5 ml of yeast extract glucose broth (YGB; see chapter 2.4) and incubated at 27° C for 24 h using a shaker at 180 rpm. To prepare the main culture,

50 µl of the incubated preculture were transferred to 5 ml YGB prewarmed to 27° C. Incubation conditions were used as described for the preculture. The main culture, in which cell numbers of 10^7 to 10^8 cfu/ml were reached, was either spotted onto the membrane in the agar-membrane screening assay or mixed with a *L. monocytogenes* culture for the co-cultivation assay.

Agar-membrane screening assay

100 µl of an overnight culture of a *Listeria* indicator strain were mixed with 10 ml of melted yeast extract glucose soft agar containing 1 % tryptone (YG^+SA) which was kept warm at 50° C until use. Ingredients of YG^+SA are:

yeast extract (Oxoid)	5.0 g
D(+)-glucose (Fluka)	20.0 g
tryptone (Oxoid)	10.0 g
agar (Oxoid)	8.0 g
distilled water	1.0 l
adjusted to pH 6.6 with HCl	
autoclaved at 121° C for 15 min	

The inoculated soft agar was immediately poured in a petri dish (9 cm in diameter). After 15 min of solidification a 4 x 5 cm piece of non-autoclaved nitrocellulose membrane (S & S PROTRAN®, Type BA 83, pore size 0.2 µm) was placed onto the YG^+SA. Five µl of a 24 h main liquid yeast culture were applied to the membrane. The anti-listerial activity of four yeasts was studied by using one membrane. The supernatant of *Lactobacillus plantarum* ALC01 containing pediocin was used as a control which was pipetted in the middle position of the membrane. After incubation at 27° C for 24 h, the assay was evaluated for inhibition zones using Henry's illumination. The inhibitory potential was classified according to a score-based system (Figure 25).

Co-cultivation of yeast and *L. monocytogenes* WSLC 1364

In the co-cultivation experiments 10^5 to 10^6 yeast cells per cm^2 were co-cultivated with around 6 *Listeria* cells per cm^2, which is a realistic contamination rate on red smear cheese (e.g. Rudolf and Scherer 2001). Cell counts of the liquid *Listeria* culture were determined by OD_{600} measurements. An OD_{600} of 0.5 corresponded to 5.8 x 10^8 cfu/ml. The culture was

diluted serially according to the cell density desired on the agar plate used in the co-cultivation experiment. An aliquot was mixed with a yeast suspension prepared by centrifuging 300 µl of the yeast main culture at 5000 rpm for 5 min and resuspending the pellet in 100 µl of ¼-Ringer solution. The *Listeria*-yeast mixture was then spread onto yeast extract glucose agar containing 1 % tryptone (YG^+A):

yeast extract (Oxoid)	5.0 g
D(+)-glucose (Fluka)	20.0 g
tryptone (Oxoid)	10.0 g
agar (Oxoid)	15.0 g
distilled water	1.0 l

adjusted to pH 6.6 with HCl
autoclaved at 121° C for 15 min

Incubation was done at 27° C for 24 h. Cell counts of *Listeria* and yeast used in the co-cultivation experiment were checked separately on YG^+A plates incubated at 27° C for 48 h in duplicates.

After co-cultivation, agar-pieces of 3.5 cm^2 were sterile cut and transferred upside-down in a 100 ml Erlenmeyer flask containing 2 ml of ¼-Ringer solution. Cell lawns were washed off by shaking the flasks for 30 min at 200 rpm. The resulting *Listeria*-yeast-suspensions were transferred to sterile tubes. Residues in the flasks were washed with another 1 ml of ¼-Ringer solution and added to the respective tube. For further examinations the agar-pieces were cooled at 4° C. The *Listeria*-yeast-suspensions were serially diluted and appropriate dilutions were plated in duplicates on YGCBA or Oxford agar (Oxoid) for determining yeast or *Listeria* counts. Two to four co-cultivation approaches were independently performed on each yeast strain. As a control, an equivalent of ¼-Ringer solution was mixed with the *Listeria* cells instead of the yeast culture. Afterwards, the same procedures followed as described above.

To test for residual cells, the cooled agar-pieces were homogenized by pressing them through a needle (20G x 1½′′, 0.9 x 40) using a 5 ml syringe. After the homogenized material was transferred in a sterile tube, the needle was washed with 2 ml ¼-Ringer solution which was added to the same tube. The homogenate was tenfold diluted and aliquots of appropriate

dilutions were plated in duplicates on YGCBA or Oxford agar (Oxoid). Yeast or *Listeria* cell counts were counted after 72 h at 27° C or 48 h at 30° C, respectively.

Assay for assessing "killer toxin activity"

This assay was performed according to Provost et al. (1995) with some modifications. $YG^{+}SA$ was inoculated with an aliquot of a *Listeria* liquid culture as described for the agar-membrane screening assay (see this chapter, p. 29). After solidification, one loop (2 mm in diameter) of yeast cells grown on YGCBA for 48 h at 27° C was streaked as a line onto the soft agar surface. Plates were incubated at 27° C for 24 h and were evaluated for inhibition zones around the yeast cells using Henry's illumination.

3 Results

3.1 Characterization of yeast populations from different European smear-ripened cheeses

A total of 2491 yeasts isolated from five European cheeses - Limburger, Reblochon, Livarot, Tilsit and Gubbeen cheese - were identified and differentiated by FTIR spectroscopy. Table 6 gives an overview of the flora components found on the individual cheeses.

Table 6: Yeast flora composition of five European red smear cheeses [%]. Cheeses obtained from different batches (B) or dairies (D). Most frequently occurring species are highlighted in yellow. *Details are discussed in chapter 4.1.1.

Species	Limburger (B 1 - 3)	Reblochon (D 1 - 3)	Livarot (D 1 - 3)	Tilsit (D 1 - 15)	Gubbeen I (B 1 - 3)	Gubbeen II (B 4 - 6)
Candida catenulata			5	< 1	33	1
Candida etchellsii				< 1		
Candida intermedia		< 1				< 1
Candida parapsilosis					< 1	
Candida tenuis		< 1				
Candida tenuis / Debaryomyces hansenii				< 1		
Candida zeylanoides				< 1		
Clavispora lusitaniae				< 1	16	
Cryptococcus humicolus			< 1			
Debaryomyces hansenii	79	3	12	89	49	98
Galactomyces geotrichum	21	27	61	< 1	-	-
Issatchenkia occidentalis				< 1		
Kluyveromyces lactis		16	9	< 1		
Pichia guilliermondii					< 1	
Pichia jadinii / Candida glabrata			< 1			
Pichia pseudocactophila / Pichia cactophila / Issatchenkia occidentalis				< 1		
Pichia triangularis				2		
Rhodotorula glutinis				< 1		
Saccharomyces cerevisiae				< 1		
Torulaspora delbrueckii		2	< 1			
Yarrowia lipolytica			11	3		
Trichosporon ovoides					< 1	
not identified		51*				

On Limburger cheese only two species were detected, *D. hansenii* and *G. geotrichum* with 79 and 21 %, respectively. The latter one dominated the flora of Reblochon cheese with 27 %. Additionally, *K. lactis, D. hansenii* and *T. delbrueckii* occurred with a frequency of 16, 3 and 2 %, respectively. *C. intermedia* as well as *C. tenuis* comprised less than 1 % of the total flora. Identification of the majority of the Reblochon yeasts (51 %) was not possible with the current version of the FTIR reference spectra database. Yeasts from Livarot cheese were mainly identified as *G. geotrichum* (61 %). Other species found on this cheese were assigned to *D. hansenii* (12 %), *Y. lipolytica* (11 %), *K. lactis* (9 %) and *C. catenulata* (5 %). Further species accounted in proportions below 1 %. Within the Tilsit yeasts *D. hansenii* clearly dominated with 89 %. *P. triangularis* and *Y. lipolytica* represented 2 and 3 % of the total yeast population, respectively, and several other species had a share below 1 %. Single isolates of Tilsit and Livarot cheese were not clearly identifiable with the current version of the FTIR reference spectra database. In batches 1 to 3 of Gubbeen cheese, *D. hansenii*, *C. catenulata* and *Cl. lusitaniae* were detected in 49, 33 and 16 %, respectively, whereas further yeast species amounted to below 1 %. The other three Gubbeen cheese batches were dominated by *D. hansenii* with 98 %. *C. catenulata* and *C. intermedia* were isolated in low numbers of 1 % or below 1 %, respectively. For details see Table 6.

3.2 Biodiversity of surface microbial consortia from Limburger cheese

The biodiversity of the yeast and bacterial flora from Limburger cheese was investigated during ripening using a polyphasic approach to characterize the microflora.

3.2.1 Cell counts

Yeast cell counts of around 10^7 cfu/cm^2 were recorded at all stages of ripening within Limburger Batches 1 and 2 (Table 7). Corresponding bacterial cell counts showed a slight increase from the early to the late stage of ripening with values around 10^6 or 10^7 cfu/cm^2 though values in Batch 2 were slightly higher. Yeast and bacterial cell counts during the early and late sampling points of Batch 3 were in the range of the other two batches but higher cell counts were detected at the middle stage of ripening. Aerotolerant catalase-negative bacteria were found in higher numbers on the agar plates inoculated with the early stage cheese

samples, especially from Batches 1 and 2. These counts were included when the total cell counts were determined. In these cases, the number of coryneform bacteria and cocci was 1 to 2 log units lower than shown in Table 7.

Table 7: Yeast and bacterial cell counts (cfu/cm^2) during the ripening within three batches. E, M, L: early, middle, late sampling point.

	Yeasts			Bacteria		
Batch	**E**	**M**	**L**	**E**	**M**	**L**
1	1,3E+07	1,3E+07	9,4E+06	1,0E+06	2,2E+06	6,6E+06
2	9,3E+06	1,3E+07	2,8E+07	6,9E+06	1,3E+07	2,1E+07
3	2,0E+07	1,4E+08	1,4E+07	8,0E+05	9,2E+08	4,3E+07

3.2.2 Identification and differentiation of yeast isolates by FTIR spectroscopy

The FTIR measurements were based on 469 not the initial 450 strains as subcultures of strains exhibiting two different colony morphologies were examined separately. Two species, *D. hansenii* and *G. geotrichum*, were found. *D. hansenii* accounted for up to or more than 90 % of the yeast populations during the different ripening stages in all three batches investigated, except for the early stage of Batch 2 where *G. geotrichum* dominated (Figure 2). There was a slight decrease of *D. hansenii* during ripening in the first and third batch but this was reversed in the second batch.

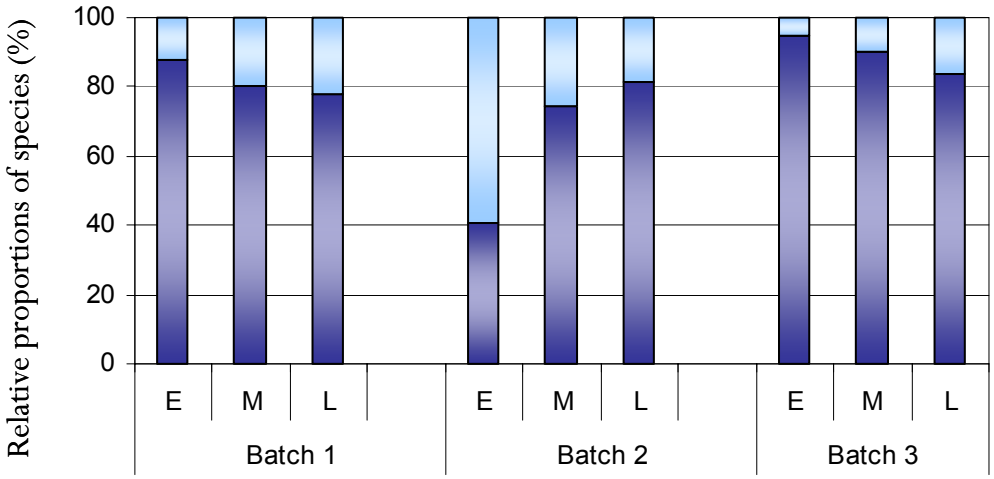

Figure 2: Composition of the yeast flora during ripening within three cheese batches. *D. hansenii* (■), *G. geotrichum* (□). E, M, L: early, middle and late sampling point.

Two different colony morphology types of *D. hansenii* were found. On YGCBA, type 1 was light blue and the surface of the colony was mat and rough. In contrast, the surface of type 2 was shiny and had a bright blue center surrounded by a white edge (Figure 3a, b).

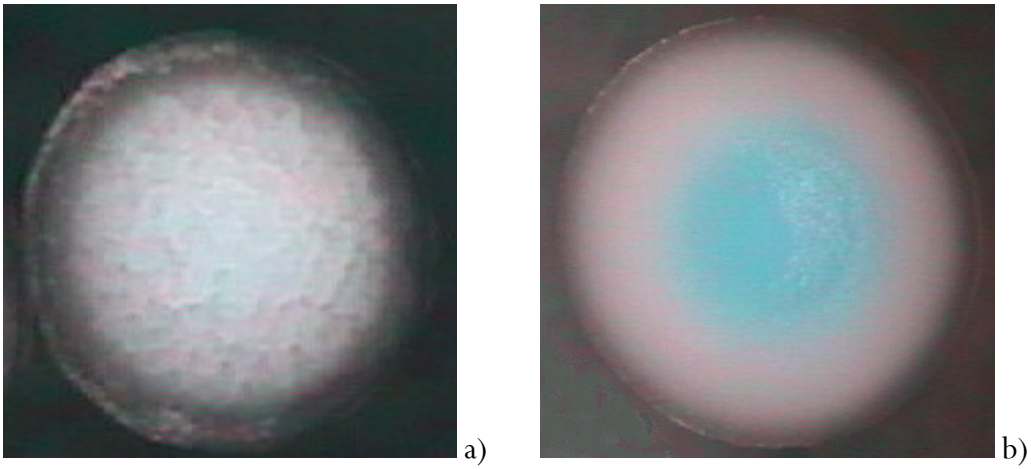

Figure 3: Colony morphology types found for *D. hansenii* cultivated on YGCBA. a) Type 1: light blue, rough colonies with a mat surface. b) Type 2: shiny colonies with a bright blue center surrounded by a white edge. 30-fold zoomed.

Figure 4 shows the cluster analysis of the recorded yeast spectra. The spectra were assigned to 3 major clusters consisting of 9 clear subclusters with 3 subclusters containing spectra of

G. geotrichum strains and 6 subclusters comprised of spectra of *D. hansenii* strains. Fourty three out of 469 yeasts were selected as representatives for further study.

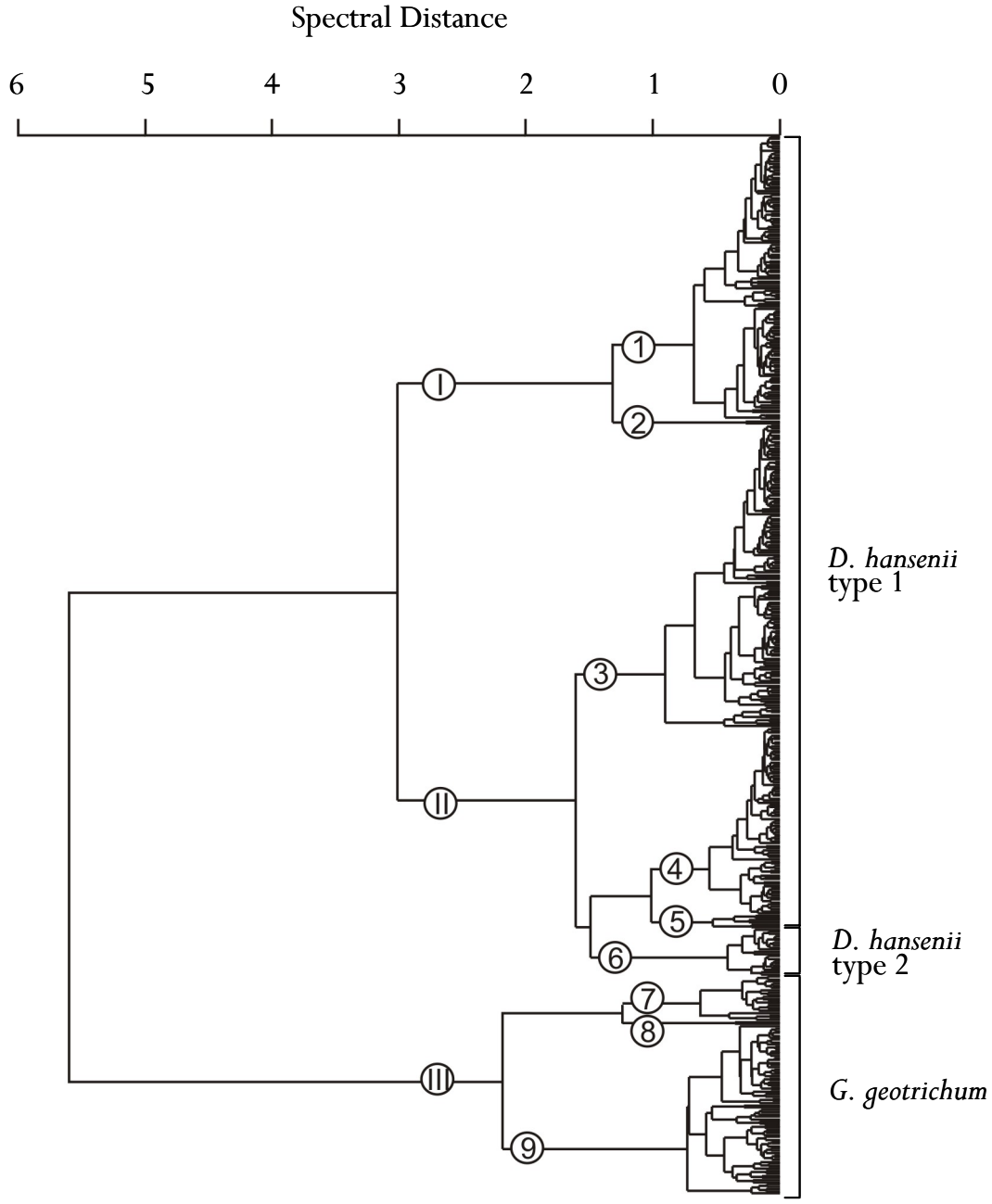

Figure 4: Dendrogram depicting FTIR spectral similarities between the yeast isolates. Major clusters are numbered (I) to (III). Subclusters (1) to (5) encompass spectra of *D. hansenii* type 1 strains and cluster 6 *D. hansenii* type 2 strains. Two colony morphological types were found on YGCBA: type 1 was characterized by the production of light blue, rough colonies with a mat surface and type 2 strains formed colonies with a bright blue center surrounded by a white edge. Spectra of *G. geotrichum* strains are assigned to subclusters 7 to 9. Average linkage, correlation with normalization to reproducibility level. Second derivative. Frequency ranges: 3030-2830 cm^{-1}; 1350-1200 cm^{-1}; 900-700 cm^{-1}. Each frequency range with weight 1 and reproducibility level 30.

3.2.3 Phenotypic characterization of yeast isolates

Physiological tests were performed on 10 (around 20 %) of the dereplicated yeast strains to confirm the results obtained by FTIR spectroscopy. The *D. hansenii* strains gave two different reaction patterns, which correlated with the two colony morphology types. Differences between the types were recorded for growth on D-glucosamine, L-arabinitol and 2-keto-D-gluconate as a sole carbon substrate and in nitrite and ethylamine as nitrogen substrates. Colony type 1 isolates were negative for these tests whereas type 2 strains were positive (Table 8). All of the *G. geotrichum* strains gave identical results (appendix, Tables 4 to 8).

Table 8: Different growth reactions in *D. hansenii* type 1 and type 2

Growth on	Type 1	Type 2
D-glucosamine (as carbon substrate)	-	+
L-arabinitol	-	+
2-keto-D-gluconate	-	+
nitrite	-	+
ethylamine	-	+

3.2.4 Identification and differentiation of bacterial isolates by molecular based methods

Four hundred and twenty three isolates were included in *rep*-studies using REP-primers[1] which revealed a high homogeneity within the REP-patterns. Twenty-seven isolates failed to grow from glycerol stock cultures. A total of 44 representative strains out of the 423 bacteria were studied further.

After the completion of the classical phenotypic tests[2], such as Gram stain, catalase and oxidase test etc., on the dereplicated isolates, further studies were restricted to the coryneform bacteria and cocci. *Rep*-PCR with BOX- or $(GTG)_5$-primers[3] was used to identify 34 rods and 2 cocci, respectively. The representative strains were assigned to species groups

[1] *rep*-PCR using REP-primers was performed by Nagamani Bora, Microbial Resources Centre, School of Biology, University of Newcastle, UK. Method used as described in Goerges et al. (submitted).
[2] Classical phenotypic tests were performed by Roberto Gelsomino, BCCM/LMG Bacteria Collection, University of Ghent, Belgium. Methods used as described in Goerges et al. (submitted).
[3] *rep*-PCR using BOX- and $(GTG)_5$-primers was performed by Roberto Gelsomino, BCCM/LMG Bacteria Collection, University of Ghent, Belgium. Method used as described in Goerges et al. (submitted).

based on 16S rDNA sequence analysis data and to the rank of species based on the results of the SDS-PAGE[4] of whole cell proteins. Representatives of the identified species were deposited in the BCCM/LMG Bacteria Collection (Figure 5 and Figure 6). The studies highlighted the presence of members of two newly described coryneform species, *Arthrobacter arilaitensis* (Irlinger et al. 2005) and *Brevibacterium aurantiacum* (Gavrish et al. 2004), among the strains isolated from all three cheese batches. The isolates assigned to each taxa gave a unique *rep*-pattern (Figure 5).

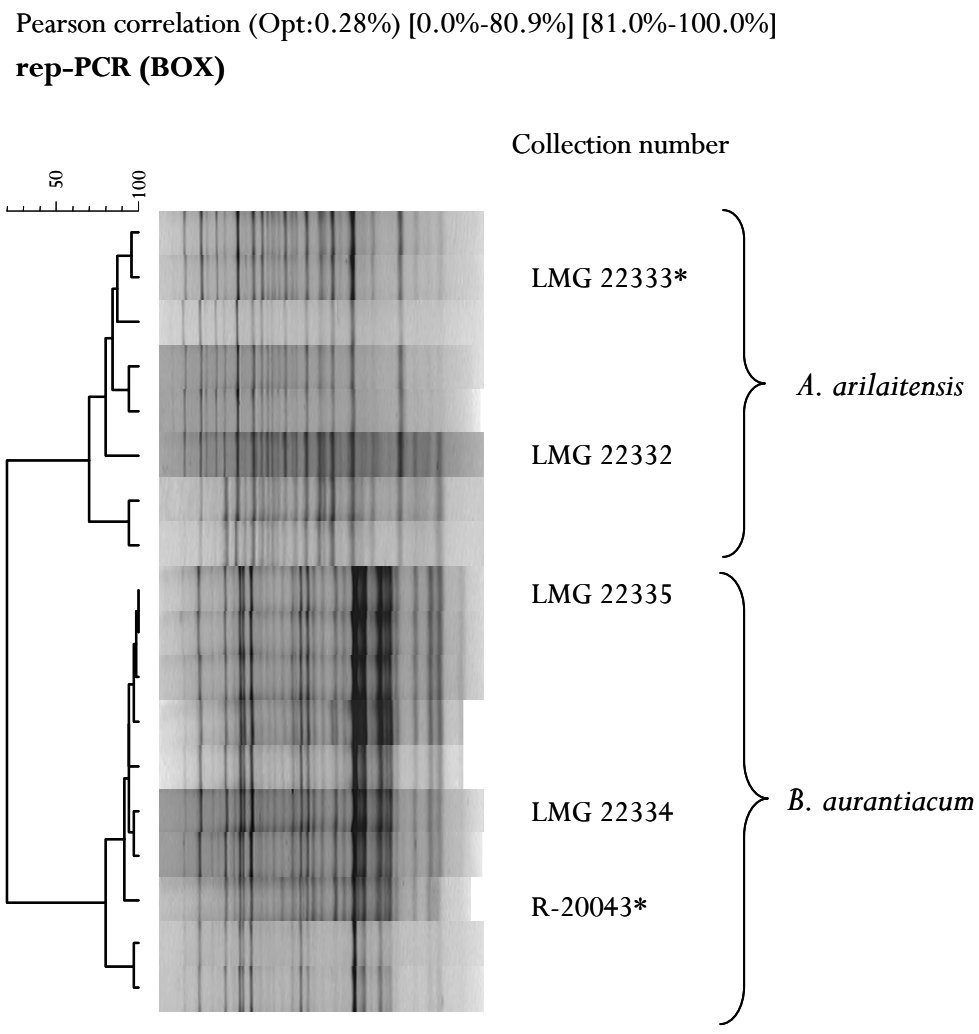

Figure 5: *rep*-PCR patterns corresponding to *A. arilaitensis* and *B. aurantiacum* on Limburger cheese. Dendrogram obtained after numerical analysis of BOX-PCR patterns of dereplicated isolates. Similarities among band patterns were calculated using the Pearson's similarity coefficient and the UPGMA algorithm. LMG 22332 – 22335: strains deposited in the BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Ghent University, Ghent, Belgium; R: Research collection, Laboratorium voor Microbiologie, Ghent University, Ghent, Belgium; *: Reference strain.

[4] 16S rDNA sequence analysis and SDS-PAGE of whole cell proteins was performed by Roberto Gelsomino, BCCM/LMG Bacteria Collection, University of Ghent, Belgium. Methods used as described in Goerges et al. (submitted).

A few isolates (n=5) were identified as *Macrococcus* spp. which showed identical *rep*-patterns (Figure 6).

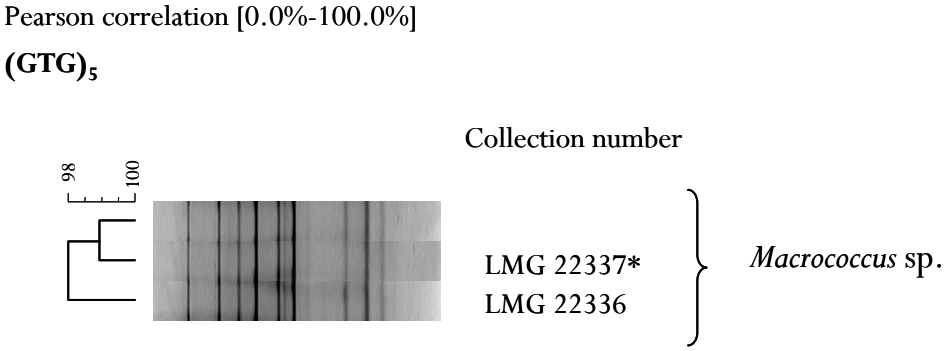

Figure 6: *rep*-PCR patterns corresponding to *Macrococcus* sp. on Limburger cheese. Dendrogram obtained after numerical analysis of $(GTG)_5$-PCR patterns of dereplicated isolates. Similarities among band patterns were calculated using the Pearson's similarity coefficient and the UPGMA algorithm. LMG 22336 – 22337: strains deposited in the BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Ghent University, Ghent, Belgium; *: Reference strain.

B. aurantiacum strains formed about 80 % of the strains isolated from the early stages of Batches 1 and 2 although some *A. arilaitensis* or *Macrococcus* strains were also found. *A. arilaitensis* was found to be the dominant organism on all of the other cheeses. The proportion of *B. aurantiacum* strains generally ranged between 16 and 30 %. Some Gram-negative bacteria and bacilli were included amongst the catalase-positive flora (Figure 7).

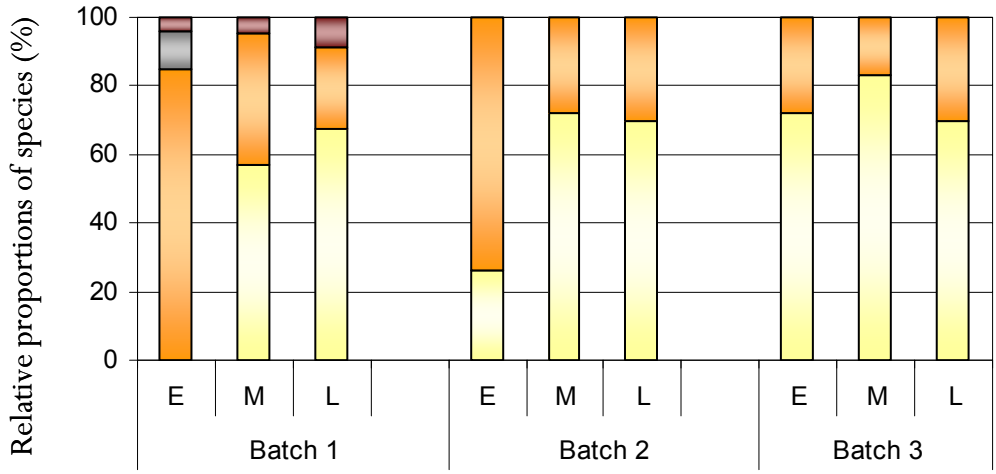

Figure 7: Composition of the catalase-positive bacterial flora during ripening within three cheese batches. *A. arilaitensis* (□), *B. aurantiacum* (□), *Macrococcus* sp. (□), bacilli and Gram-negative bacteria (□). E, M, L: early, middle and late sampling point.

3.2.5 Identification and differentiation of coryneform isolates by FTIR spectroscopy

A total of 414 coryneform bacteria out of the 423 isolates subjected to REP-PCR studies were also investigated by FTIR spectroscopy. Sixty percent of the coryneform bacteria were identified as *A. nicotianae* and 40 % as *B. linens*. The spectral cluster analysis of the 414 coryneform bacteria revealed two major clusters which were subdivided into 9 subclusters (Figure 8). Five of the subclusters encompassed spectra assigned to *A. nicotianae* and the other four subclusters were assigned to *B. linens*.

Figure 8: Dendrogram depicting FTIR spectral similarities between the coryneform isolates. Major clusters are numbered (I) and (II). Subclusters (1) to (4) encompass spectra of *B. linens.* Spectra of *A. nicotianae* strains are assigned to subclusters 5 to 9. Average linkage, correlation with normalization to reproducibility level. First derivative. Frequency ranges with weights and reproducibility levels: 3000-2800 cm^{-1}/0.8/3.3; 1800-1500 cm^{-1}/0.8/5; 1500-1200 cm^{-1}/0.9/20; 1200-900 cm^{-1}/0.9/33; 900-700 cm^{-1}/0.9/116.

3.2.6 Pulsed field gel electrophoresis on coryneform isolates[5]

Nineteen out of the 44 dereplicated isolates were subjected to PFGE analysis after chromosomal DNA was digested with enzymes *Spe*I, *Asc*I and *Xba*I. In all cases two different band patterns corresponding to *A. arilaitensis* and *B. aurantiacum* were found. As an example, Figure 9 shows the patterns obtained when the chromosomal DNA was cut with *Spe*I.

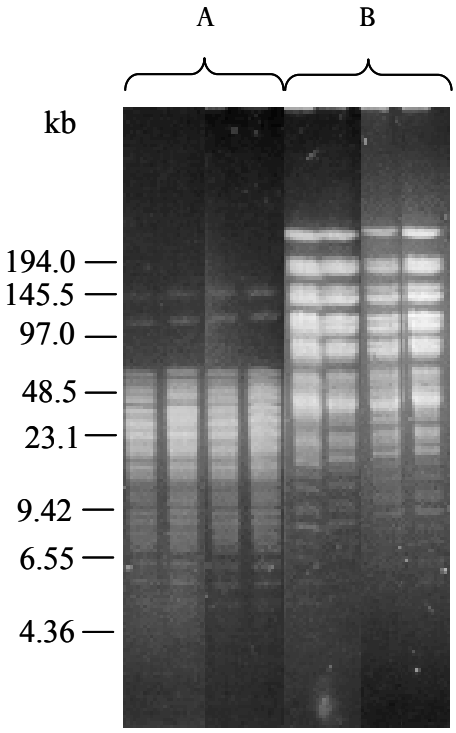

Figure 9: PFGE band pattern of *A. arilaitensis* and *B. aurantiacum* isolates. A: *A. arilaitensis*; B: *B. aurantiacum*; restriction digest of chromosomal DNA with *Spe*I; Low Range PFG Marker (BioLabs® Inc., New England).

[5] PFGE studies were performed by Mary Rea, Biotechnology Department, Moorepark Food Research Centre, Teagasc, Fermoy, Co. Cork, Ireland. Method used as described in Brennan et al. (2002).

3.3 Incidence of commercial surface starter microorganisms during ripening of a Limburger cheese

Chapter 3.2 described the results on the biodiversity of the surface microbial consortia from three batches of a German Limburger cheese during different stages of ripening. The isolates collected for this purpose were also used to study the occurrence of the commercial smear starter microorganisms added to the cheese milk. One representative of each of the four starter organisms, *D. hansenii*, *Geotrichum candidum*, *A. nicotianae* and *B. linens*, was available.

For each starter organism at least 12 FTIR spectra were independently recorded. The spectral similarities between 469 yeast or 414 coryneform isolates and the yeast or bacterial starters were studied by cluster analysis. Separate clusters resulted for the *G. geotrichum* starter spectra. Figure 10 shows a dendrogram depicting the similarity between the spectra of the surface isolates and starters assigned to *G. geotrichum*.

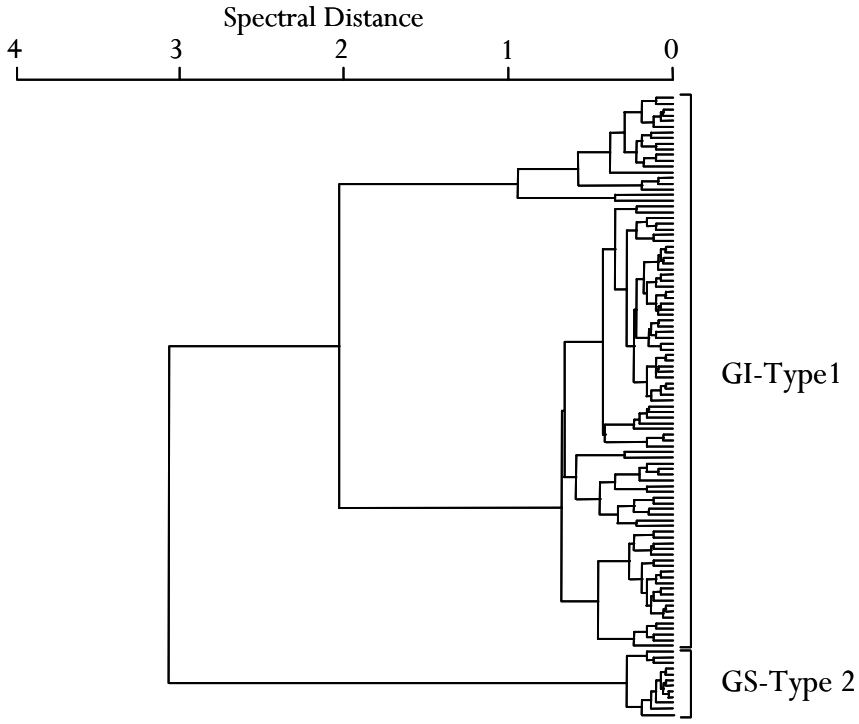

Figure 10: Dendrogram depicting FTIR spectral similarities between *G. geotrichum* surface isolates and *G. geotrichum* starters. GI: *G. geotrichum* isolated from cheese smear; GS: *G. geotrichum* from smear starter culture. Colony morphology types found on YGCBA: type 1: turquoise to blue colored colony surface; type 2: light green, fluffy colony surface. Average linkage, correlation with normalization to reproducibility level. Second derivative. Frequency ranges: 3030-2830 cm^{-1}; 1350-1200 cm^{-1}; 900-700 cm^{-1}. Each frequency range with weight 1 and reproducibility level 30.

Two colony morphology types were found for *G. geotrichum* (Figure 11). The *G. geotrichum* isolates appeared with a turquoise to blue colored colony surface (type 1) when cultivated on YGCBA whereas the *G. geotrichum* starter had a light green, fluffy colony surface (type 2).

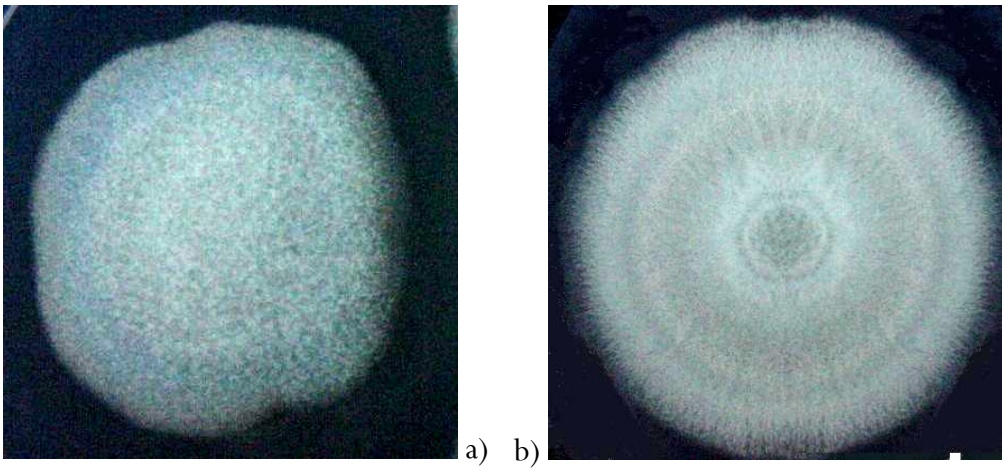

Figure 11: Colony morphology types found for *G. geotrichum* cultivated on YGCBA. a) Type 1: turquoise to blue colored colony surface. b) Type 2: light green, fluffy colony surface.

As observed for *G. geotrichum*, spectra of the bacterial smear starters clustered separately from those of the surface isolates. As an example, Figure 12 shows a dendrogram depicting FTIR spectral similarities between smear starters and isolates of *A. nicotianae*.

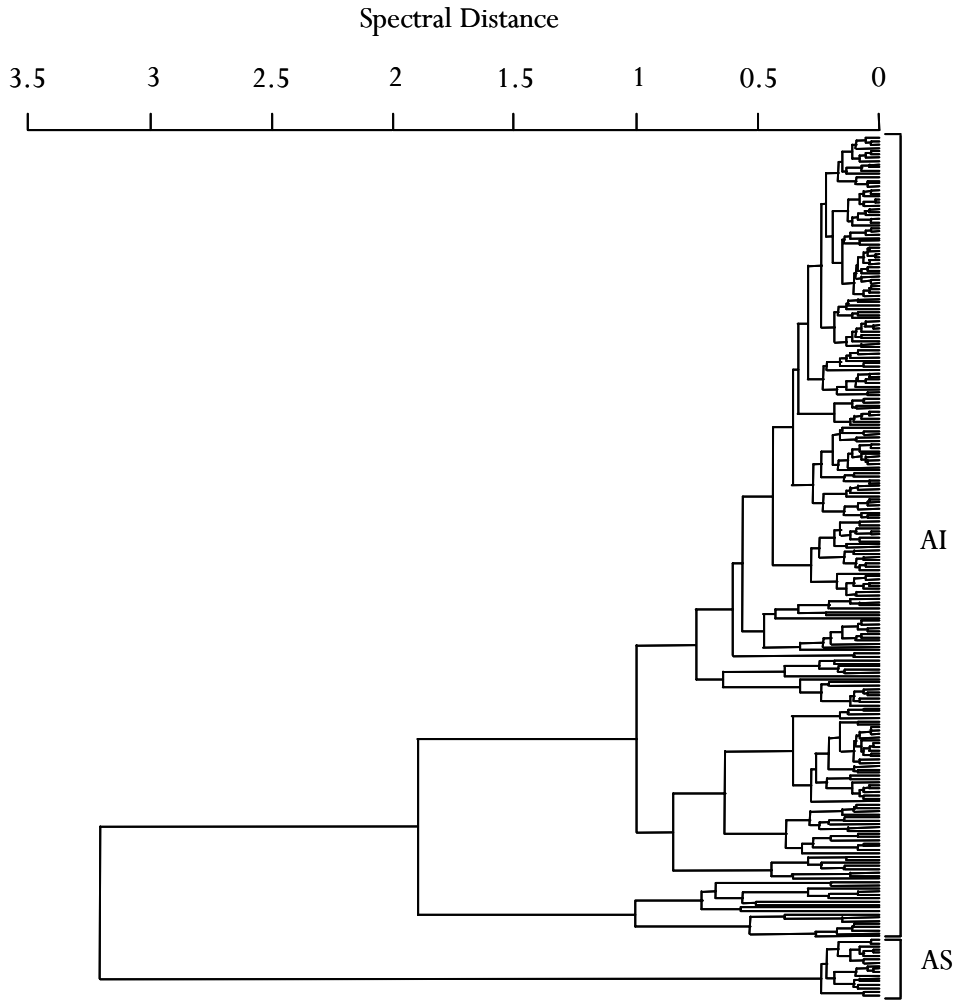

Figure 12: Dendrogram depicting FTIR spectral similarities between *A. nicotianae* surface isolates and *A. nicotianae* starters. AI: *A. nicotianae* isolated from cheese smear; AS: *A. nicotianae* from smear starter culture. Average linkage, correlation with normalization to reproducibility level. First derivative. Frequency ranges with weights and reproducibility levels: 3000-2800 cm^{-1}/0.8/3.3; 1800-1500 cm^{-1}/0.8/5; 1500-1200 cm^{-1}/0.9/20; 1200-900 cm^{-1}/0.9/33; 900-700 cm^{-1}/0.9/116.

In contrast to the *G. geotrichum* and both bacterial starters, the *D. hansenii* smear starter spectra clustered together with a minority of surface isolates spectra assigned to this species (Figure 13) showing type 2 colony morphology (see chapter 3.2.2).

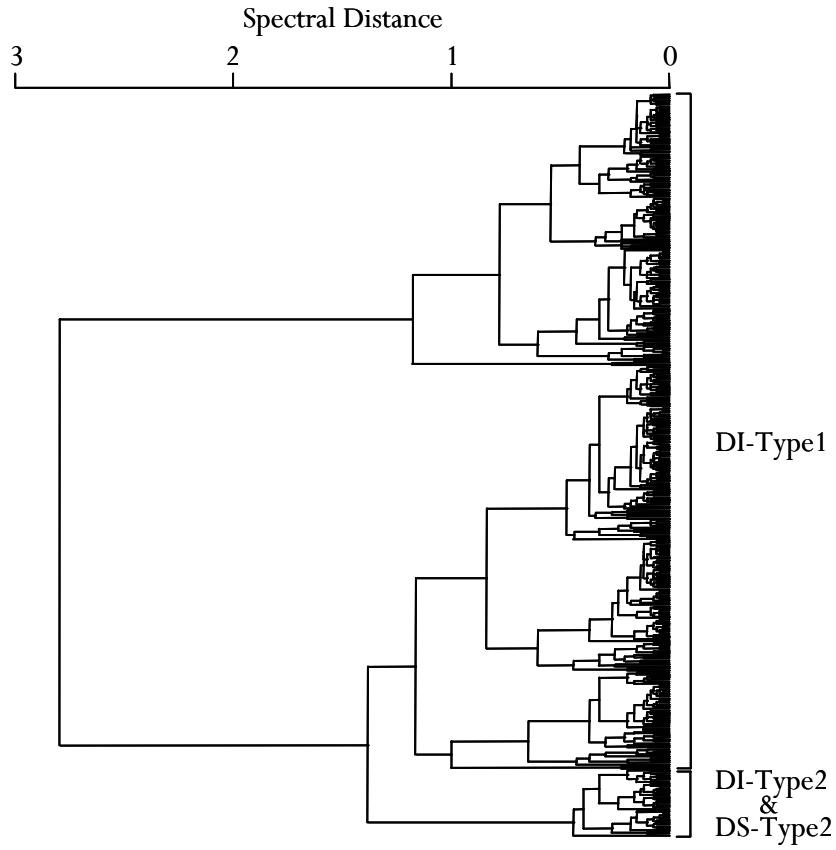

Figure 13: Dendrogram depicting FTIR spectral similarities between *D. hansenii* surface isolates and *D. hansenii* starters. DI: *D. hansenii* isolated from cheese smear; DS: *D. hansenii* from smear starter culture. Colony morphology types found on YGCBA: type 1: light blue, rough colonies with a mat surface; type 2: colonies with a bright blue center surrounded by a white edge. Average linkage, correlation with normalization to reproducibility level. Second derivative. Frequency ranges: 3030-2830 cm^{-1}; 1350-1200 cm^{-1}; 900-700 cm^{-1}. Each frequency range with weight 1 and reproducibility level 30.

The results obtained in these FTIR studies indicated that from the four starter organisms added only the *D. hansenii* starter was reisolated from the surface of the Limburger cheese investigated, albeit in low numbers. Therefore, more detailed examinations were done on fresh isolates obtained from an additional Limburger batch and on the four smear starters using FTIR spectroscopy and several molecular based methods.[6]

[6] Where necessary for a better understanding, surface isolates and starters of the first and second isolation series are marked with indices IS1 and IS2, respectively, in the following chapters.

3.3.1 Detailed analysis of the smear starter organisms

Variability of the smear starter organisms of isolation series 2

It is expected that different strains of the same species can establish differently on the cheese surface and, therefore, are isolated in different numbers from the smear. To investigate intraspecies variability a random sampling of 50 colonies was taken for each of the four starter organisms. The similarities between the representatives of each smear starter organism were studied based on FTIR spectra. Figure 14 shows a cluster analysis of *D. hansenii* starter spectra which formed one single cluster indicating that all 50 representatives belonged to the same strain. A similar cluster structure was obtained for the *G. geotrichum* starter as well as for both bacterial IS2-starters.

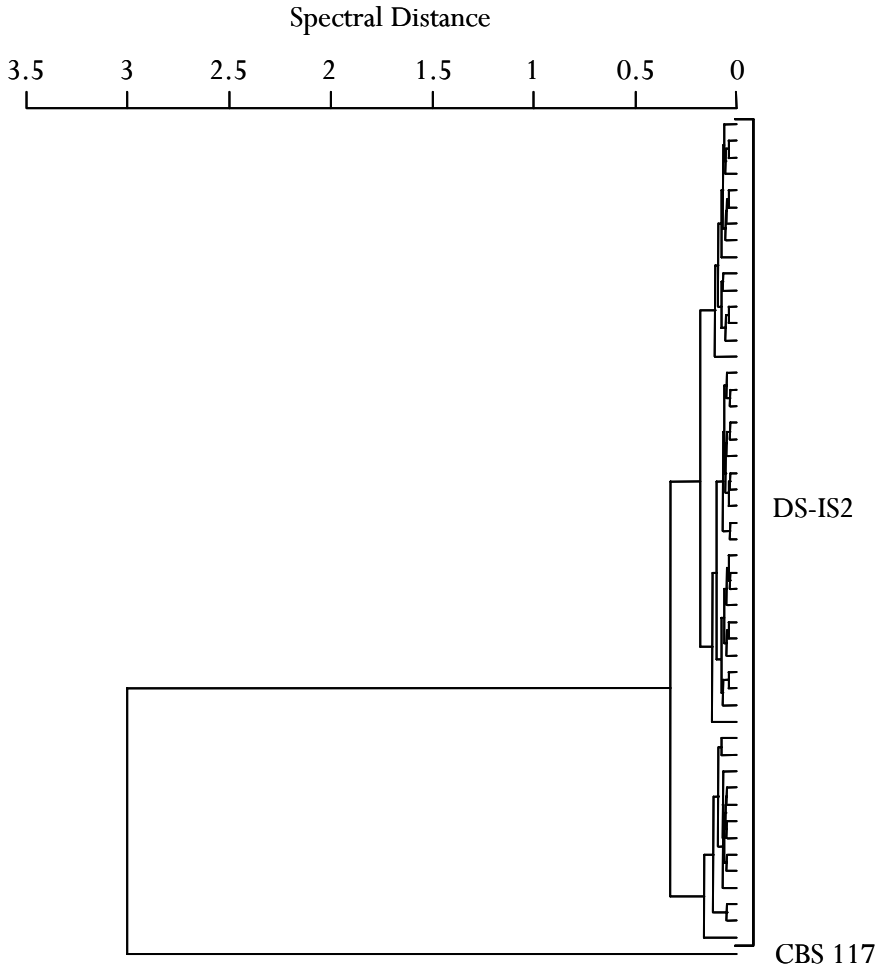

Figure 14: Dendrogram depicting FTIR spectral similarities between 50 representatives of the *D. hansenii* IS2-starter. DS: *D. hansenii* from smear starter culture; IS2: isolation series 2. Outgroup: *D. hansenii* CBS 117. Average linkage, correlation with normalization to reproducibility level. Second derivative. Frequency ranges: 3030-2830 cm^{-1}; 1350-1200 cm^{-1}; 900-700 cm^{-1}. Each frequency range with weight 1 and reproducibility level 30.

Similarity between smear starters of isolation series 1 and 2[7]

Smear starters of isolation series 1 and 2 were analyzed in an approximately three years interval. As shown by analyses of IS2-smear starters (see above), the commercial mixed culture was composed of one strain of each of the four species. Similarities between the starters of both isolation series were investigated using cluster analyses based on FTIR spectra. For *D. hansenii* as well as *G. geotrichum* and *B. linens* a high similarity between IS1- and IS2 starters was revealed since their spectra were mixed in one cluster. As an example, the similarity analysis obtained for spectra of the *D. hansenii* IS1- and IS2-starters is shown in Figure 15.

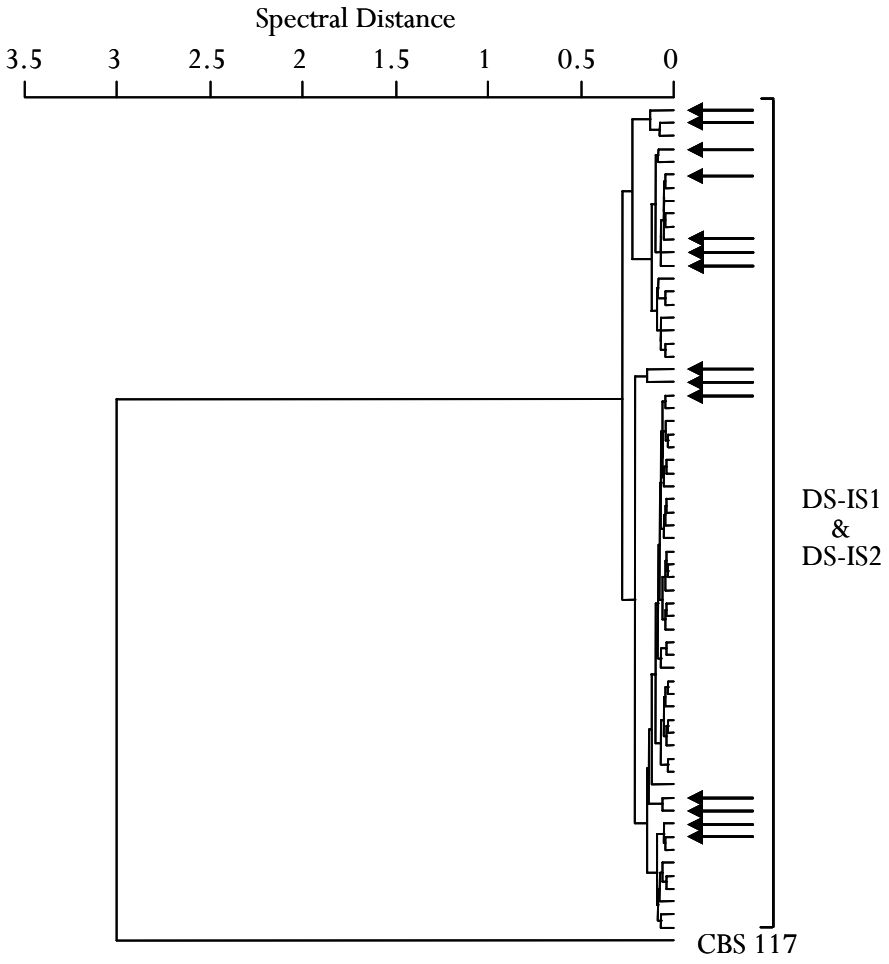

Figure 15: Dendrogram depicting FTIR spectral similarities between *D. hansenii* IS1- and IS2-starters. Arrows indicate spectra of IS1-starters. DS: *D. hansenii* from smear starter culture; IS1, IS2: isolation series 1, 2. Outgroup: *D. hansenii* CBS 117. Average linkage, correlation with normalization to reproducibility level. Second derivative. Frequency ranges: 3030-2830 cm^{-1}; 1350-1200 cm^{-1}; 900-700 cm^{-1}. Each frequency range with weight 1 and reproducibility level 30.

[7] Smear starters of isolation series 1 and 2 are marked with indices IS1 and IS2, respectively.

Spectra of IS1- and IS2-starters assigned to *A. nicotianae* also formed one cluster indicating a high similarity. However, within this cluster IS1-smear starters were arranged in one block (Figure 16) and were not mixed with IS2-smear starters as it was observed for *D. hansenii* and both bacterial starters (Figure 15).

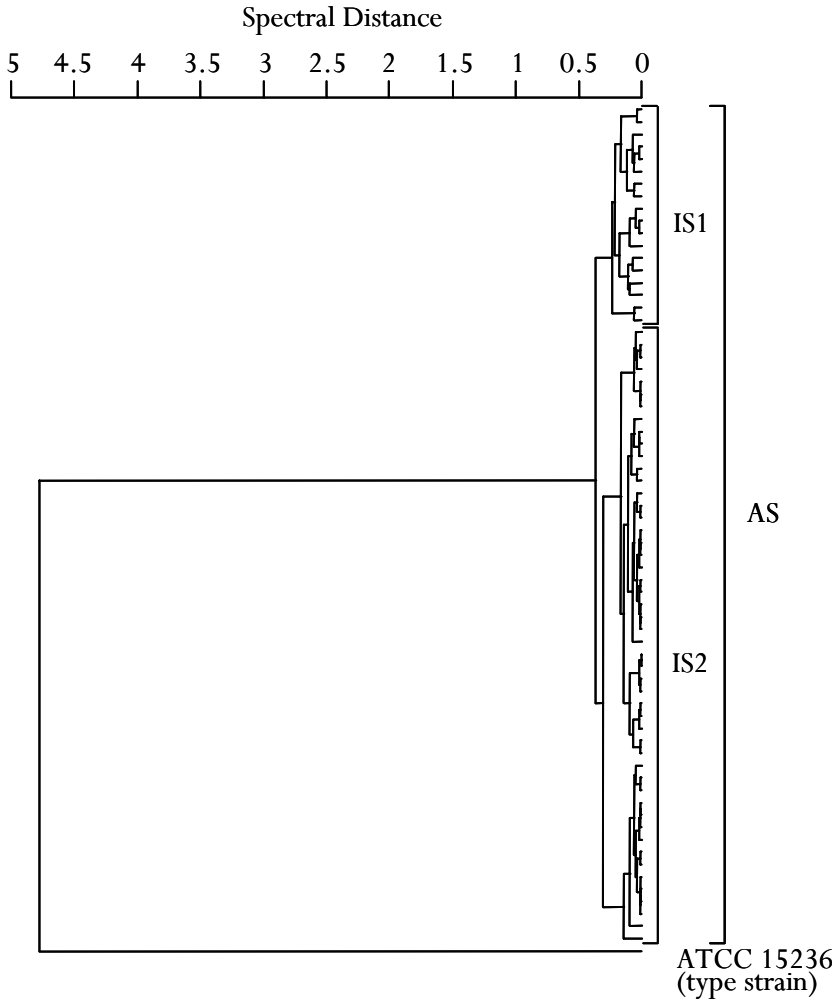

Figure 16: Dendrogram depicting FTIR spectral similarities between *A. nicotianae* IS1- and IS2-starters. AS: *A. nicotianae* from smear starter culture; IS1, IS2: isolation series 1, 2. Outgroup: *A. nicotianae* ATCC 15236, type strain. Average linkage, correlation with normalization to reproducibility level. First derivative. Frequency ranges with weights and reproducibility levels: 3000-2800 cm^{-1}/0.8/3.3; 1800-1500 cm^{-1}/0.8/5; 1500-1200 cm^{-1}/0.9/20; 1200-900 cm^{-1}/0.9/33; 900-700 cm^{-1}/0.9/116.

3.3.2 Incidence of the smear starters in isolates of a further Limburger batch[8]

To study the incidence of the smear starter organisms at different stages of ripening in more detail a fourth batch of the same Limburger cheese was used. FTIR measurements were done on 170 yeast and 311 coryneform bacteria, followed by similarity analyses of these spectra with those of the respective smear starters.

Yeast

FTIR measurements were performed on 166 out of the initially isolated 170 yeasts due to problems in cultivating some of the *G. geotrichum* isolates. A total of 38 % were identified as *D. hansenii*. All of these isolates showed the previously described type 2 colony morphology (see chapter 3.2.2). Within *G. geotrichum* the two previously mentioned colony morphology types occurred (see chapter 3.3, p. 43). The first type was represented by 49 % of the total yeast isolates whereas the other type comprised 13 %. In contrast to type 2, characterized by a creamy texture, type 1 was difficult to handle due to its adhesive properties on solid media causing the above mentioned cultivation problems. *D. hansenii* dominated (88 %) in the early stage of ripening whereas *G. geotrichum* amounted for up to 85 % on all the other cheeses (Table 9).

Table 9: Fractions [%] of *D. hansenii* and *G. geotrichum* within the yeast isolates. Sampling after 3, 6, 9, and 28 days of ripening.

	3 d	6 d	9 d	28 d
D. hansenii	88	16	15	18
G. geotrichum	12	84	85	82

Figure 17 shows a cluster analysis including the spectra of 166 yeast isolates and of 50 *D. hansenii* and *G. geotrichum* starters, respectively. One cluster existed for *D. hansenii* which included the starters spectra mixed with spectra of the surface isolates identified as this yeast

[8] Results presented in this chapter are restricted to surface isolates and smear starters of isolation series 2. Therefore no indices are placed.

species. In *G. geotrichum* two different clusters were obtained corresponding to the two different colony morphological types observed. One of the clusters encompassed all starters spectra and a minority of surface isolates spectra whereas the other cluster contained the majority of the surface isolates spectra.

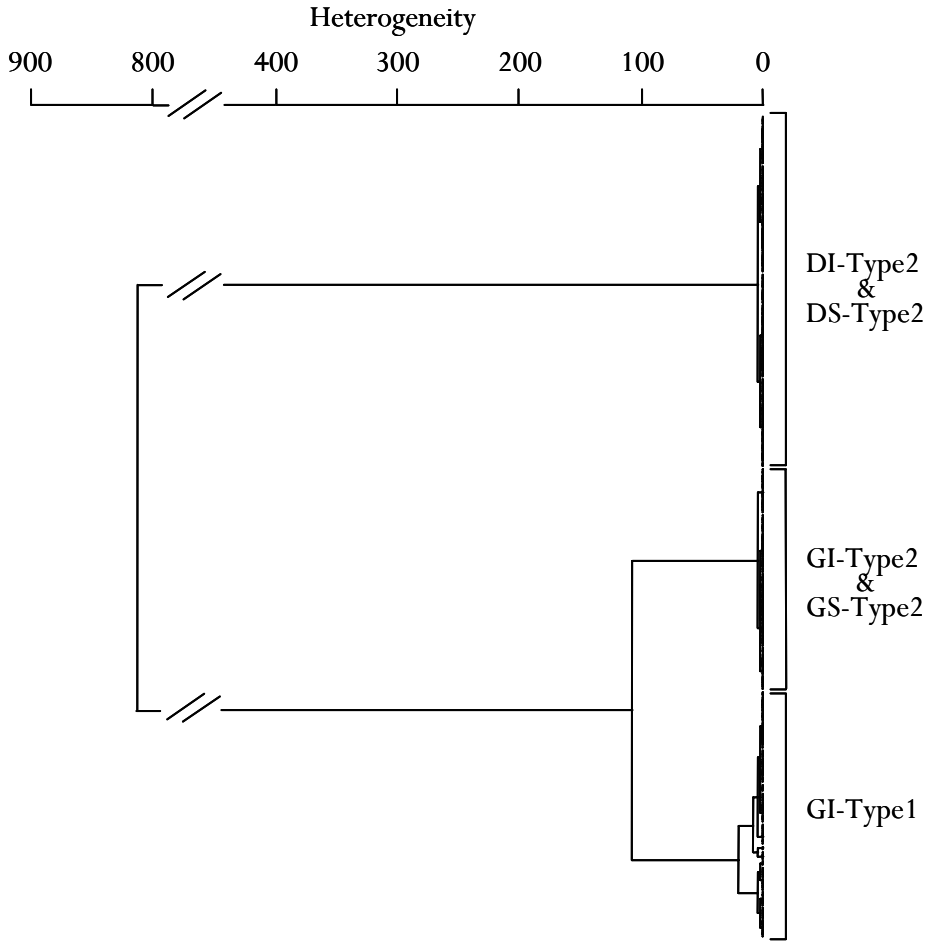

Figure 17: Dendrogram depicting FTIR spectral similarities between the yeast surface isolates and the yeast starters. DI: *D. hansenii* isolated from cheese smear; DS: *D. hansenii* from smear starter culture; GI: *G. geotrichum* isolated from cheese smear; GS: *G. geotrichum* from cheese smear. Colony morphology type found for *D. hansenii* on YGCBA: type 2: colonies with a bright blue center surrounded by a white edge; Colony morphology types found for *G. geotrichum*: type 1: turquoise to blue colored colony surface; *G. geotrichum* type 2: light green and fluffy colony surface. Ward´s algorithm, correlation with normalization to reproducibility level. Second derivative. Frequency ranges: 3030-2830 cm^{-1}; 1350-1200 cm^{-1}; 900-700 cm^{-1}. Each frequency range with weight 1 and reproducibility level 30.

Coryneform bacteria

According to the FTIR measurements performed on 211 coryneform isolates collected from cheeses during three different stages of ripening, 78 % of these isolates were identified as *A. nicotianae* and 22 % as *B. linens*. A cluster analysis on spectra of the surface isolates and

smear starters of isolation series 2 is shown in Figure 18. The spectra of each bacterial starter were arranged in separate clusters. They were both clearly distinguishable from clusters formed by the surface isolates spectra. One cluster contained spectra of surface isolates assigned to *B. linens* and two clusters consisted of spectra assigned to *A. nicotianae* surface isolates.

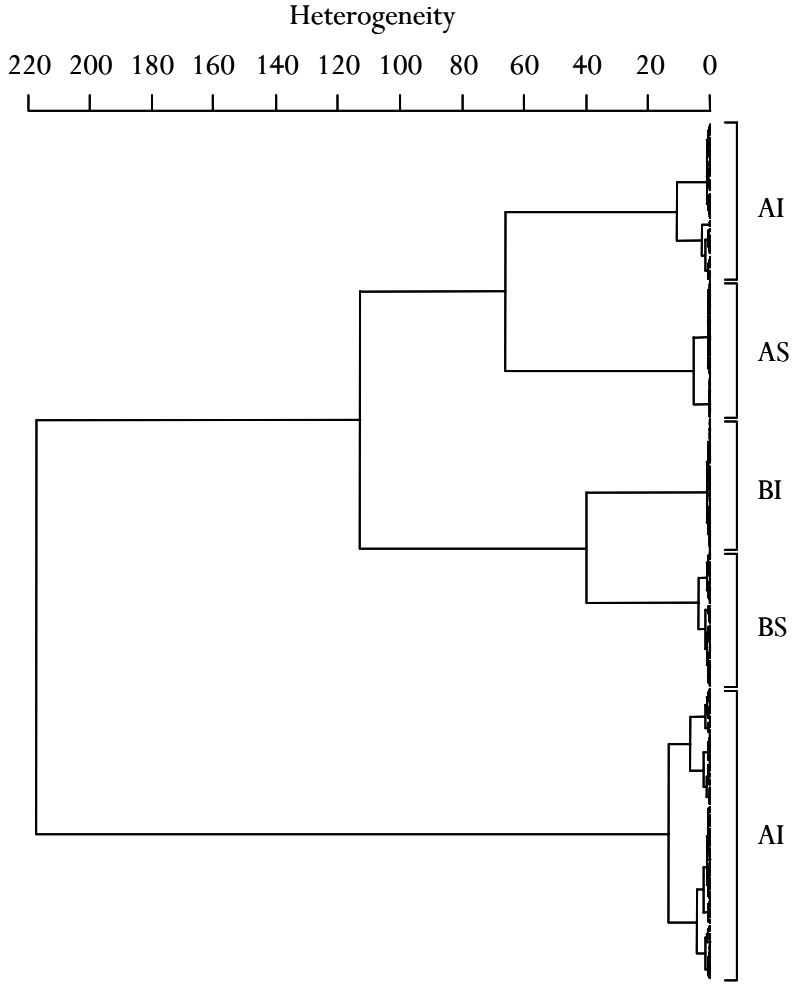

Figure 18: Dendrogram depicting FTIR spectral similarities between 211 coryneform surface isolates and the coryneform starters. AI: *A. nicotianae* isolated from cheese smear; AS: *A. nicotianae* from smear starter culture; BI: *B. linens* isolated from cheese smear; BS: *B. linens* from smear starter culture. Ward's algorithm, correlation with normalization to reproducibility level. First derivative. Frequency ranges with weights and reproducibility levels: 3000-2800 cm^{-1}/0.8/3.3; 1800-1500 cm^{-1}/0.8/5; 1500-1200 cm^{-1}/0.9/20; 1200-900 cm^{-1}/0.9/33; 900-700 cm^{-1}/0.9/116.

Since spectra of both bacterial starters did not show any similarity to the spectra of the surface isolates, the random sampling was extended by collecting additional 100 isolates from the cheese preparations used before. The same two species were found in similar fractions.

Within a total of 311 isolates *A. nicotianae* accounted for 72 % and *B. linens* for 28 %. Both species occurred in equal fractions within the isolates picked from the cheese surface examined after the second smearing (6 d). However, in the later stages *A. nicotianae* clearly dominated (Table 10). A similarity analysis was performed on 311 surface isolates spectra and on 50 *A. nicotianae* as well as *B. linens* starters spectra. The cluster structure in the updated dendrogram remained about the same as shown in Figure 18, demonstrating that no similarity between the starters and isolates of the respective species was found.

Table 10: Fractions [%] of *A. nicotianae* and *B. linens* within the coryneform isolates. Fractions within 211/311 coryneform isolates. Sampling after 6, 9 and 28 days of ripening; $_T$: isolation from preparations on TSA; $_S$: isolation from preparations on PCA^{3+} using a spatula technique (for details see chapter 2.3); n.d.: not determined.

	6 d_T	9 d	9 d_S	9 d_T	28 d
A. nicotianae	48/45	91/n.d.	80/79	86/83	94/93
B. linens	52/55	9/n.d.	20/21	14/17	6/7

3.3.3 Typing of yeast isolates by molecular based methods

Mitochondrial DNA restriction fragment length polymorphism (RFLP)[9]

For yeast typing a total of 47 isolates from *D. hansenii* and *G. geotrichum* were subjected to mitochondrial DNA RFLP analyses. Cluster analyses on FTIR spectra resulted in selecting 30 representatives out of 469 IS1-surface isolates and 17 representatives out of 166 IS2-surface isolates. Furthermore, representatives of IS1- and IS2-starters assigned to both species were analyzed. Two different profiles were detected for *D. hansenii*. Both profiles were present within IS1-surface isolates whereas only one of these profiles could be assigned to IS2-surface isolates. The latter one also corresponded with those of IS1- and IS2-starters (Figure 19).

[9] Mitochondrial DNA RFLP studies were performed by Jérôme Mounier, Dairy Products Research Centre, Teagasc, Fermoy, Co. Cork, Ireland. Method used as described in Mounier et al. (2005).

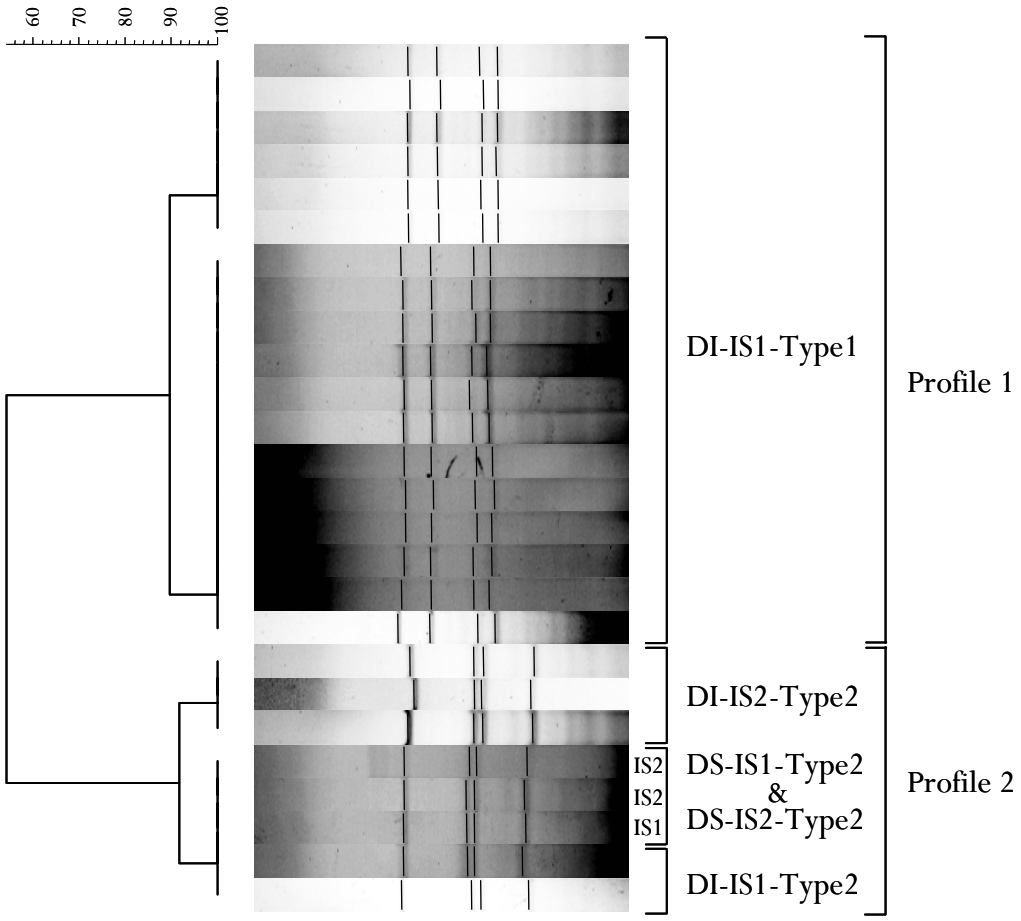

Figure 19: Mitochondrial DNA RFLP profiles of *D. hansenii* IS1- and IS2-surface isolates as well as *D. hansenii* IS1- and IS2-starters. Purified DNA was digested with *Hae*III. Similarities among band patterns were calculated using the Dice's similarity coefficient and the UPGMA algorithm. DI: *D. hansenii* isolated from cheese smear; DS: *D. hansenii* from smear starter culture; IS1, IS2: isolation series 1, 2. Colony morphology types found on YGCBA: type 1: light blue, rough colonies with a mat surface; type 2: colonies with a bright blue center surrounded by a white edge.

RFLP analysis on *G. geotrichum* surface isolates also resulted in two different profiles. Variations in one or two bands were not considered to be significant enough to split in different patterns. Representatives of IS1-surface isolates could be assigned to one of these profiles whereas IS2-isolates to both of them. The second profile was also found for representatives of IS1- and IS2-starters. For details see Figure 20.

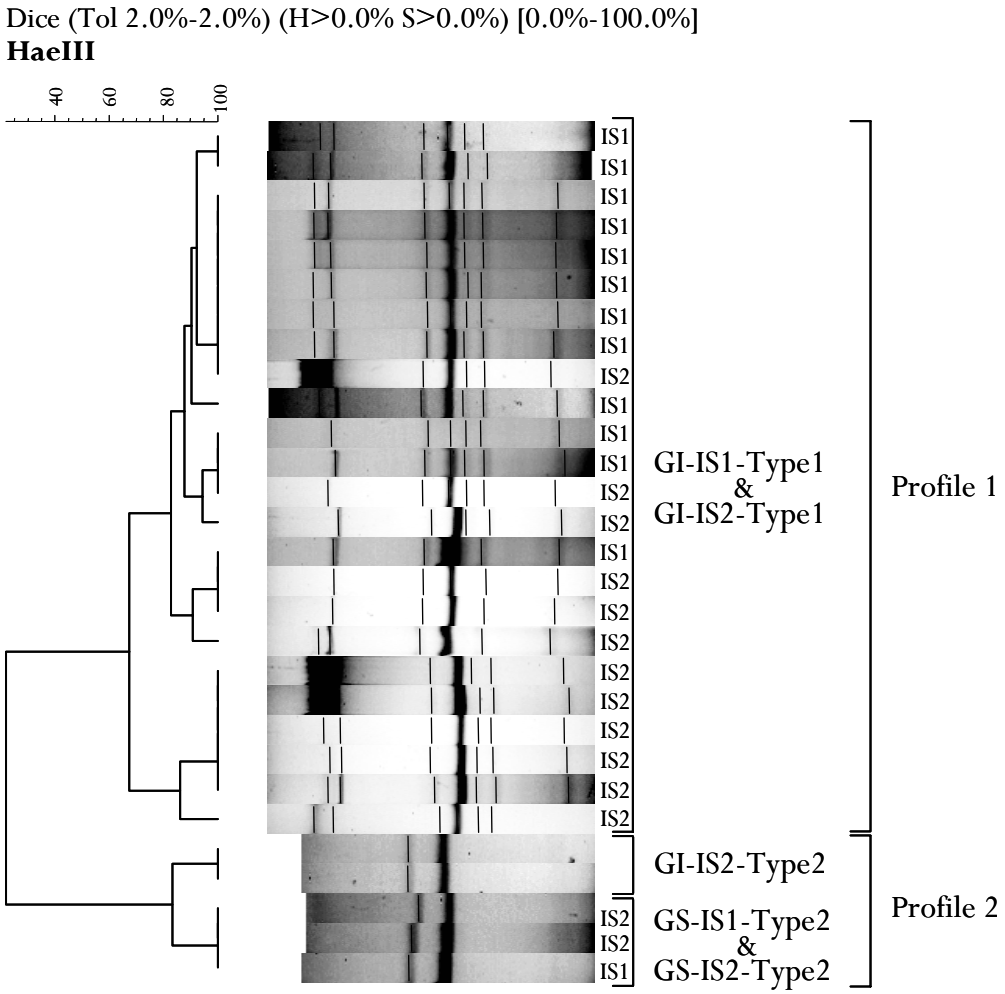

Figure 20: Mitochondrial DNA RFLP profiles of *G. geotrichum* IS1- and IS2-surface isolates as well as *G. geotrichum* IS1- and IS2-starters. Purified DNA was digested with *Hae*III. Similarities among band patterns were calculated using the Dice′s similarity coefficient and the UPGMA algorithm. GI: *G. geotrichum* isolated from cheese smear; GS: *G. geotrichum* from smear starter culture; IS1, IS2: isolation series 1, 2. Colony morphology types found on YGCBA: type 1: turquoise to blue colored colony surface; type 2: light green and fluffy colony surface.

Random amplification of polymorphic DNA (RAPD)-PCR[10]

In addition to the mtRFLP analysis, RAPD-PCR studies were performed on selected *G. geotrichum* IS1-surface isolates (10), IS2-surface isolates (11), IS1-starters (1) and IS2-starters (2). Two RAPD patterns could be distinguished which corresponded to the different colony morphology types observed for *G. geotrichum*. The first pattern was found within IS1- and IS2-surface isolates, the other one in IS2-surface isolates as well as in IS1 -and IS2-starters (Figure 21).

[10] RAPD studies were performed by Jérôme Mounier, Dairy Products Research Centre, Teagasc, Fermoy, Co. Cork, Ireland. Method used as described in Brennan et al. (2002).

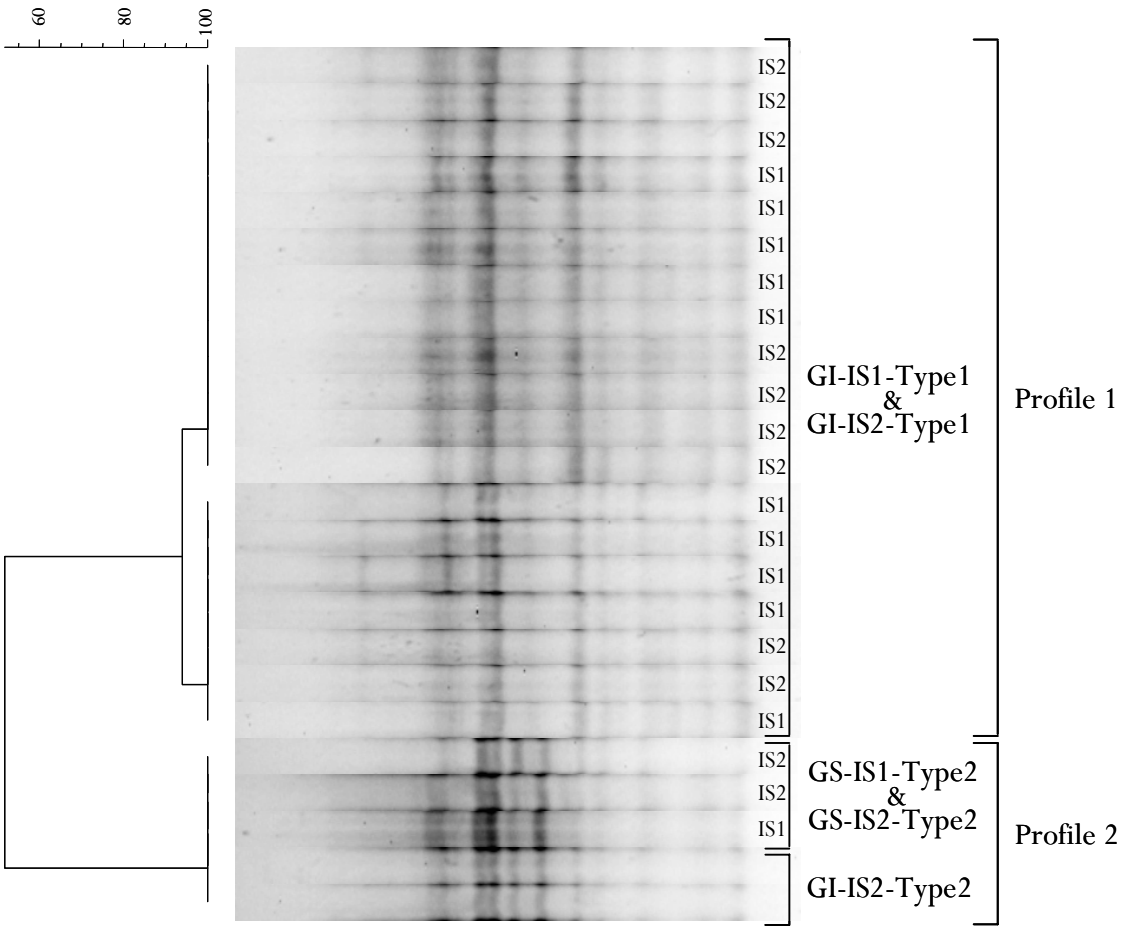

Figure 21: RAPD profiles of *G. geotrichum* IS1- and IS2-surface isolates as well as *G. geotrichum* IS1- and IS2-starters. M13 forward primer [5′-GTAAAACGACGGCCAGT-3′]. Similarities among band patterns were calculated using the Dice′s similarity coefficient and the UPGMA algorithm. GI: *G. geotrichum* isolated from cheese smear; GS: *G. geotrichum* from smear starter culture; IS1, IS2: isolation series 1, 2. Colony morphology types found on YGCBA: type 1: turquoise to blue colored colony surface; type 2: light green and fluffy colony surface.

3.3.4 Typing of bacterial isolates by molecular based methods

rep-PCR[11]

For studies on the bacterial biodiversity, BOX-PCR was used for identification of the IS1-surface isolates. This method was also performed on the bacterial smear IS1-starters. Just as the IS1-surface isolates, these smear starters, originally added as *A. nicotianae* and *B. linens*, were identified as the newly described species *A. arilaitensis* or *B. aurantiacum* (Gavrish et al.

[11] *rep*-PCR using BOX-primers was performed by Roberto Gelsomino, BCCM/LMG Bacteria Collection, University of Ghent, Belgium. Method used as described in Goerges et al. (submitted).

2004; Irlinger et al. 2005). However, their *rep*-patterns were different when compared to these of the respective surface IS1-isolates (Figure 22).

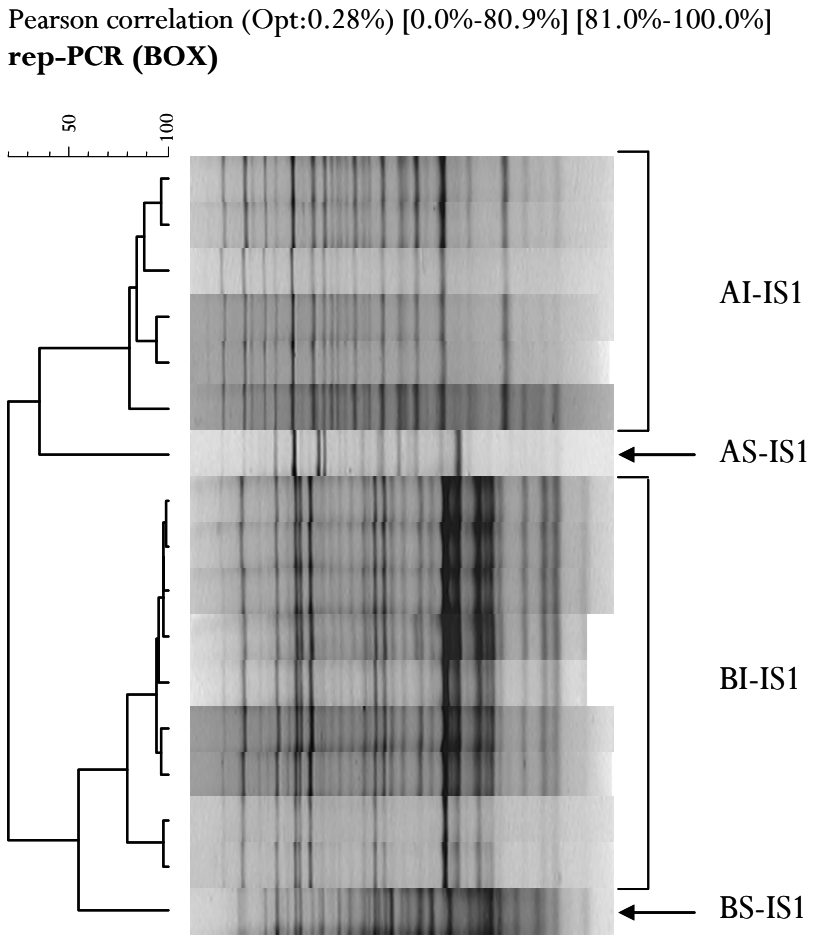

Figure 22: BOX-PCR patterns corresponding to *A. arilaitensis* IS1-surface isolates and *A. arilaitensis* IS1-starters as well as *B. aurantiacum* IS1-surface isolates and *B. aurantiacum* IS1-starters. Dendrogram obtained after numerical analysis of BOX-PCR patterns. Similarities among band patterns were calculated using the Pearson´s similarity coefficient and the UPGMA algorithm. AI: *A. arilaitensis* isolated from cheese smear; AS: *A. arilaitensis* from smear starter culture; BI: *B. aurantiacum* isolated from cheese smear; BS: *B. aurantiacum* from smear starter culture. IS1, IS2: isolation series 1, 2.

PFGE[12]

To further clarify the relationships between the bacterial surface isolates and smear starters, PFGE analyses were performed. Restriction digest of chromosomal DNA of 19 IS1-surface isolates and one representative of each IS1-starter was performed using the enzymes *Spe*I, *Asc*I and *Xba*I. For each enzyme two profiles were obtained within IS1-surface isolates which

[12] PFGE studies were performed by Mary Rea, Biotechnology Department, Moorepark Food Research Centre, Teagasc, Fermoy, Co. Cork, Ireland. Method used as described in Brennan et al. (2002).

corresponded to *A. arilaitensis* and *B. aurantiacum* (see chapter 3.2.6). Their profiles were different from these of the smear IS1-starters (Figure 23).

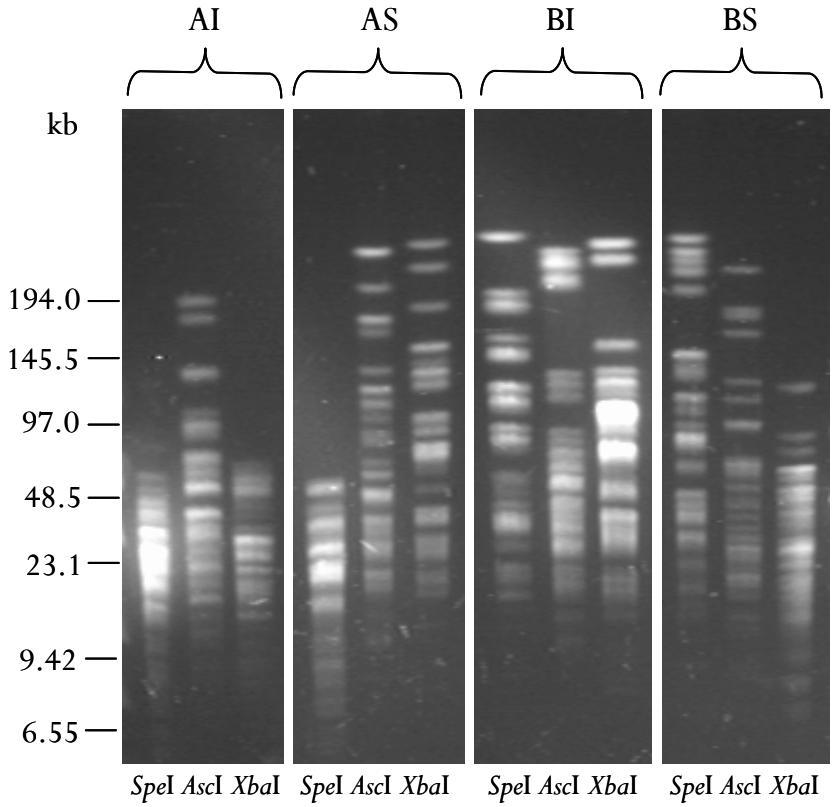

Figure 23: PFGE band patterns of *A. arilaitensis* IS1-surface isolates and *A. arilaitensis* IS1-starters as well as *B. aurantiacum* IS1-isolates and *B. aurantiacum* IS1-starters. AI: *A. arilaitensis* isolated from cheese smear; AS: *A. arilaitensis* from smear starter culture; BI: *B. aurantiacum* isolated from cheese smear; BS: *B. aurantiacum* from smear starter culture. Chromosomal DNA of the surface isolates and smear starters was independently digested using three enzymes, *Spe*I, *Asc*I and *Xba*I. Low Range PFG Marker (BioLabs® Inc., New England).

Further PFGE studies were done on 18 IS2-surface isolates and seven IS2-smear starters which were selected as representatives of different clusters obtained in similarity analyses based on FTIR spectra. According to the FTIR measurements, 15 representatives of IS2-surface isolates and three representatives of IS2-starters were assigned to *A. nicotianae*, the other representatives were identified as *B. linens*. Restriction digest on representatives of *A. nicotianae* and *B. linens* were performed using enzymes *Spe*I and *Asc*I, respectively, since these enzymes were considered to be most suitable for the respective species. As shown in Figure 24, four different PFGE profiles were obtained. For both species the profiles of IS2-surface isolates and IS2-starters did not correspond to each other. Identical profiles were

found for the isolates of both isolation series as well as for the starters of both isolation series (Figure 23 and Figure 24). Thus, isolates and smear starters of isolation series 2 could also be identified as the newly described species *A. arilaitensis* and *B. aurantiacum* (Figure 24).

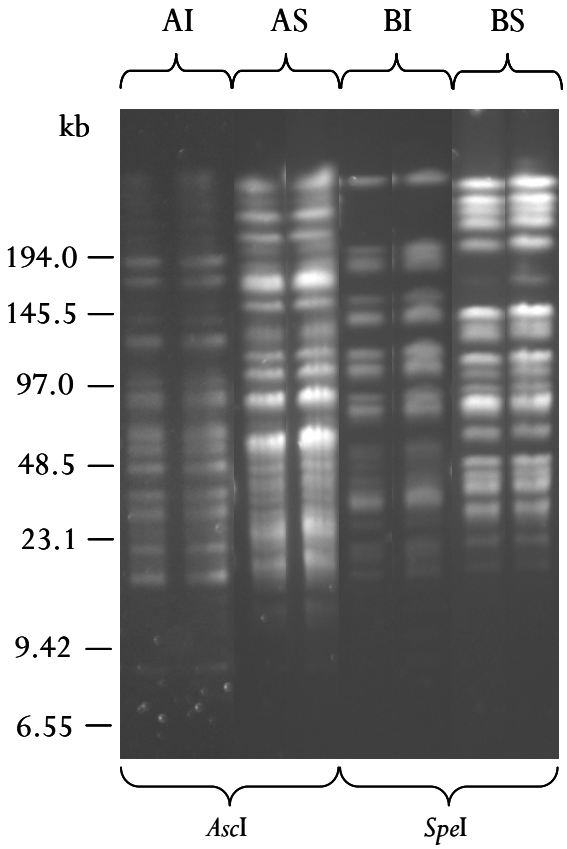

Figure 24: PFGE band patterns of *A. arilaitensis* IS2-surface isolates and *A. arilaitensis* IS2-starters as well as *B. aurantiacum* IS2-surface isolates and *B. aurantiacum* IS2-starters. AI: *A. arilaitensis* isolated from cheese smear, restriction digest of chromosomal DNA with *Asc*I; AS: *A. arilaitensis* from smear starter culture, restriction digest of chromosomal DNA with *Asc*I; BI: *B. aurantiacum* isolated from cheese smear, restriction digest of chromosomal DNA with *Spe*I; BS: *B. aurantiacum* from smear starter culture, restriction digest of chromosomal DNA with *Spe*I. Each pattern is shown for two representatives. Low Range PFG Marker (BioLabs® Inc., New England).

3.4 Anti-listerial activity of food-borne yeast

First, a membrane based screening assay was developed which was easy and fast to screen a high number of isolates within a short period of time. For selected isolates co-cultivation experiments and an assay for assessing killer toxin activity were carried out.

3.4.1 Agar-membrane screening assay

Preliminary experiments PE1 – PE3

For experiment PE1, 100 yeasts selected from the Weihenstephan yeast collection were screened against two *L. monocytogenes* indicator strains, WSLC 1001 and 1364. According to the different inhibition zones obtained a score-based evaluation system was developed ranging from zero (no inhibition) to five (very clear inhibition) (Figure 25).

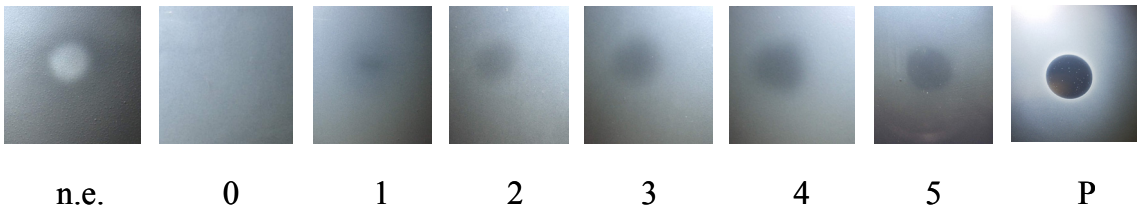

Figure 25: Score based evaluation system for anti-listerial activity of yeasts in an agar-membrane screening assay. Score 0: no inhibition; score 1: very weak inhibition; score 2: weak inhibition; score 3: moderate inhibition; score 4: clear inhibition; score 5: very clear inhibition; n.e.: not evaluable, white staining in the soft agar under the yeast spot grown on the nitrocellulose membrane; P: supernatant of *Lb. plantarum* containing pediocin applied onto the soft agar.

In PE1 the main culture was incubated 12, 24, and 48 h before spotted onto the autoclaved membrane lying on the solidified soft agar. Cultures incubated for 12 h spotted on the membrane often caused a white staining of the soft agar but no inhibition zone. Main cultures used after 48 h incubation resulted in too big macrocolonies on the membrane. Therefore, the 24 h incubation time was considered to be most suitable for this assay. Depending on the *Listeria* strain tested about 7 % of the yeast showed a clear and 1 % a very clear inhibition whereas around 47 % exhibited low to moderate inhibition. The other yeast strains could not be analyzed either due to poor growth on the membrane or white staining of the soft agar (Figure 25).

Two further screenings, PE2 and PE3, were performed to monitor the anti-listerial activity against different yeast strains and to check, at the same time, for reproducibility of the assay. Eighteen yeasts out of the 100 yeast strains examined before were selected for these approaches: Six with no or weak activity, six with moderate inhibitory potential and six with clear or very clear anti-listerial activity (appendix, Table 9). Their inhibitory properties were tested against six *L. monocytogenes* indicator strains, WSLC 1001, 1039, 1211, 1364, 1416 and 1685. In contrast to experiments PE1 and PE3 the membrane was not autoclaved in PE2. Depending on the *Listeria* indicator strain 58 % of the yeasts tested in PE2 showed a low to moderate anti-listerial activity, 22 % had a clear and 6 % a very clear inhibitory potential. In experiment PE3, a low to moderate inhibitory effect was ascribed to 56 % of the yeast strains, 10 % displayed a clear inhibition. None of the yeasts had a very clear anti-listerial potential. For the other yeasts screened in experiments PE2 an PE3 either no anti-listerial activity was observed or no evaluation was possible due to a white staining found on the soft agar surface. Five yeasts, *K. marxianus* WSYC 1, *C. parapsilosis* WSYC 51, *P. membranifaciens* WSYC 223, *I. orientalis* WSYC 263 and *I. occidentalis* WSYC 312, expressed the strongest inhibitory activity in both experiments.

Acidification test

To exclude an effect of acidification of the yeast liquid culture used for the agar-membrane screening assay the liquid medium for the cultivation of a selection of yeasts was buffered with Bis-Tris adjusted to pH 6.5. Without any buffering the liquid cultures reached about pH 4.5. However, there were no significant differences in inhibition detectable between buffered and unbuffered yeast cultures spotted on the membrane. The buffer solutions itself did not influence the listerial growth. Furthermore, YGB for the yeast cultivation acidified to pH 4.3 did not show any inhibitory effect when spotted on the membrane.

Reproducibility test

The reproducibility of the screening method was tested using a set of 22 yeasts consisting of strains used in the preliminary experiments and of isolates from different European cheeses (appendix, Table 10). The inhibitory potential of these yeasts against six *L. monocytogenes* indicator strains WSLC 1001, 1039, 1211, 1364, 1416 and 1685 was determined in four

repetitions. Table 11a - d list the anti-listerial activity of the ten most inhibitory yeasts against the six *Listeria* indicator strains in four repetitions. In four repetitions the same seven yeast strains showed the best anti-listerial activity five of which were the top five yeasts from the preliminary tests and did not originate from cheese. The other two strains, H-AG43 and YIV1L8, were isolated from an Austrian Graukäse and French Livarot cheese, respectively. The detailed results of the reproducibility experiment are listed in the appendix, Tables 11 to 13.

Table 11a-d: Anti-listerial activity of yeast against six different *L. monocytogenes* indicator strains, WSLC 1001, 1039, 1211, 1364, 1416 and 1685, in four independent experiments of the reproducibility test of the agar-membrane screening assay. Maximum value: 30; minimum value: 0; Seven yeasts with the best anti-listerial activity are highlighted in yellow. *Yeasts with a clear inhibitory activity in the preliminary experiments.

a)

No.	Strain	Species	Values in I
1	WSYC 223*	*P. membranifaciens*	28
2	WSYC 263*	*I. orientalis*	27
3	WSYC 312*	*I. occidentalis*	27
4	H-AG43	*I. occidentalis*	25
5	WSYC 51*	*C. parapsilosis*	22
6	WSYC 1*	*K. marxianus*	19
7	IV1L8	*Y. lipolytica*	18
8	IV2M20	*C. catenulata*	16
9	WSYC 122	*G. geotrichum*	14
10	2.23S2Y	*C. catenulata*	12

b)

Strain	Species	Values in II
WSYC 263*	*I. orientalis*	25
WSYC 51*	*C. parapsilosis*	23
WSYC 223*	*P. membranifaciens*	22
WSYC 312*	*I. occidentalis*	22
IV1L8	*Y. lipolytica*	21
WSYC 1*	*K. marxianus*	18
H-AG43	*I. occidentalis*	17
IV2M20	*C. catenulata*	17
WSYC 122	*G. geotrichum*	16
LM1E36	*D. hansenii*	12

c)

No.	Strain	Species	Values in III
1	WSYC 223*	*P. membranifaciens*	25
2	WSYC 312*	*I. occidentalis*	25
3	WSYC 263*	*I. orientalis*	22
4	WSYC 1*	*K. marxianus*	22
5	WSYC 51*	*C. parapsilosis*	22
6	IV1L8	*Y. lipolytica*	15
7	H-AG43	*I. occidentalis*	14
8	2.23S2Y	*C. catenulata*	13
9	LM1E36	*D. hansenii*	10
10	IV2M20	*C. catenulata*	9

d)

Strain	Species	Values in IV
WSYC 312*	*I. occidentalis*	26
WSYC 223*	*P. membranifaciens*	25
WSYC 263*	*I. orientalis*	24
H-AG43	*I. occidentalis*	24
WSYC 1*	*K. marxianus*	22
WSYC 51*	*C. parapsilosis*	21
IV1L8	*Y. lipolytica*	19
IV2M20	*C. catenulata*	16
WSYC 184	*D. hansenii*	13
LM1E36	*D. hansenii*	13

By summarizing the four values obtained for each yeast the scores of the total anti-listerial activity were calculated (maximum value 120). Values from zero to 100 were reached. The top seven yeasts got total scores from 73 to 100 (Table 12).

Table 12: Total anti-listerial activity of yeast against six different *L. monocytogenes* strains, WSLC 1001, 1039, 1211, 1364, 1416 and 1685, in the reproducibility test of the agar-membrane screening assay. Total anti-listerial activity given as sum of inhibition values obtained in four independent experiments (see Table 11). Maximum total score: 120; minimum total score: 0. Seven yeasts with the best anti-listerial activity are highlighted in yellow. *Yeasts with a clear inhibitory activity in the preliminary experiments.

No.	Strain	Species	Source	Scores of total anti-listerial activity
1	WSYC 223*	*Pichia membranifaciens*	yoghurt with fruit	100
2	WSYC 312*	*Issatchenkia occidentalis*	soil	100
3	WSYC 263*	*Issatchenkia orientalis*	fruit juice or berries	98
4	WSYC 51*	*Candida parapsilosis*	yoghurt	88
5	WSYC 1*	*Kluyveromyces marxianus*	milk products	81
6	H-AG43	*Issatchenkia occidentalis*	Graukäse	80
7	IV1L8	*Yarrowia lipolytica*	Livarot	73
8	IV2M20	*Candida catenulata*	Livarot	58
9	2.23S2Y	*Candida catenulata*	Gubbeen	50
10	WSYC 122	*Galactomyces geotrichum*	unknown	46
11	LM1E36	*Debaryomyces hansenii*	Limburger	40
12	LM1M24	*Galactomyces geotrichum*	Limburger	34
13	LM2E21	*Debaryomyces hansenii*	Limburger	24
14	WSYC 184	*Debaryomyces hansenii*	feta	21
15	G1077	*Pichia triangularis*	unknown	17
16	IV1M1	*Galactomyces geotrichum*	Livarot	17
17	WSYC 22	*Candida zeylanoides*	throat	16
18	WSYC 215	*Kluyveromyces marxianus*	feta	15
19	3.4S22Y	*Debaryomyces hansenii*	Gubbeen	12
20	H-2a21	*Trichosporon ovoides*	Austrian red smear cheese	11
21	1.37.10Y	*Clavispora lusitaniae*	Gubbeen	10
22	TS3M4	*Pichia triangularis*	Tilsit	0

Screening of different cheese isolates

Since the focus of the present study was set on finding anti-listerial yeast that originate from red smear cheeses, 87 yeasts from Limburger cheese and 100 yeasts from a German Almkäse were screened against the *Listeria* strains WSLC 1039, 1211, 1364, 1416 and 1685. None of

the Limburger yeasts and only one of the Almkäse isolates identified as *Y. lipolytica* showed a significant anti-listerial potential. Four out of five *Listeria* indicator strains were strongly inhibited in three independent screening approaches. To get a widespread overview of the anti-listerial potential from cheese yeasts, 117 yeasts isolated from 19 different European smear-ripened cheeses were screened against the same *Listeria* strains as mentioned above. Ten yeasts out of 117 were able to inhibit two or more *L. monocytogenes* indicator strains significantly in four repetitions, all ten isolates belonged to *C. intermedia* (Table 13). The ten yeasts were isolated from three different cheeses two of which were produced in the same dairy. One of them showed very strong activity against one *Listeria* indicator strain and strong activity against three others. Among the remaining nine yeasts one had a strong inhibitory potential against all *Listeria* strains tested, six against three *Listeria* strains and two yeasts against two different indicator strains. In the other cases they were moderate inhibitory. The isolates of most cheeses did not show a significant anti-listerial activity.

Table 13: Anti-listerial activity of ten *C. intermedia* strains isolated from different red smear cheeses against five *L. monocytogenes* indicator strains in the agar-membrane screening assay. Anti-listerial activity of each yeast strain against the individual *L. monocytogenes* indicator strains given as a sum of scores (see Figure 25) obtained in four independent experiments. Maximum value: 20; minimum value: 0. Sum of scores: 11 to 14 = moderate inhibition; 15 to 18 = strong inhibition; 19 and 20 = very strong inhibition. Total anti-listerial activity of each yeast given as a sum of inhibition values obtained for the different *L. monocytogenes* indicator strains. Maximum value: 100; minimum value: 0. Yeast code: Cheese number (see appendix, Table 3)_isolate number, WSYC: Weihenstephan Yeast Collection.

	L. monocytogenes indicator strain WSLC					
Yeast code	1039	1211	1364	1416	1685	Score of total anti-listerial activity
3_8	16	16	17	15	15	79
9_4 (WSYC 523)	15	16	20	15	13	79
3_4	14	16	16	16	14	76
3_5 (WSYC 517)	13	17	17	15	14	76
3_6	15	16	16	14	14	75
3_9 (WSYC 518)	15	15	17	14	14	75
9_7	14	15	17	16	13	75
5_10	16	13	16	15	14	74
3_19	16	14	15	14	12	71
3_20	15	14	15	13	11	68

3.4.2 Inhibition of *L. monocytogenes* in a yeast-co-cultivation assay

A total of 14 yeasts were selected from 117 European red smear cheese isolates for co-cultivation with *L. monocytogenes* WSLC 1364. This indicator strain proved to be very sensitive in the agar-membrane screening assay. The yeasts selected were from various species and showed different anti-listerial activity according to the agar-membrane screening assay. The influence of different yeasts on the growth of *L. monocytogenes* was examined on agar-pieces of which the *Listeria*-yeast lawn was removed and cell counts of both organisms were determined. Residual yeast and *Listeria* cell counts on the agar-pieces were also assayed. Since cell residues of both organisms were negligible, they were not determined in further experiments performed for reproducing the results and therefore were not included in the data presented below.

As shown in Figure 26, all yeasts tested in a 24 h co-cultivation with *L. monocytogenes* WSLC 1364 showed some inhibition compared to the controls where *Listeria* cells always grew up to around 5 x 10^7 cfu/cm^2. In the presence of up to 10^8 yeast cells per cm^2, *Listeria* cell counts were one to five log units lower than in the controls (Figure 26). In seven out of 14 cases, a variation in the *Listeria* cell counts per cm^2 of one log unit was observed in comparison to the control. Yeasts were assigned to *C. intermedia* (1), *Cl. lusitaniae* (1), *D. hansenii* (3), *S. unisporus* (1) and *Y. lipolytica* (1). One yeast, identified as *C. anglica*, repressed listerial growth by two log units (cfu/cm^2) and another yeast, identified as *C. intermedia*, caused a reduction by three log units (cfu/cm^2) when compared to the control. Four yeasts, belonging to *C. intermedia* (3) and *K. marxianus* (1) inhibited *Listeria* by around four log units (cfu/cm^2). The strongest inhibitory effect was observed in a yeast identified as *C. intermedia*. *Listeria* cell counts showed a difference of five log units (cfu/cm^2) to the control. Details are shown in Figure 26.

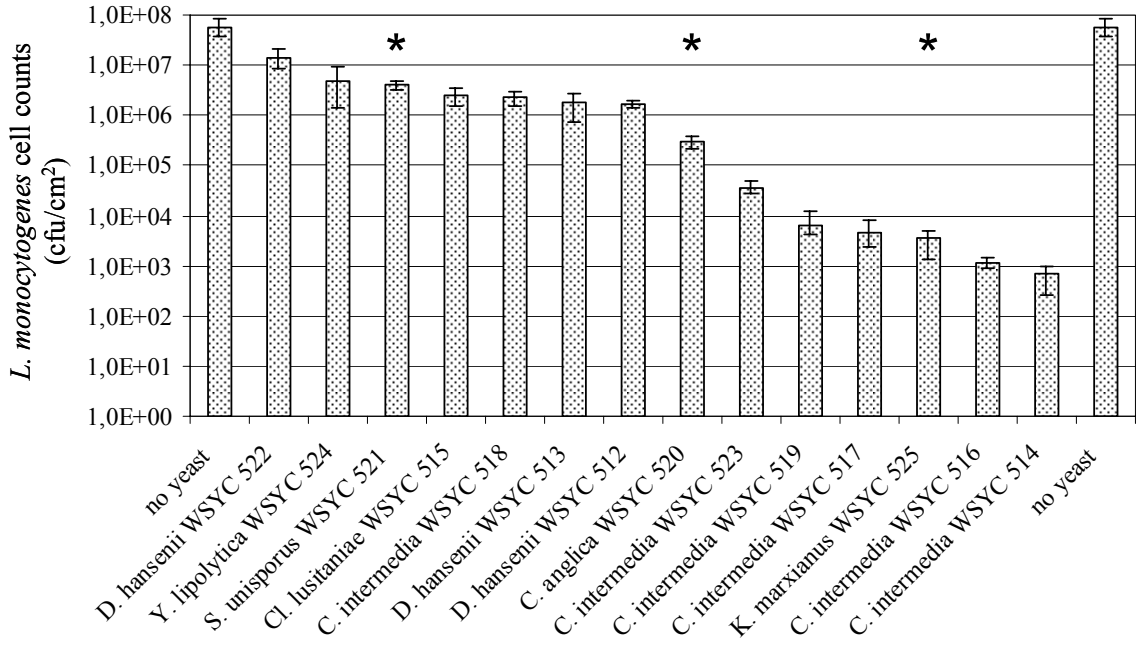

Figure 26: Inhibition of *L. monocytogenes* WSLC 1364 when co-cultured with yeast strains isolated from different European smear-ripened cheeses. Cell counts given as mean values of two to four independent experiments with error bars. *Yeasts in which a marginal inhibition zone was obtained in the "killer-toxin assay" (see chapter 3.4.3).

3.4.3 Test for killer activity

The 14 yeasts co-cultivated with *L. monocytogenes* WSLC 1364 were tested in an assay used for assessing killer toxin activity (Provost et al. 1995). In three yeasts (Figure 26), *C. anglica*, *K. marxianus* and *S. unisporus*, a very small inhibition area of 1 mm around the yeast cells grown on the soft agar surface was reproducibly observed.

4 Discussion

4.1 Characterization of yeast populations from different European smear-ripened cheeses

Within the SCM EU project 2491 yeasts isolated from Limburger, Reblochon, Livarot, Tilsit and Gubbeen cheese were identified and differentiated by FTIR spectroscopy. Based on these data, the composition of the yeast flora from these five cheeses was compared.

4.1.1 Species diversity on the individual cheeses

Only a few species occurred in significant numbers on the five red smear cheeses. Most species were isolated sporadically which was also observed in other studies (Bockelmann 2002a; Eliskases-Lechner and Ginzinger 1995b). All yeast species found are typically detectable on various cheeses, in cheese brines and in the dairy environment (Eliskases-Lechner and Ginzinger 1995b; Petersen et al. 2001; Prillinger et al. 1999; Rohm et al. 1992; Seiler 2002). *D. hansenii* was the only yeast found on all cheese surfaces. It was dominant on all cheeses with up to 98 % except on the French cheeses where *G. geotrichum* was the dominant yeast with 27 % and 61 %, respectively. The high prevalence of *D. hansenii* on different red smear cheeses has been often reported (Bockelmann 2002a; Bockelmann and Hoppe-Seyler 2001; Eliskases-Lechner and Ginzinger 1995b; Rohm et al. 1992; Valdés-Stauber et al. 1997). *G. geotrichum* is considered to play an important role especially on French smear-ripened cheeses (Busse 1989). Many studies involving this organism were initiated by French research groups (e.g. Aldarf et al. 2004; Boutrou and Gueguen 2005; Dubernet et al. 2002; Gente et al. 2002; Guichard and Bonnarme 2005; Marcellino et al. 2001) confirming its relevance in red smear cheese production or cheese production in general. Relevant fractions of *C. catenulata*, *Cl. lusitaniae*, *K. lactis* and *Y. lipolytica* were only found on some of the cheeses investigated (Table 6).

Compared to the other cheeses the lowest species diversity was found on Limburger cheese since only two species, *D. hansenii* and *G geotrichum*, were isolated from the cheese surfaces of all three batches. Also within the isolates obtained from Gubbeen cheese Batches 4 to 6 a high homogeneity was observed with *D. hansenii* accounting for 98 %. In Batches 1 to 3 *D. hansenii*

was one of the most prevalent yeast, but additionally, *C. catenulata* (33 %) and *Cl. lusitaniae* (16 %) were isolated, showing that species diversity on Gubbeen cheese has significantly changed within the six years interval between the production of the first and second three batches. The species diversity on Livarot, Reblochon and Tilsit cheese was considered to be rather high since in other studies, involving Tilsit, Limburger, Romadur and Weinkäse, a maximum of four species was commonly isolated (Eliskases-Lechner and Ginzinger 1995b; Valdés-Stauber et al. 1997). Livarot and Reblochon cheeses investigated in the present study were each produced from three different dairies which consequently led to more diversity as if several cheese batches from one dairy were examined as it was the case with Limburger and Gubbeen cheese. Tilsit yeasts were collected from cheeses produced in 15 different dairies resulting in an even higher diversity, although other species among *D. hansenii* only occurred in low fractions.

A particular problem became apparent in identifying Reblochon yeasts since no valid or definite identification result was obtained for around 51 % of the isolates using the current version of the FTIR reference spectra database. French project partners arranged for an additional identification by physiological tests (Robert et al. 1994; Robert et al. 1997) and/or sequencing of the D1/D2 region of the 26S rDNA (Decock et al. 2005) at BCCM/MUCL Mycothèque de l'Université catholique de Louvain. Due to this measure, approximately 13 % and 11 % of the 404 Reblochon isolates could be identified as *Saccharomyces servazzii* and *S. unisporus*, respectively, species which were recently assigned to the genus *Kazachstania* (Kurtzman 2003). Both species are typically isolated from kefir (Seiler 2002), however, *S. unisporus* is also occasionally isolated from cheese (Corsetti et al. 2001; Seiler 2002). These species were only represented by four or 23 spectra, respectively, in the FTIR reference database. In contrast to this, the database encompassed 327 spectra of *D. hansenii*. Oberreuter et al. (2002b) showed a correlation between the number of strains per species in the database and the percentage of correct identification. A correct identification is therefore more likely the better a species is represented in the library. Thirteen percent of all Reblochon yeasts belonged to *Saturnispora* sp.. No identification was possible on species level using physiological as well as molecular based methods. The majority of the *Saturnispora* sp. isolates were obtained from cheeses produced in dairy no. 2 showing gene sequence similarities to a

Saturnispora strain (CBS 5621; www.cbs.knaw.nl/databases/index.htm) isolated from sauerkraut. A small number of isolates from Reblochon cheeses produced in dairy no. 3 could also be identified as *Saturnispora* sp., however, it differed from the other *Saturnispora* sp. in its physiological reaction profile. Spectra of this genus were not at all represented in the current version of the FTIR reference library which explains the identification problem. Until now, species of this genus were not isolated from cheese surfaces. Through additional identification methods a minority of Reblochon isolates which could not be identified by FTIR spectroscopy were assigned to *C. anglica*, *C. atlantica*, *K. lactis* and *T. delbrueckii*. *C. anglica* was initially isolated from apple cider (Kurtzman et al. 2001) and *C. atlantica*, synonym *Trichosporon atlanticum* (Barnett et al. 2000), from a North-Atlantic shrimp egg (Siepmann and Höhnk 1962). Both species were not at all represented in the FTIR spectra database since the reference library was initially constructed for identifying yeast isolated from yoghurt and fruit. Apparently, there were no suitable reference strains included in the reference database to ensure a proper identification for the above mentioned representatives of *K. lactis* and *T. delbrueckii*. Approximately 10 % of the Reblochon yeasts still remain unidentified. A main part of the Reblochon yeast flora proved to be completely different compared to the other cheeses investigated and a particularly high species diversity was found. In contrast to Limburger, Tilsit and Gubbeen cheeses, Reblochon cheeses were exclusively made from raw milk which might explain the high diversity within the yeast flora since raw milk is an important source for microbial colonization of the cheese surface (Bockelmann and Hoppe-Seyler 2001). Significant differences in the microflora were also observed between Reblochon cheeses from the three dairies indicating that a specific house microflora of the manufacturers established on these cheese surfaces (Table 14). The house microflora significantly influences the composition of the surface flora of smear-ripened cheeses (Bockelmann 2002a; Mounier et al. submitted).

Table 14: Yeast species found on Reblochon cheeses produced by three different dairies. 1, 2, 3: dairies 1, 2, 3. *Yeasts especially isolated from cheeses produced in dairies 1 and 3 spectra of which clustered together.

Species	Cheeses obtained from dairy no.
Candida anglica	1, 3
Candida atlantica	2
Candida intermedia	2
Candida tenuis	2
Debaryomyces hansenii	2
Galactomyces geotrichum	1, 2, 3
Kluyveromyces lactis	1, 2, 3
Saccharomyces servazzii	2, 3
Saccharomyces unisporus	1
Saturnispora sp.	2, 3
Torulaspora delbrueckii	1, 2, 3
not identified*	1, 2, 3

Dairy dependent differences concerning the species composition were also obtained for Livarot cheeses of the present study. Interestingly, Livarot cheeses made from raw or pasteurized milk showed a comparable yeast flora complexity which emphasizes the influence of the house microflora. The relevance of an intact house microflora and starter cultures increased since the microflora from cheese milk has lost its importance due to pasteurization (Bockelmann and Hoppe-Seyler 2001).

4.1.2 Intraspecies variability

It was shown in chapter 4.1.1 that the species flora composition of the five cheeses varied in diversity. By evaluating the flora composition of the different cheeses aspects of intraspecies diversity were also included. Since *D. hansenii* and *G. geotrichum* were the most frequently occurring species on the cheeses investigated, studies on the intraspecies diversity were restricted to these two species. For this reason, cluster analyses were performed on a selection of FTIR spectra representing the *D. hansenii* as well as *G. geotrichum* flora of the individual cheeses.

D. hansenii

Table 15 shows a matrix illustrating the cluster structure in a dendrogram depicting the spectral similarities of *D. hansenii* representatives selected from all cheeses. Most often, cheese specific clusters were formed. Tilsit cheese spectra only formed five common clusters whereas 30 spectra appeared single clustered. A reason for this might be the high diversity within the Tilsit yeasts since isolates were collected from Tilsit produced by 15 different manufacturers. Generally, representatives for each cheese (Limburger, Reblochon, Livarot, Tilsit and Gubbeen cheese) were selected per cluster from a dendrogram including all yeast spectra of one cheese. The number of representatives increased by cluster size. Tilsit spectra were arranged in numerous small clusters or were even single clustered demonstrating a high diversity. Thus, only one or two representatives per cluster were chosen. In contrast to this, for Gubbeen or Limburger yeast which proved to be very homogeneous up to six spectra per cluster were selected. Depending on this procedure, the number of cheese-specific clusters varied in the dendrogram depicting FTIR spectral similarities of *D. hansenii* representatives from all cheeses.

As shown in Table 15 one single cluster contained spectra from three cheeses, Limburger, Tilsit and Livarot. Although *D. hansenii* was dominant on Limburger, Tilsit and Gubbeen cheese, only one common cluster including spectra from Limburger and Tilsit or Tilsit and Gubbeen cheese occurred. Both clusters contained two spectra, one spectrum assigned to each cheese. No similarity was found between isolates from Limburger and Gubbeen cheese. Interestingly, the representatives from the French cheeses did not show any similarity to each other.

Table 15: Cluster structure in a dendrogram depicting FTIR spectral similarities between *D. hansenii* representatives of five European red smear cheeses shown in a matrix. LM: Limburger; RB: Reblochon; IV: Livarot; TS: Tilsit; GB: Gubbeen. Number in black: number of clusters in which isolates spectra assigned to one cheese (grey background color), two or three cheeses (orange background color) are arranged; number in blue: number of single clustered isolates spectra assigned to the individual cheeses.

	LM	RB	IV	TS	GB	IV + TS
LM	6 + 4	-	1	1	-	1
RB	-	1	-	1	-	-
IV	1	-	1	-	-	-
TS	1	1	-	5 + 30	1	-
GB	-	-	-	1	16 + 12	-
IV + TS	1	-	-	-	-	-

G. geotrichum

Except for one cluster in which spectra from Livarot and Reblochon clustered together separate clusters were formed for spectral representatives of *G. geotrichum* from the different cheeses (Table 16). Representatives from Limburger cheese showed completely different colony morphologies compared to those obtained from Livarot and Reblochon. The single spectrum of a *G. geotrichum* isolate originating from Tilsit cheese did not show any similarity to the other spectra.

Table 16: Cluster structure in a dendrogram depicting FTIR spectral similarities between *G. geotrichum* representatives of five European red smear cheeses shown in a matrix. LM: Limburger; RB: Reblochon; IV: Livarot; TS: Tilsit; GB: Gubbeen. Number in black: number of clusters in which isolates spectra assigned to one cheese (grey background color) or two cheeses (orange background color) are arranged; number in blue: number of single clustered isolates spectra assigned to the individual cheeses.

	LM	RB	IV	TS	GB
LM	3 + 1	-	-	-	-
RB	-	1 + 2	1	-	-
IV	-	1	4 + 4	-	-
TS	-	-	-	1	-
GB	-	-	-	-	-

D. hansenii and *G. geotrichum* clearly play an important role on various red smear cheeses, but interestingly, isolates from individual cheeses rarely showed spectral similarity to each other indicating a certain individuality of each flora based on phenotypic characteristics. Generally, a high diversity was found within *D. hansenii* using phenotypic (Kümmerle et al. 1998; Wenning et al. 2002) as well as genotypic methods (Petersen and Jespersen 2004; Petersen et al. 2001). A high diversity was also attested for *Geotrichum candidum*, the anamorphic form of *G. geotrichum* (Gente et al. 2002; Marcellino et al. 2001).

4.2 Biodiversity of surface microbial consortia from Limburger cheese

A polyphasic approach was used in this study to get an overall picture of the intraspecies and interspecies diversity of the Limburger cheese ripening flora. The polyphasic approach builds upon the advantages of the different methods and, hence, provides round taxonomies (Goodfellow et al. 1997; Lombardi et al. 2002; Vandamme et al. 1996).

4.2.1 Yeast and bacterial cell counts

The yeast counts were similar to those recorded in earlier studies although the corresponding bacterial counts on the mature cheeses were lower (Bockelmann 2002a; Brennan et al. 2002; Carnio et al. 1999; Eliskases-Lechner and Ginzinger 1995b; Valdés-Stauber et al. 1997). The bacterial and yeast cell counts recorded for the middle stage of Batch 3 were one log unit higher than those from the other batches whereas values from the early and late stages were comparable. The cheeses showed different intensities of smear color on their upper and lower faces indicating slight differences in the smear development. This phenomenon may be attributed to more anaerobic conditions and consequently less growth of the obligate aerobes on the cheeses lying on the ripening shelfs. Perhaps the smear rind of the cheese investigated in the middle ripening stage of Batch 3 was better developed than in the other cases leading to a higher number of organisms being transferred in the cheese sampling.

Generally, ripening is characterized by an initial dominance of yeasts followed by bacterial dominance in the later stages of ripening (Busse 1989; Eliskases-Lechner and Ginzinger 1995b). However, in the present study yeasts were also dominant during the middle and late stages of the Batch 1 samples and were of the same order of magnitude as the bacteria within

corresponding stages of the Batch 2 samples. Bacterial dominance after the initial ripening phase was only observed in Batch 3 samples. Compared to the second and especially the third batch, the ripening of Batch 1 seemed to be slower since the cheese investigated at the first sampling point was less colored and had a more yeasty smell. A delayed pH increase might have caused a decreased development of the bacterial community.

4.2.2 Biodiversity within the yeast flora

The only yeast species detected were *D. hansenii* and *G. geotrichum*, a lower species diversity than found in previous studies (Rohm et al. 1992; Valdés-Stauber et al. 1997). Bockelmann (2002a) reported additional species on the surfaces of Limburger, Romadur and similar cheese varieties, but their numbers were quite low. It is possible that additional yeast species were present on the Limburger cheese investigated albeit at an order of magnitude that was not detectable using the current methods.

D. hansenii dominated each stage of ripening in all three batches, except for the early stage of Batch 2. This organism has been repeatedly found to be the most commonly isolated yeast on various cheeses (Bockelmann 2002a; Bockelmann and Hoppe-Seyler 2001; Eliskases-Lechner and Ginzinger 1995b; Rohm et al. 1992; Valdés-Stauber et al. 1997) and it is known to be a highly salt tolerant yeast that is often found in cheese brine (Kammerlehner 2003). Similarly, *G. geotrichum* is detected on cheeses (Rohm et al. 1992), especially on soft red smear cheeses such as Limburger and Romadur (Bockelmann 2002a). According to Bockelmann (2002a) commercial preparations of *G. geotrichum* are not commonly used as starter cultures in the production of Limburger or Romadur in Germany. However, in the present study a smear starter culture including *D. hansenii* and *Geotrichum candidum* (anamorphic form of *G. geotrichum*) among other organisms was added to the cheese milk.

Based on the analysis of FTIR measurements, the 469 yeast isolates were assigned to 3 major clusters which encompassed 9 subclusters (Figure 4), a result indicating a comparatively high degree of homogeneity. The *D. hansenii* isolates were assigned to two different groups, types 1 and 2, based on colony morphology. Type 2 was restricted to subcluster 6. These taxa

were also recognized on the basis of the physiological data. It seems likely from the FTIR data (Figure 4) that there exist at least five groups within *D. hansenii* type 1.

4.2.3 Biodiversity within the bacterial flora

Most of the bacterial strains isolated from the surface flora of the Limburger cheese samples were identified either as *A. arilaitensis* (Irlinger et al. 2005) or *B. aurantiacum* strains (Gavrish et al. 2004). Within the flora of another German red smear soft cheese, *B. linens* and *A. nicotianae* were the only coryneform species detectable (Maoz et al. 2003). This is comparable to the results from the present study since isolates today identified as *B. aurantiacum* were previously often assigned to *B. linens* because it was regarded to be the only orange-pigmented species within the genus *Brevibacterium* (Gavrish et al. 2004). Isolates in this study identified as *A. arilaitensis* were previously assigned to *A. nicotianae*. Additional investigations must clarify whether isolates from other red smear cheeses should be identified as *B. aurantiacum* and *A. arilaitensis* as well. Maoz et al. (2003) analyzed two different varieties of mature cheeses. *A. nicotianae* formed up to 90 % of the total bacterial population on one of them. Other groups have reported that *A. nicotianae* is an important member of ripening consortia, but none of them found this organism in these high numbers (Bockelmann and Hoppe-Seyler 2001; Eliskases-Lechner and Ginzinger 1995a; Valdés-Stauber et al. 1997). In the present study, *A. arilaitensis* dominated the middle and late stages of ripening in all of the cheese batches, as well as in the early stage of the third batch where it accounted for up to 82 % of the isolates. Feurer et al. (2004a) found *A. arilaitensis* to be the dominant species on a French smeared soft cheese made from pasteurized milk.

The only coryneform bacteria found on cheese 1E were *B. aurantiacum* strains. Members of this taxon were still dominant in the early stage of Batch 2 though *A. arilaitensis* strains formed 25 % of the bacterial community. *B. linens* strains usually represent 0 to 20 % of the bacterial populations found on smear-ripened cheeses (Bockelmann 2002a; Eliskases-Lechner and Ginzinger 1995a; Maoz et al. 2003). Previous reports of *B. linens* or *Brevibacterium* strains in the cheese microflora may well have been members of *B. aurantiacum*. Hence, the number of *B. aurantiacum* strains detected in the early stage of the first and second batches in the present study can be considered to be very high. *B. aurantiacum* strains seem to be well adapted to the

early ripening environment. However, in the third batch *A. arilaitensis* strains dominated from the earliest ripening stage.

The REP-studies[13] indicated a low intraspecies diversity amongst *B. aurantiacum* strains. In general, a high *B. linens* intraspecies diversity has been reported using 16S rDNA sequence data (Oberreuter et al. 2002a). However, since new orange-pigmented *B. linens*-like strains, including *B. aurantiacum*, have been described (Gavrish et al. 2004) it can be assumed that members of this species were also included in the study reported by Oberreuter et al. (2002a), thereby adding to the observed high heterogeneity. Similarly, *A. arilaitensis* isolates formed a homogeneous taxon based on the *rep*-PCR data.

The two BOX-PCR[14] patterns corresponded to the species *A. arilaitensis* and *B. aurantiacum*. The BOX-technique is generally considered to be highly discriminatory as strain specific patterns are generated (Hermans et al. 1995; Ivanova et al. 2002) hence the results of the present study provide further evidence that the *A. arilaitensis* and *B. aurantiacum* strains formed homogeneous groups. Similarly, strain-specific patterns were found when restriction digests of chromosomal DNA of 19 representative *A. arilaitensis* and *B. aurantiacum* isolates were subjected to PFGE studies, implying that the isolates represented single clones of the respective species. Comparable results were reported by Brennan et al. (2002) in a similar analysis of organisms isolated from an Irish smear-ripened cheese. Physiological investigations based on commercially available test kits, such as Biotype 100 (Biomerieux, France)[15], on the same set of isolates as for PFGE also revealed a high degree of homogeneity as there were only slight differences in physiological reactions.

Isolates assigned to the genus *Macrococcus* were only found on the cheese surface in the early stage of Batch 1 where they formed 10 % of the isolates. Little is known about the numbers or activities of members of *Macrococcus* spp. on cheese surfaces. Members of this taxon have

[13] *rep*-PCR with REP-primers was performed by Nagamani Bora, Microbial Resources Centre, School of Biology, University of Newcastle, UK. Method used as described in Goerges et al. (submitted).
[14] *rep*-PCR with BOX-primers was performed by Roberto Gelsomino, BCCM/LMG Bacteria Collection, University of Ghent, Belgium. Method used as described in Goerges et al. (submitted).
[15] Physiological tests using Biotype 100 (Biomerieux) were performed by Françoise Irlinger, INRA, Laboratoire de Génie et de Microbiologie des Procédés Alimentaire, Thiverval Grignon, France.

been reported in the curd of an Italian cheese (Randazzo et al. 2002) which was not smear-ripened.

Corynebacterium species were not detected in samples from any of the Limburger cheese batches even though corynebacteria have been reported to be the dominant organisms on surfaces of red smear cheeses (Bockelmann and Hoppe-Seyler 2001; Brennan et al. 2002; Maoz et al. 2003; Mayr et al. 2004; Oberreuter et al. 2003). Further studies are required to explain these apparently conflicting results.

4.2.4 Characterization of the microflora by a polyphasic approach

Polyphasic approaches have often been used to study microbial communities in food (Baruzzi et al. 2002; Cocolin et al. 2004; Corsetti et al. 2003; Ercolini et al. 2003; Nigatu et al. 2001; Randazzo et al. 2004; Randazzo et al. 2002) but these investigations dealt with lactic acid bacteria. Little is known about a polyphasic investigation of ripening organisms isolated from red smear cheeses (Brennan et al. 2002; Feurer et al. 2004a; Prillinger et al. 1999). The present study is the first comprehensive taxonomic study of the culturable bacterial and yeast flora isolated from smeared cheeses (Hohenegger et al.; Larpin et al.; Rea et al.; Thomas et al., manuscripts submitted). It was encouraging that in general the results from the applications of the different methods showed a high degree of congruence, as exemplified by FTIR spectroscopy and physiological data obtained on the yeasts. Similarly, the classification of the bacterial isolates as *A. arilaitensis* and *B. aurantiacum* was supported by genotypic and phenotypic data. The comparatively low taxonomic biodiversity of the microbial flora on Limburger cheese contrasts with corresponding data from European red smear cheeses (Hohenegger et al.; Larpin et al.; Rea et al.; Thomas et al., manuscripts submitted).

4.3 Incidence of commercial surface starter microorganisms during ripening of a Limburger cheese

For the production of the Limburger cheese a modified version of the traditional old-young smearing was used but, additionally, a commercial smear starter culture was added to the cheese milk to support the ripening process. According to the culture supplier, the

commercial mixed culture consisted of *D. hansenii*, *Geotrichum candidum*, *A. nicotianae* and *B. linens*. A total of four batches of that Limburger cheese were examined during different stages of ripening for the incidence of the four smear starter organisms. In the first three Limburger batches only one representative of each smear starter organism was available[16], whereas in the fourth batch 50 representatives of each starter[17] were available.

4.3.1 Incidence of the yeast starters

D. hansenii starter and a minority of IS1-isolates (21 isolates) from *D. hansenii* were both of type 2 colony morphology. They showed a high spectral similarity to each other which indicates that 21 representatives of the *D. hansenii* smear starter were recovered from the cheese surface of Limburger Batches 1 to 3. It was confirmed by mtDNA RFLP studies[18] that the *D. hansenii* starter strain was represented by these 21 isolates since the same profile was obtained (Figure 19). The dominant yeast found on Limburger cheeses of the three batches differed from the starter strain in its colony morphology (type 1) and its FTIR clusters were clearly separated from that of the starter strain (Figure 13). One RFLP pattern was found for all yeasts exhibiting type 1 colony morphology yielding one strain clearly distinguishable from the starter strain. In contrast to *D. hansenii*, no spectral similarities were found between the *G. geotrichum* starter and surface isolates from isolation series 1 identified as this species. This was confirmed by mtDNA RFLP as well as RAPD[19] studies which gave different profiles for the starter and for the surface isolates resulting in two different strains (Figure 20 and Figure 21). Furthermore, the two strains corresponded to the two different colony morphology types observed in *G. geotrichum*. Table 17 shows the fractions of the individual strains during ripening. The *D. hansenii* starter strain was represented by 4 % within all IS1-isolates or by around 6 % within IS1-isolates identified as *D. hansenii*. Although this strain was added to the cheese milk in high numbers, it was never able to compete successfully with the other yeasts in all of the three Limburger batches investigated. The non-starter strain of *D. hansenii* dominated the flora in all three batches during all stages of ripening with up to 95 % except

[16] Isolates and starters of the first isolation series are marked with the index IS1.
[17] Isolates and starters of the second isolation series are marked with the index IS2.
[18] Mitochondrial DNA RFLP studies were performed by Jérôme Mounier, Dairy Products Research Centre, Teagasc, Fermoy, Co. Cork, Ireland. Method used as described in Mounier et al. (2005).
[19] RAPD studies were performed by Jérôme Mounier, Dairy Products Research Centre, Teagasc, Fermoy, Co. Cork, Ireland. Method used as described in Brennan et al. (2002).

the early stage of Batch 2 where the non-starter strain of *G. geotrichum* dominated. The latter strain usually occurred in fractions of 5 to 25 % (Table 17).

Table 17: Fractions [%] of the *D. hansenii* and *G. geotrichum* strains found within IS1-isolates. 1, 2, 3: Batches 1, 2, 3; E, M, L: early, middle, late sampling point.

	1E	1M	1L	2E	2M	2L	3E	3M	3L
D. hansenii starter strain	14	2	12	4	-	4	-	-	5
other *D. hansenii* strain	74	78	66	37	75	78	95	90	79
G. geotrichum starter strain	-	-	-	-	-	-	-	-	-
other *G. geotrichum* strain	12	20	22	59	25	18	5	10	16

In the fourth Limburger batch, yeasts were isolated from cheeses after each smearing to see the influence of these treatments on the prevalence of the smear starters. Additionally, a cheese after 28 days of ripening was examined which corresponded to the time point chosen as middle stage of ripening for investigations on Limburger Batches 1 to 3. In contrast to the first three batches all *D. hansenii* isolates from the fourth batch represented the *D. hansenii* smear starter strain since FTIR spectra of IS2-isolates and -starters assigned to this species formed one common cluster and RFLP profiles corresponded to each other. A minority of IS2-isolates (22 isolates) represented the *G. geotrichum* starter strain as spectra of these isolates clustered together with IS2-starters spectra and at the same time their RFLP and RAPD profiles corresponded. The *G. geotrichum* starter strain belonging to the type 2 colony morphology showed a different RFLP as well as RAPD pattern than *G. geotrichum* IS2-isolates assigned to type 1 colony morphology indicating the affiliation to another strain. Also FTIR spectra of both *G. geotrichum* strains clustered separately from each other. Table 18 lists the fractions of the *D. hansenii* and *G. geotrichum* strains during different stages of ripening in the fourth Limburger batch. The *D. hansenii* starter strain was represented by 88 % within isolates from the cheese sample after the first smearing (3 d). It was isolated in 15 to 18 % in the following ripening stages. A similar order of magnitude was detected for the *G. geotrichum* starter strain. The *G. geotrichum* non – starter strain was only found from the second smearing (6 d) on and dominated with 62 to 78 %.

Table 18: Fractions [%] of the *D. hansenii* and *G. geotrichum* strains found within IS2-isolates. Sampling after 3, 6, 9 and 28 days of ripening.

	3 d	6 d	9 d	28 d
D. hansenii starter strain	88	16	15	18
other *D. hansenii* strain	-	-	-	-
G. geotrichum starter strain	12	6	15	20
other *G. geotrichum* strain	-	78	70	62

A succession in the yeast flora was also observed by Feurer et al. (2004b) in studies on a French soft red smear cheese. These authors, however, did not examine at the strain level. The yeasts of the smearing inoculum, *D. hansenii* and *Rhodotorula mucilaginosa*, established differently on the cheese surface although they were inoculated in about equal intensities. *D. hansenii* became dominant during ripening whereas *R. mucilaginosa* tended to disappear and was not detectable any more ten days after wrapping. Instead, *Geotrichum candidum* was found in minority although it was not inoculated. Petersen et al. (2002) investigated the microbial succession of *D. hansenii* strains on the cheese surface of a Danish red smear cheese during ripening. In contrast to the present study three *D. hansenii* strains were found on cheeses after the first smearing. Two of these strains were also detected in the inoculation slurry among them the *D. hansenii* starter strain which always occurred in minority. The dominant strain in the inoculation slurry and on cheeses after the first smearing was exclusively isolated from cheese surfaces during later stages of ripening. On an Irish smear-ripened cheese studied by Mounier et al. (submitted) the same *D. hansenii* strain highly dominated throughout the ripening period that was most abundant in the dairy environment. The yeast starter strain, however, was never reisolated from the cheese surface.

FTIR-, RFLP- as well as RAPD studies showed that both yeast starter strains remained the same within the three years in which the examinations on the four cheese batches were performed. But interestingly, the starter strains established differently on the cheese surfaces investigated in isolation series 1 and 2. The flora composition of the fourth batch has changed significantly compared to that of the first three batches: (i) the *D. hansenii* non-starter strain

which dominated IS1-isolates was not isolated from the cheese surfaces, (ii) no further *D. hansenii* strain was found among the starter strain, (iii) the *G. geotrichum* starter strain was isolated, though in minority and (iv) the *G. geotrichum* non-starter strain which remained the same over the three years interval dominated the flora of all cheeses investigated except after the first smearing. During this stage of ripening the *D. hansenii* starter strain dominated the flora with 88 %. In this case no comparison was possible to the first three batches since the earliest time point chosen for isolation series 1 was set only after the second smearing (Figure 1). From cheese surfaces during later ripening stages of Batch 4 the *D. hansenii* starter strain was isolated in 15 to 18 % which is in about the same order of magnitude as found for cheeses 1E and 1L in isolation series 1. Commonly found fractions of the *D. hansenii* starter strain in isolation series 1 were lower or this strain was not at all detectable with the used method.

4.3.2 Incidence of the bacterial starters

The surface isolates and starters of isolation series 1 and 2 were identified as *A. nicotianae* and *B. linens* using FTIR spectroscopy which was in agreement with the culture supplier. By *rep*-PCR studies[20] the surface isolates and the two bacterial smear starters of isolation series 1 were identified as two newly described species, *A. arilaitensis* (Irlinger et al. 2005) and *B. aurantiacum* (Gavrish et al. 2004). For the starters and surface isolates of isolation series 2 no *rep*-studies were enabled. However, according to comparison of PFGE profiles[21], starters and surface isolates of the second isolation series could also be assigned to *A. arilaitensis* and *B. aurantiacum*. Discrepancies between the results obtained by FTIR spectroscopy and molecular based methods occurred since no spectra of these new species were represented in the current FTIR references spectra database.

In contrast to the yeast smear starters the two bacterial smear starters were not found on any of the cheese batches investigated. It was apparent from similarity analyses of the FTIR spectra that the bacterial smear starter organisms were different from the surface isolates assigned to the same species. This was confirmed by *rep*- as well as PFGE studies since

[20] *rep*-PCR using BOX-primers was performed by Roberto Gelsomino, BCCM/LMG Bacteria Collection, University of Ghent, Belgium. Method used as described in Goerges et al. (submitted).
[21] PFGE studies were performed by Mary Rea, Biotechnology Department, Moorepark Food Research Centre, Teagasc, Fermoy, Co. Cork, Ireland. Method used as described in Brennan et al. (2002).

separate profiles were obtained for starters and surface isolates of the same species indicating the presence of two different strains. Brennan et al. (2002) reported an inhibitory effect of staphylococci and coryneforms against a starter culture strain which could not be recovered from the cheese surface at any time during ripening. In the present study, this aspect was not further examined. However, it is an interesting result that the starter culture did not succeed in the cheese environment. Mounier et al. (2005) examined the microflora of four Irish farmhouse smear-ripened cheeses at the mid-stage of ripening. Three of these cheeses were inoculated with commercial smear starters none of which were reisolated from the cheese surfaces. The findings of the different studies highlight the relevance of the adventitious, resident microflora. In studies of Mounier et al. (submitted) on sources of the adventitious microflora of an Irish smear-ripened cheese, the dairy environment and the skin of the cheese personnel proved to be important.

Since no starter organisms were found within IS1- and IS2-isolates the fractions of the two bacterial strains correspond to these given in chapters 3.2.4 and 3.3.2 with the difference that the surface isolates belong to the new species *A. arilaitensis* and *B. aurantiacum* instead of *A. nicotianae* and *B. linens*. The microflora of the four Limburger batches was characterized by one *A. arilaitensis* strain dominating all ripening stages examined except the earliest time points in Batches 1, 2 and 4 where a *B. aurantiacum* strain occurred in majority or in approximately equal fractions. In contrast to the yeast microflora no significant changes in the strain composition were noticed in isolation series 2 compared to isolation series 1.

4.3.3 Congruence and suitability of the phenotypic and genotypic methods used

By using phenotypic as well as genotypic methods, representatives of the four starter strains could be clearly distinguished from the respective surface isolates. However, it was not unusual that FTIR spectra of several clusters could be assigned to the same strain based on molecular methods (e.g. Figure 4, Figure 8 and Figure 18). FTIR spectra are whole-cell fingerprints that represent a total physico-chemical cell analysis of the individual organisms (Helm et al. 1991). Micro-evolutionary changes of the cellular overall characters recorded by FTIR spectroscopy result in certain variations of the spectra which may therefore cause a

splitting in several subclusters within one species (Kümmerle et al. 1998; Oberreuter et al. 2002b). It has been shown previously that the FTIR technique is not a reliable method to establish taxonomic relationships (Kümmerle et al. 1998; Oberreuter et al. 2002b). Nevertheless, FTIR spectroscopy is a suitable method to determine the incidence of smear starter organisms within isolates obtained from cheese surfaces. Cluster analyses using Ward′s cluster algorithm instead of Average linkage proved to be more informative for this purpose since the variance within each cluster is minimized leading to most homogeneous groups (Ward 1963). To distinguish strains based on genotypic markers, further methods have to be employed. Therefore, mtDNA RFLP, RAPD, BOX-PCR and PFGE analyses were additionally used to investigate yeast and bacteria. Petersen et al. (2002) examined the microbial succession of *D. hansenii* strains during the production of a Danbo-type cheese by mtDNA RFLP analysis. It was considered to be a suitable method to control the presence and growth of starter cultures. Mitochondrial DNA RFLP studies were also performed by Romano et al. (1996) for typing representatives of *C. zeylanoides* and *D. hansenii*. As shown in several studies, RAPD analysis also proved to be a reliable method for investigating genetic diversity within a species (Marcellino et al. 2001; Romano et al. 1996; Vasdinyei and Deak 2003). Romano et al. (1996) considered the mtDNA RFLP technique to be more discriminating. However, in the present study both methods lead to the same result. The suitability of BOX-PCR and PFGE analysis for identification on strain level was already discussed and shown in chapter 4.2.3.

4.3.4 Development and application of defined starter cultures

As shown in the fourth Limburger batch only the *D. hansenii* starter strain from the smear culture added to the cheese milk seemed to play a significant role, at least at the beginning of ripening. During later ripening stages the yeast starters at most played an inferior role and the bacterial starter strains were not recovered at any time point with the method used. Therefore, the application of this smear culture appeared not to be optimal. The relevance of this culture for the ripening process still has to be investigated. It might act as a booster when the commercial culture preparation is additionally added to the smear water. According to Bockelmann et al. (2005) appropriate surface cultures should be added to the cheese milk, the cheese brines and the smear water. Bockelmann et al. (2005) developed a defined culture

for soft cheeses composed of *Geotrichum candidum*, *D. hansenii*, *St. equorum*, *Microbacterium gubbeenense* or *A. nicotianae* and *B. linens*. As shown in the present study *St. equorum* is not a regularly found member of the soft cheese flora. However, the presence of *St. equorum* enables an accelerated deacidification and smear development (Bockelmann et al. 2005). For defined soft cheese cultures but also for semi-soft cheese cultures it proved to be advantageous for the deacidification to use a brine inoculated with *D. hansenii* and *St. equorum* and to exclude these organisms from the smear preparations (Bockelmann et al. 2005; Jaeger et al. 2002). Moreover, this reduces the strain number in the frequently applied smear inoculum which thus lowers the costs. In contrast to semi-soft cheeses *G. geotrichum* / *Geotrichum candidum* is a relevant component of the soft cheese microflora (Bockelmann 2002b; Bockelmann et al. 2005). Its presence in the cheese milk was found to be important for appearance and aroma development of Limburger cheese (Bockelmann 2003). In general, which is in contrast to the present study, no commercial preparations of this organism are used. Therefore, it is considered to be a typical yeast of the house flora (Bockelmann 2002a; Bockelmann et al. 2005).

Due to hygienic risks, e.g. contamination with pathogens such as *L. monocytogenes*, and increasing hygienic demands of European guidelines and regulations the old-young smearing process should be completely avoided and replaced by the application of defined starter cultures (Bockelmann 2002a,2002b; Bockelmann et al. 1997a; Bockelmann et al. 2005). These cultures have to be composed of strains that are able to compete with the adapted house flora so that they establish fast on the cheese surface and are still detectable on the surface of mature cheeses (Bockelmann 2002a, 2002b). Certain strains may not be suitable for smear cheese ripening (Bockelmann et al. 2005) as it was observed e.g. by Brennan et al. (2002) since they were not able to reisolate a commercial *B. linens* strain, nowadays identified as *B. aurantiacum* (Vancanneyt, personal communication), from green or mature cheeses. Another *B. linens* strain, however, was successfully used in a defined culture (Bockelmann 2002a; Bockelmann et al. 1997a; Bockelmann et al. 2005).

In general, most experiences exist about establishing defined smear starters for semi-soft cheeses (Bockelmann 2002b). Several studies were performed on Tilsit-type cheeses

concerning this topic (Bockelmann 2002a; Bockelmann et al. 2000; Bockelmann et al. 1997a; Hannon et al. 2004; Rademaker et al. 2005). Smear starter cultures for semi-soft cheeses should encompass *D. hansenii*, *St. equorum*, *Corynebacterium casei*, *M. gubbeenense* or *A. nicotianae*, and *B. linens* (Bockelmann et al. 2005). Production of Tilsit-type cheese using defined smear cultures was successfully performed under lab conditions and on pilot scale (Bockelmann et al. 2005; Hannon et al. 2004). In studies of Bockelmann and co-workers (Bockelmann 2002b; Bockelmann et al. 2005) it was shown that a culture composed of suitable strains is able to compete against the house microflora and to establish on the cheese surface. Defined smear cultures for semi-soft cheeses proved to be unsuitable for the ripening of soft cheeses which shows that an individual flora has to be developed for each cheese type (Bockelmann 2002b).

4.4 Anti-listerial activity of food-borne yeast

Since red smear cheeses are risk products for listerial contamination (Rudolf and Scherer 2001; Terplan et al. 1986), suitable counter measures are needed. One possibility is to combat *Listeria* directly on the cheese surface by ripening organisms producing anti-listerial substances. In contrast to previous studies (e.g. Boucabeille et al. 1997; Carnio et al. 1999; Carnio et al. 2000; Eppert et al. 1997; Maisnier-Patin and Richard 1995; Maoz et al. 2003; Motta and Brandelli 2002; Ryser et al. 1994; Valdés-Stauber et al. 1991; Valdés-Stauber and Scherer 1994) the present study was focused on detecting yeast with an inhibitory activity, especially those isolated from smear-ripened cheeses, since their application could antagonize *Listeria* in their initial stages of multiplication on cheese surfaces during the early ripening phases. Anti-listerial properties of yeast were evaluated using two different methods developed for this purpose.

4.4.1 Inhibition of *L. monocytogenes* in the agar-membrane based screening assay

Preliminary experiments PE1 - PE3

Strains showing a significant anti-listerial activity in experiment PE1 retained their inhibitory potential in all experiments. However, results obtained in approach PE2 were slightly better

than those of the same strains tested in PE1 and PE3. For experiment PE2 the nitrocellulose membrane was not autoclaved before use. By autoclaving, a shrinking of the membrane was observed which probably resulted in a reduction of the pore size, thus lowering the inhibitory effect. Further, yeast liquid precultures for experiments PE1 and PE2 were inoculated from fresh cultures grown on agar plates whereas precultures for PE3 were started from agar slant cultures stored at 4° C for two weeks. Possibly, this was the reason that some yeast strains showed less growth in the liquid pre- and main cultures as well as onto the membrane compared to the other two experiments. According to the preliminary experiments it seemed most suitable to use unautoclaved membrane for the rest of the experiments and to always start from fresh cultures grown on agar plates to insure proper yeast growth which is required to get a reproducible anti-listerial activity.

Reproducibility of the screening assay

The reproducibility of the screening assay was studied by investigating the anti-listerial activity of a test set of isolates containing yeasts from different sources against six different *L. monocytogenes* indicator strains. In four repetitions a high repeatability was found which was also confirmed by three further project partners. As shown in Table 19a - d screenings from all four partners yielded similar results. Identical strains were found under the top ten yeasts with only slight differences in the order in investigations of partner C (Table 19c) compared to the results of the present study (Table 19d). Six or eight strains from the top ten yeasts obtained in the present study also ranked among the ten yeasts expressing the strongest anti-listerial activity in examinations of the other two partners (Table 19a, b). The top five yeasts from the preliminary experiments as well as Graukäse isolate H-AG43 were represented among the yeasts possessing the best anti-listerial properties in all four partners′ screenings. Furthermore, in three of four cases, Livarot cheese isolate IV1L8 was in the top ten yeasts.

Table 19a-d: Total anti-listerial activity of yeast against six different *L. monocytogenes* strains in the agar-membrane screening assay. Total anti-listerial activity given as sum of inhibition values obtained for the individual yeast strains against different *L. monocytogenes* strains (WSLC 1001, 1039, 1211, 1364, 1416 and 1685) in four independent experiments. Maximum total score: 120; minimum total score: 0. a) to c): Results of the reproducibility test obtained by three partners of the SCM EU project; d) Results of the reproducibility test obtained in the present study. Strains ranking among the top ten yeasts in the present study are highlighted in yellow in tables listing the results of the different partners. *Yeasts with a clear inhibitory activity in the preliminary experiments.

a)

No.	Strain	Scores of total anti-listerial activity
1	**WSYC 312***	67
2	**WSYC 1***	66
3	**WSYC 263***	64
4	**WSYC 223***	59
5	**LM1E36**	53
6	**WSYC 51***	52
7	**H-AG43**	44
8	**1.37.10Y**	40
9	**IV2M20**	38
10	**IV1L8**	35

b)

Strain	Scores of total anti-listerial activity
WSYC 51*	105
WSYC 312*	101
WSYC 263*	97
WSYC 184*	96
WSYC 223*	95
WSYC 1*	92
H-AG43	91
LM1M24	74
LM2E21	69
LM1E36	62

c)

No.	Strain	Scores of total anti-listerial activity
1	**IV2M20**	83
2	**2.23S2Y**	65
3	**WSYC 1***	63
4	**WSYC 263***	60
5	**WSYC 312***	56
6	**WSYC 51***	54
7	**H-AG43**	50
8	**IV1L8**	50
9	**WSYC 223***	49
10	**WSYC 122**	33

d)

Strain	Scores of total anti-listerial activity
WSYC 223*	100
WSYC 312*	100
WSYC 263*	98
WSYC 51*	88
WSYC 1*	81
H-AG43	80
IV1L8	73
IV2M20	58
2.23S2Y	50
WSYC 122	46

Anti-listerial activity of different cheese yeasts

None of 87 Limburger isolates and only one out of 100 Almkäse yeasts exhibited anti-listerial activity. Compared to previously performed analyses, the fraction of inhibitory yeasts was quite low. The Limburger cheese investigated showed a very little diversity in its complement of yeasts as it contained only two species, *D. hansenii* and *G. geotrichum* (see chapters 3.1

and 3.2.2). In contrast, the Almkäse microbial flora consisted of four different species, *D. hansenii*, *Cl. lusitaniae*, *Tr. ovoides* and *Y. lipolytica*. However, in both cases *D. hansenii* clearly dominated within the tested isolates (appendix, Table 2). The high frequency by which *D. hansenii* occurs on red smear cheeses (Bockelmann 2002a; Bockelmann and Hoppe-Seyler 2001; Eliskases-Lechner and Ginzinger 1995b; Rohm et al. 1992; Valdés-Stauber et al. 1997), on cheeses in general (Pereira-Dias et al. 2000; Welthagen and Viljoen 1998) or in different dairy products (Rohm et al. 1992; Seiler 2002) has been often described. In total, 50 % of the 413 yeast strains examined in this study were assigned to *D. hansenii* and only one strain showed a noteworthy inhibition against two *L. monocytogenes* strains tested. The only isolate from Almkäse that effected the growth of *Listeria* was identified as *Y. lipolytica* and this species had a merely 2 % share from these cheese isolates investigated. Ten yeasts from 117 yeasts out of 19 different smeared cheeses were able to inhibit two or more *L. monocytogenes* indicator strains significantly in four repetitions. All ten yeasts belonged to *C. intermedia*. The ten yeasts were isolated from three different cheeses two of which were produced in the same dairy. The isolates of most cheeses did not show a significant anti-listerial activity. Ryser et al. (1994) investigated bacteria for their anti-listerial activity and found *Listeria* inhibitory isolates only on 15 out of 105 red smear cheeses.

In all experiments performed only a minority of screened yeast isolates showed a significant anti-listerial activity. The same is also known about cheese ripening bacteria inhibiting *Listeria* (Carnio et al. 1999; Ryser et al. 1994; Valdés-Stauber et al. 1991). However, within the inhibition zone caused by the yeast, growth of *L. monocytogenes* was still observed. A higher inhibition rate would have resulted in a transparent zone as it is observed when the supernatant of a *Lb. plantarum* strain including the anti-listerial pediocin is applied onto the soft agar surface (Figure 25).

Generally, it became clear in the agar-membrane based screening assay that an antagonistic potential could be ascribed to different species which also resulted from screenings of ripening bacteria (Carnio et al. 1999; Ryser et al. 1994; Valdés-Stauber et al. 1991). The inhibitory potential of yeasts was clearly strain dependent (compare the two *K. marxianus* strains in Table 12). Similar results were obtained for ripening bacteria (Carnio et al. 1999;

Valdés-Stauber et al. 1991). The *Listeria* indicator strains showed different sensitivity against the inhibitory substance(s) as also observed by Carnio et al. (1999) and Valdés-Stauber et al. (1991) in studies on anti-listerial coryneform bacteria. One *L. monocytogenes* indicator strain (WSLC 1685) proving to be most sensitive against the inhibitory substance(s) produced by different yeast lost its sensitive properties after several experiments.

4.4.2 Inhibition of *L. monocytogenes* in the co-cultivation assay

Fourteen yeasts out of 117 European red smear cheese isolates were co-cultivated with *L. monocytogenes* WSLC 1364. All yeasts tested showed some inhibition of *L. monocytogenes* compared to the controls where *Listeria* reached 5 x 10^7 cfu/cm^2. In the presence of up to 10^8 yeast cells per cm^2, *Listeria* cell counts of one to five log units lower than in the controls were found, thus differences in the anti-listerial potential were not due to different yeast cell numbers. Half of the yeasts investigated repressed listerial growth by one log unit per cm^2 compared to the control which might be due to competition for nutrients. This weak growth reduction has to be taken into account in evaluating the inhibitory potential of the individual yeasts so that an inhibition of four instead of five log units per cm^2 was obtained as the strongest inhibitory effect in these experiments expressed by a *C. intermedia*. Further four yeasts, belonging to *C. intermedia* and *K. marxianus*, inhibited *Listeria* by three log units (cfu/cm^2).

The inhibitory potential of yeast against *Listeria* was clearly pronounced in the co-cultivation experiment in which conditions (direct contact of yeast and *Listeria* cells) and contamination rates were chosen as they are found on smear cheese surfaces (e.g. Rudolf and Scherer 2001). This is important for a potential application of anti-listerial yeast in red smear cheese microbial consortia.

4.4.3 Inhibition of *L. monocytogenes* by using a "killer-toxin" assay

An assay according to Provost et al. (1995) was performed for assessing a killer-toxin activity. In three out of 14 yeast isolates a slight inhibition zone around the yeast cells grown on the agar was observed. However, Provost et al. (1995) reported very large, clearly visible

inhibition zones surrounding the killer-toxin producing yeast. The marginal inhibition zones obtained in the "killer-toxin assay" did not correlate with the inhibition found in the agar-membrane assay or with the co-cultivation assay (Table 20).

Table 20: The inhibitory potential of three yeasts in the agar-membrane screening assay, co-cultivation assay and "killer-toxin" assay. [a]Average scores obtained in four independent experiments according to the score based system (Figure 25). [b]Average reduction of *Listeria* cell counts obtained in two to three independent experiments compared to the controls and minus a reduction of 1 log unit which was most probably caused due to competition for nutrients. [c]Inhibition zone around the yeast cells [mm].

Strain	Species	Agar-membrane assay[a]	Co-cultivation assay[b]	"Killer-toxin" assay[c]
WSYC 520	*C. anglica*	score 3	1 log units/cm^2	1
WSYC 521	*S. unisporus*	score 2 - 3	-	1
WSYC 525	*K. marxianus*	score 2/n.e.	3 log units/cm^2	1

Since the active substance did not show a visible capacity for diffusion it is suggested that the inhibitory principle is not of a bacteriocin- or killer-toxin-type.

4.5 Outlook

Red smear cheeses are often contaminated with *L. monocytogenes*. Food safety has to be increased in order to avoid health risks for consumers, but also to protect cheese producers from financial losses jeopardizing their existence. It would be useful to develop a defined ripening culture including anti-listerial GRAS (generally recognized as safe) organisms. Knowledge about the flora composition and dynamics during the ripening of various smear-ripened cheeses obtained in this study might contribute to this project. Using a defined, anti-listerial ripening culture would be a promising tool for prevention as well as crisis intervention in red smear cheese producing companies. The risky old-young smearing technique could be replaced by a safe procedure also insuring a high quality product. If a listerial contamination occurs in spite of this precaution, the anti-listerial organisms included in the ripening culture will be able to promptly antagonize it. Anti-listerial yeasts are especially suitable for this application since yeasts are dominant during the early ripening stages and are therefore able to combat *Listeria* from the time of contamination usually caused

by the first smear treatments. However, comprehensive studies are required to test the suitability of the anti-listerial yeast to be included in a defined culture. The anti-listerial yeast has to establish sufficiently on the cheese surface but without a negative effect on the ripening process or the growth of other ripening organisms. For an application of anti-listerial yeast in food it is further important to characterize the compound(s) acting against *L. monocytogenes*. Since the efficiency of a protective culture could be significantly reduced by mutants resistant to the inhibitory substance(s) investigations on the frequency of their formation and their stability are necessary to estimate their ability to assert itself in the cheese ripening environment.

5 References

Abee T., Krockel L. and Hill C. 1995. Bacteriocins: modes of action and potentials in food preservation and control of food poisoning. Int. J. Food Microbiol. 28: 169-185.

Aldarf M., Fourcade F., Amrane A. and Prigent Y. 2004. Diffusion of lactate and ammonium in relation to growth of *Geotrichum candidum* at the surface of solid media. Biotechnol. Bioeng. 87: 69-80.

Altenburger P., Kämpfer P., Akimov V.N., Lubitz W. and Busse H.-J. 1997. Polyamine distribution in actinomycetes with group B peptidoglycan and species of the genera *Brevibacterium*, *Corynebacterium*, and *Tsukamurella*. Int. J. Syst. Bacteriol. 49: 270-277.

Andrighetto C., Psomas E., Tzanetakis N., Suzzi G. and Lombardi A. 2000. Randomly amplified polymorphic DNA (RAPD) PCR for the identification of yeasts isolated from dairy products. Lett. Appl. Microbiol. 30: 5-9.

Arias C.R., Burns J.K., Friedrich L.M., Goodrich R.M. and Parish M.E. 2002. Yeast species associated with orange juice: evaluation of different identification methods. Appl. Environ. Microbiol. 68: 1955-1961.

Baleiras Couto M.M., van der Vossen J.M.B.M., Hofstra H. and Huis in 't Veld J.H.J. 1994. RAPD analysis: a rapid technique for differentiation of spoilage yeasts. Int. J. Food Microbiol. 24: 249-260.

Barefoot S.F. and Klaenhammer T.R. 1983. Detection and activity of lactacin B, a bacteriocin produced by Lactobacillus acidophilus. Appl. Environ. Microbiol. 45: 1808-1815.

Barker C. and Park S.F. 2001. Sensitization of *Listeria monocytogenes* to low pH, organic acids, and osmotic stress by ethanol. Appl. Environ. Microbiol. 67: 1594-1600.

Barnett J.A. 1992. The taxonomy of the genus *Saccharomyces* Meyen *ex* Reess: a short review for non-taxonomists. Yeast 8: 1-23.

Barnett J.A., Payne R.W. and Yarrow D. 2000. Yeasts: Characteristics and identification. Cambridge University Press, Cambridge, UK.

Baruzzi F., Matarante A., Morea M. and Cocconcelli P.S. 2002. Microbial community dynamics during the Scamorza Altamurana cheese natural fermentation. J. Dairy Sci. 85: 1390-1397.

Bereksi N., Gavini F., Benezech T. and Faille C. 2002. Growth, morphology and surface properties of *Listeria monocytogenes* Scott A and LO28 under saline and acid environments. J. Appl. Microbiol. 92: 556-565.

Beresford T. and Williams A. 2004. The microbiology of cheese ripening. In: Fox PF, McSweeney PLH, Cogan TM & Guinee TP (eds.), Cheese - chemistry, physics and microbiology. Elsevier Academic Press, Amsterdam, pp. 287-317.

Bernard K., Bellefeuille M., Hollis D.G., Daneshvar M.I. and Moss C.W. 1994. Cellular fatty acid composition and phenotypic and cultural characterization of CDC fermentative coryneform groups 3 and 5. J. Clin. Microbiol. 32: 1217-1222.

Bilinski C.A., Innamorato G. and Stewart G.G. 1985. Identification and characterization of antimicrobial activity in two yeast genera. Appl. Environ. Microbiol. 50: 1330-1332.

Bockelmann W. 2002a. Development of defined surface starter cultures for the ripening of smear cheeses. Int. Dairy J. 12: 123-131.

Bockelmann W. 2002b. Kulturenentwicklung geschmierter Käse (Schnittkäse, Weichkäse, Sauermilchkäse). DMZ Lebensmittel und Milchwirtschaft 3: 93-99.

Bockelmann W. 2003. The production of smear cheeses. In: Smit G & Dodds F (eds.), Maximising quality. Woodhead Publishing Ltd., Cambridge, GB, pp. 470-497.

Bockelmann W. and Hoppe-Seyler T. 2001. The surface flora of bacterial smear-ripened cheeses from cow's and goat's milk. Int. Dairy J. 11: 307-314.

Bockelmann W., Hoppe S.T., Jaeger B. and Heller K.J. 2000. Small scale cheese ripening of bacterial smear cheeses. Milchwissenschaft 55: 621-624.

Bockelmann W., Hoppe S.T., Krusch U., Hoffmann W. and Heller K.J. 1997a. The microflora of Tilsit cheese. Part 2. Development of a surface smear starter culture. Nahrung 41: 213-218.

Bockelmann W., Krusch U., Engel G., Klijn N., Smit G. and Heller K.J. 1997b. The microflora of Tilsit cheese. Part 1. Variability of the smear flora. Nahrung 41: 208-212.

Bockelmann W., Willems K.P., Neve H. and Heller K.J. 2005. Cultures for the ripening of smear cheeses. Int. Dairy J. 15: 719-732.

Boucabeille C., Mengin-Lecreulx D., Henckes G., Simonet J.M. and van Heijenoort J. 1997. Antibacterial and hemolytic activities of linenscin OC2, a hydrophobic substance produced by *Brevibacterium linens* OC2. FEMS Microbiol. Lett. 153: 295-301.

Boutrou R. and Gueguen M. 2005. Interests in *Geotrichum candidum* for cheese technology. Int. J. Food Microbiol. 102: 1-20.

Bredholt S., Nesbakken T. and Holck A. 2001. Industrial application of an antilisterial strain of *Lactobacillus sakei* as a protective culture and its effect on the sensory acceptability of cooked, sliced, vacuum-packaged meats. Int. J. Food Microbiol. 66: 191-196.

Brennan N.M., Cogan T.M., Loessner M. and Scherer S. 2004. Bacterial surface-ripened cheeses. In: Fox PF, McSweeney PLH, Cogan TM & Guinee TP (eds.), Cheese - chemistry, physics and microbiology. Elsevier Academic Press, Amsterdam, pp. 199-225.

Brennan N.M., Ward A.C., Beresford T.P., Fox P.F., Goodfellow M. and Cogan T.M. 2002. Biodiversity of the bacterial flora on the surface of a smear cheese. Appl. Environ. Microbiol. 68: 820-830.

Brown J.M., Frazier R.P., Morey R.E., Steigerwalt A.G., Pellegrini G.J., Daneshvar M.I., Hollis D.G. and McNeil M.M. 2005. Phenotypic and Genetic Characterization of Clinical Isolates of CDC Coryneform Group A-3: Proposal of a New Species of *Cellulomonas*, *Cellulomonas denverensis* sp. nov. J. Clin. Microbiol. 43: 1732-1737.

Buchanan R.E., Gibbons N.E., Cowan S.T., Holt J.G., Liston J., Murray R.G.E., Niven C.F., Ravin A.W. and Stanier R.Y. (Editors), 1974. Bergey′s Manual of Determinative Bacteriology. 8th edition. Williams & and Wilkins, Baltimore, Maryland, USA.

Budak A., Sahl H.G. and Brandis H. 1982. Antibacterial activities of *Candida* yeasts. Partial purification and characterization of the active substance of *Candida guilliermondii*. Zentralbl. Bakteriol. Mikrobiol. Hyg. [A] 253: 390-396.

Busse H.J. and Schumann P. 1999. Polyamine profiles within genera of the class Actinobacteria with LL-diaminopimelic acid in the peptidoglycan. Int. J. Syst. Bacteriol. 49: 179-184.

Busse M. 1989. Die Oberflächenflora von geschmiertem Käse. Milchwissenschaft 99: 137-141.

Carnio M.C., Eppert I. and Scherer S. 1999. Analysis of the bacterial surface ripening flora of German and French smeared cheeses with respect to their anti-listerial potential. Int. J. Food Microbiol. 47: 89-97.

Carnio M.C., Höltzel A., Rudolf M., Henle T., Jung G. and Scherer S. 2000. The macrocyclic peptide antibiotic micrococcin P_1 is secreted by the food-borne bacterium *Staphylococcus equorum* WS 2733 and inhibits *Listeria monocytogenes* on soft cheese. Appl. Environ. Microbiol. 66: 2378-2384.

Cleveland J., Montville T.J., Nes I.F. and Chikindas M.L. 2001. Bacteriocins: safe, natural antimicrobials for food preservation. Int. J. Food Microbiol. 71: 1-20.

Cocolin L., Rantsiou K., Iacumin L., Urso R., Cantoni C. and Comi G. 2004. Study of the ecology of fresh sausages and characterization of populations of lactic acid bacteria by molecular methods. Appl Environ Microbiol 70: 1883-1894.

Corsetti A., De Angelis M., Dellaglio F., Paparella A., Fox P.F., Settanni L. and Gobbetti M. 2003. Characterization of sourdough lactic acid bacteria based on genotypic and cell-wall protein analyses. J Appl Microbiol 94: 641-654.

Corsetti A., Rossi J. and Gobbetti M. 2001. Interactions between yeasts and bacteria in the smear surface-ripened cheeses. Int. J. Food Microbiol. 69: 1-10.

Cotter P.D. and Hill C. 2003. Surviving the acid test: responses of gram-positive bacteria to low pH. Microbiol. Mol. Biol. Rev. 67: 429-453, table of contents.

Cure G.L. and Keddie R.M. 1973. Methods for the morphological examination of aerobic coryneform bacteria. In: Board RG & Lovelock DW (eds.), Sampling - microbiological monitoring of environments - 1973. Academic Press, London, United Kingdom, pp. 123-135.

Daneshvar M.I., Hollis D.G., Weyant R.S., Jordan J.G., MacGregor J.P., Morey R.E., Whitney A.M., Brenner D.J., Steigerwalt A.G., Helsel L.O., Raney P.M., Patel J.B., Levett P.N. and Brown J.M. 2004. Identification of Some Charcoal-Black-Pigmented CDC Fermentative Coryneform Group 4 Isolates as *Rothia dentocariosa* and Some as *Corynebacterium aurimucosum*: Proposal of Rothia dentocariosa emend. Georg and Brown 1967, *Corynebacterium aurimucosum* emend. Yassin et al. 2002, and *Corynebacterium nigricans* Shukla et al. 2003 pro synon. *Corynebacterium aurimucosum*. J. Clin. Microbiol. 42: 4189-4198.

de Briel D., Couderc F., Riegel P., Jehl F. and Minck R. 1992. High-performance liquid chromatography of corynomycolic acids as a tool in identification of *Corynebacterium* species and related organisms. J. Clin. Microbiol. 30: 1407-1417.

Deák T. and Beuchat L.R. 1995. Evaluation of the MicroScan enzyme-based system for the identification of foodborne yeasts. J. Appl. Bacteriol. 79: 439-446.

Decock C., Bitew A. and Castillo G. 2005. *Fomitiporia tenuis* and *Fomitiporia aethiopica* (Basidiomycetes, Hymenochaetales), two undescribed species from the Ethiopian highlands: taxonomy and phylogeny. Mycologia 97: 121-129.

Dieuleveux V., Rarah R.A.M., Chataud J. and Gueguen M. 1997. Inhibition of *Listeria monocytogenes* by *Geotrichum candidum*. Microbiol. Aliment. Nutrit. 15: 147-156.

Dlauchy D., Tornai-Lehoczki J. and Peter G. 1999. Restriction enzyme analysis of PCR amplified rDNA as a taxonomic tool in yeast identification. Syst. Appl. Microbiol. 22: 445-453.

Dubernet S., Panoff J.M., Thammavongs B. and Gueguen M. 2002. Nystatin and osmotica as chemical enhancers of the phenotypic adaptation to freeze-thaw stress in *Geotrichum candidum* ATCC 204307. Int. J. Food Microbiol. 76: 215-221.

Dussurget O., Pizarro-Cerda J. and Cossart P. 2004. Molecular determinants of *Listeria monocytogenes* virulence. Annu. Rev. Microbiol. 58: 587-610.

Eijsink V.G., Skeie M., Middelhoven P.H., Brurberg M.B. and Nes I.F. 1998. Comparative studies of class IIa bacteriocins of lactic acid bacteria. Appl. Environ. Microbiol. 64: 3275-3281.

Eliskases-Lechner F. and Ginzinger W. 1995a. The bacterial flora of surface-ripened cheeses with special regard to coryneforms. Lait 75: 571-583.

Eliskases-Lechner F. and Ginzinger W. 1995b. The yeast flora of surface-ripened cheeses. Milchwissenschaft 50: 458-462.

Ennahar S., Aoude-Werner D., Sorokine O., Van Dorsselaer A., Bringel F., Hubert J.C. and Hasselmann C. 1996. Production of pediocin AcH by *Lactobacillus plantarum* WHE 92 isolated from cheese. Appl. Environ. Microbiol. 62: 4381-4387.

Ennahar S., Sashihara T., Sonomoto K. and Ishizaki A. 2000. Class IIa bacteriocins: biosynthesis, structure and activity. FEMS Microbiol. Rev. 24: 85-106.

Eppert I., Valdes-Stauber N., Götz H., Busse M. and Scherer S. 1997. Growth reduction of *Listeria* spp. caused by undefined industrial red smear cheese cultures and bacteriocin-producing *Brevibacterium linens* as evaluated in situ on soft cheese. Appl. Environ. Microbiol. 63: 4812-4817.

Ercolini D., Hill P.J. and Dodd C.E. 2003. Bacterial community structure and location in Stilton cheese. Appl. Environ. Microbiol. 69: 3540-3548.

Esteve-Zarzoso B., Belloch C., Uruburu F. and Querol A. 1999. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. Int. J. Syst. Bacteriol. 49: 329-337.

Farber J.M. and Peterkin P.I. 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Rev. 55: 476-511.

Feurer C., Irlinger F., Spinnler H.E., Glaser P. and Vallaeys T. 2004a. Assessment of the rind microbial diversity in a farmhouse-produced vs a pasteurized industrially produced soft red-smear cheese using both cultivation and rDNA-based methods. J. Appl. Microbiol. 97: 546-556.

Feurer C., Vallaeys T., Corrieu G. and Irlinger F. 2004b. Does smearing inoculum reflect the bacterial composition of the smear at the end of the ripening of a French soft, red-smear cheese? J. Dairy Sci. 87: 3189-3197.

Fleet G.H. 1990. Yeasts in dairy products. J. Appl. Bacteriol. 68: 199-211.

Funke G., Peters K. and Aravena-Roman M. 1998. Evaluation of the RapID CB plus system for identification of coryneform bacteria and *Listeria* spp. J. Clin. Microbiol. 36: 2439-2442.

Funke G., von Graevenitz A., Clarridge J.E., 3rd and Bernard K.A. 1997. Clinical microbiology of coryneform bacteria. Clin. Microbiol Rev. 10: 125-159.

Gavrish E.Y., Krauzova V.I., Potekhina N.V., Karasev S.G., Plotnikova E.G., Altyntseva O.V., Korosteleva L.A. and Evtushenko L.I. 2004. Three new species of brevibacteria, *Brevibacterium antiquum* sp. nov., *Brevibacterium aurantiacum* sp. nov., and *Brevibacterium permense* sp. nov. Microbiology 73: 176-183.

Gente S., Desmasures N., Panoff J.M. and Gueguen M. 2002. Genetic diversity among *Geotrichum candidum* strains from various substrates studied using RAM and RAPD-PCR. J. Appl. Microbiol. 92: 491-501.

Goerges S., Bora N., Gelsomino R., Rea M., Irlinger F., Beduhn R., Cogan T.M., Goodfellow M., Swings J., Vancanneyt M., Ward A.C. and Scherer S. Biodiversity of surface microbial consortia from Limburger cheese. Submitted.

Goodfellow M., Manfio G.P. and Chun J. 1997. Towards a practical species concept for culturable bacteria. In: Claridge MF, Dawah HA & Wilson MR (eds.), Species: The units of biodiversity. Chapman and Hall, London, pp. 25-59.

Guichard H. and Bonnarme P. 2005. Development and validation of a plate technique for screening of microorganisms that produce volatile sulfur compounds. Anal. Biochem. 338: 299-305.

Hannon J.A., Sousa M.J., Lillevang S., Sepulchre A., Bockelmann W. and McSweeney P.L.H. 2004. Effect of defined-strain surface starters on the ripening of Tilsit cheese. Int. Dairy J. 14: 871-880.

Helm D., Labischinski H., Schallehn G. and Naumann D. 1991. Classification and identification of bacteria by Fourier-transform infrared spectroscopy. J. Gen. Microbiol. 137 (Pt 1): 69-79.

Hermans P.W.M., Sluijter M., Hoogenboezem T., Heersma H., van Belkum A. and de Groot R. 1995. Comparative study of five different DNA fingerprint techniques for molecular typing of *Streptococcus pneumoniae* strains. J. Clin. Microbiol. 33: 1606-1612.

Hipp S.S., Lawton W.D., Chen N.C. and Gaafar H.A. 1974. Inhibition of *Neisseria gonorrhoeae* by a factor produced by *Candida albicans*. Appl. Microbiol. 27: 192-196.

Hof H. 2003. History and epidemiology of listeriosis. FEMS Immunology and Medical Microbiology 35: 199.

Hohenegger M., Bora N., Gelsomino R., Goerges S., Goodfellow M., Ward A.C., Swings J., Vancanneyt M., Scherer S. and Sebastiani H. Surface microflora of Tilsit cheese. Submitted.

Holt J.G., Krieg N.R., Sneath P.H.A., Staley J.T. and Williams S.T. 1994. Bergey′s Manual of Determinative Bacteriology, 9th edition. Williams & Wilkins, Baltimore, Maryland, USA.

Holzapfel W.H., Geisen R. and Schillinger U. 1995. Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. Int. J. Food Microbiol. 24: 343-362.

Hoppe-Seyler T.S., Jaeger B., Bockelmann W., Noordman W.H., Geis A. and Heller K.J. 2003. Identification and differentiation of species and strains of *Arthrobacter* and *Microbacterium barkeri* isolated from smear cheeses with amplified ribosmal DNA restriction analysis (ARDRA) and pulsed field gel electrophoresis (PFGE). Syst. Appl. Microbiol. 26: 438-444.

Hoppe-Seyler T.S., Jaeger B., Bockelmann W., Noordman W.H., Geis A. and Heller K.J. 2004. Molecular identification and differentiation of *Staphylococcus* species and strains of cheese origin. Syst. Appl. Microbiol. 27: 211-218.

Hou X.G., Y. K., Sultana F., Hirose K., Miyake M., Otsuka Y., Misawa S., Oguri T., Yamamoto H. and Ezaki T. 1997. Genetic identification of members of the genus *Corynebacterium* at genus and species levels with 16S rDNA-targeted probes. Microbiol. Immunol. 41: 453-460.

Irlinger F., Bimet F., Delettre J., Lefevre M. and Grimont P.A. 2005. *Arthrobacter bergerei* sp. nov. and *Arthrobacter arilaitensis* sp. nov., novel coryneform species isolated from the surfaces of cheeses. Int. J. Syst. Evol. Microbiol. 55: 457-462.

Irlinger F., Morvan A., El Solh N. and Bergere J.L. 1997. Taxonomic characterization of coagulase-negative staphylococci in ripening flora from traditional French cheeses. Syst. Appl. Microbiol. 20: 319-328.

Ivanova E.P., Matte G.R., Matte M.H., Coenye T., Huq A. and Colwell R.R. 2002. Characterization of *Pseudoalteromonas citrea* and *P. nigrifaciens* isolated from different ecological habitats based on REP-PCR genomic fingerprints. Syst. Appl. Microbiol. 25: 275-283.

Jaeger B., Hoppe-Seyler T., Bockelmann W. and Heller K.J. 2002. The influence of the brine microflora on the ripening of smear cheeses. Milchwissenschaft 57: 645-648.

Kammerlehner J. 1986. Labkäse-Technologie, Band I. Th. Mann, Gelsenkirchen-Buer.

Kammerlehner J. 2003. Käsetechnologie. Freisinger Künstlerpresse W. Bode, Freising, Germany.

Kämpfer P. and Kroppenstedt R.M. 1996. Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. Can. J. Microbiol. 42: 989-1005.

Kämpfer P., Seiler H. and Dott W. 1993. Numerical classification of coryneform bacteria and related taxa. J. Gen. Appl. Microbiol. 39: 135-214.

Kayser F.H., Bienz K.A., Eckert J. and Zinkernael R.M. 1998. Medizinische Mikrobiologie, 9. überarbeitete und neugestaltete Auflage. Thieme, Stuttgart.

Klaenhammer T.R. 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. 12: 39-85.

Kollöffel B., Burri S., Meile L. and Teuber M. 1997. Development of 16S-rRNA oligonucleotide probes for *Brevibacterium*, *Micrococcus/Arthrobacter* and *Microbacterium/Aureobacterium* used in dairy starter cultures. Syst. Appl. Microbiol. 20: 409-417.

Krämer J. 1992. Lebensmittelmikrobiologie, 2. Auflage. Ulmer, Stuttgart.

Kreger-van Rij N.J.W. (Editor), 1984. The yeasts - a taxonomic study. third revised and enlarged edition. Elsevier Science Publishers B. V., Amsterdam.

Kümmerle M., Scherer S. and Seiler H. 1998. Rapid and reliable identification of food-borne yeasts by Fourier-transform infrared spectroscopy. Appl. Environ. Microbiol. 64: 2207-2214.

Kurtzman C.P. 2003. Phylogenetic circumscription of *Saccharomyces*, *Kluyveromyces* and other members of the *Saccharomycetaceae*, and the proposal of the new genera *Lachancea*, *Nakaseomyces*, *Naumovia*, *Vanderwaltozyma* and *Zygotorulaspora*. FEMS Yeast Res. 4: 233-245.

Kurtzman C.P. and Robnett C.J. 1998. Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Antonie van Leeuwenhoek 73: 331-371.

Kurtzman C.P. and Robnett C.J. 2003. Phylogenetic relationships among yeasts of the '*Saccharomyces* complex' determined from multigene sequence analyses. FEMS Yeast Res. 3: 417-432.

Kurtzman C.P., Robnett C.J. and Yarrow D. 2001. Three new species of *Candida* from apple cider: *C. anglica*, *C. cidri* and *C. pomicola*. Antonie van Leeuwenhoek 80: 237.

Larpin S., Bonaïti C., Bora N., Gelsomino R., Goerges S., Irlinger F., Goodfellow M., Ward A.C., Vancanneyt M., Swings J., Scherer S., Guéguen M. and Desmasures N. Biodiversity of surface microbial consortia from Livarot, a French traditional smear ripened cheese. Submitted.

Lee Wong A.C. 1998. Biofilms in food processing environments. J. Dairy Sci. 81: 2765-2770.

Leistner L. 2000. Basic aspects of food preservation by hurdle technology. Int. J. Food Microbiol. 55: 181-186.

Leistner L. and Gorris L.G.M. 1995. Food preservation by hurdle technology. Trends in Food Science & Technology 6: 41-46.

Loessner M., Guenther S., Steffan S. and Scherer S. 2003. A pediocin-producing *Lactobacillus plantarum* strain inhibits *Listeria monocytogenes* in a multispecies cheese surface microbial ripening consortium. Appl. Environ. Microbiol. 69: 1854-1857.

Loessner M. and Scherer S. 2002. Listerien Teil II - Aktuelle Lage und Entwicklung neuer Schutz- und Reifungskulturen. Deutsche Molkereizeitung 123: 217-221.

Lombardi A., Maistro L.D., De Dea P., Gatti M., Giraffa G. and Neviani E. 2002. A polyphasic approach to highlight genotypic and phenotypic diversities of *Lactobacillus helveticus* strains isolated from dairy starter cultures and cheeses. J. Dairy Res. 69: 139-149.

Low J.C. and Donachie W. 1997. A review of *Listeria monocytogenes* and listeriosis. Vet. J. 153: 9.

Magliani W., Conti S., Gerloni M., Bertolotti D. and Polonelli L. 1997. Yeast killer systems. Clin. Microbiol. Rev. 10: 369-400.

Maisnier-Patin S., Forni E. and Richard J. 1996. Purification, partial characterisation and mode of action of enterococcin EFS2, an antilisterial bacteriocin produced by a strain of *Enterococcus faecalis* isolated from a cheese. Int. J. Food Microbiol. 30: 255-270.

Maisnier-Patin S. and Richard J. 1995. Activity and purification of linenscin OC2, an antibacterial substance produced by *Brevibacterium linens* OC2, an orange cheese coryneform bacterium. Appl. Environ. Microbiol. 61: 1847-1852.

Maoz A., Mayr R. and Scherer S. 2003. Temporal stability and biodiversity of two complex antilisterial cheese-ripening microbial consortia. Appl. Environ. Microbiol. 69: 4012-4018.

Marcellino N., Beuvier E., Grappin R., Gueguen M. and Benson D.R. 2001. Diversity of *Geotrichum candidum* strains isolated from traditional cheesemaking fabrications in France. Appl. Environ. Microbiol. 67: 4752-4759.

Mayr R., Fricker M., Maoz A. and Scherer S. 2004. Anti-listerial activity and biodiversity of cheese surface cultures: influence of the ripening temperature regime. Eur. Food Res. Technol. 218: 242-247.

McLauchlin J., Mitchell R.T., Smerdon W.J. and Jewell K. 2004. *Listeria monocytogenes* and listeriosis: a review of hazard characterisation for use in microbiological risk assessment of foods. Int. J. Food Microbiol. 92: 15.

Moghaddas J., Truant A.L., Jordan C. and Buckley H.R. 1999. Evaluation of the RapID Yeast Plus System for the identification of yeast. Diagn. Microbiol. Infect. Dis. 35: 271-273.

Motta A.S. and Brandelli A. 2002. Characterization of an antibacterial peptide produced by *Brevibacterium linens*. J. Appl. Microbiol. 92: 63-70.

Mounier J., Gelsomino R., Goerges S., Vancanneyt M., Vandemeulebroecke K., Hoste B., Scherer S., Swings J., Fitzgerald G.F. and Cogan T.M. 2005. Surface microflora of four smear-ripened cheeses. Appl. Environ. Microbiol., in press.

Mounier J., Goerges S., Gelsomino R., Vancanneyt M., Vandemeulebroecke K., Hoste B., Brennan N.M., Scherer S., Swings J., Fitzgerald G.F. and Cogan T.M. Sources of the adventitious microflora of a smear-ripened cheese. Submitted.

Nigatu A., Ahrne S. and Molin G. 2001. Randomly amplified polymorphic DNA (RAPD) profiles for the distinction of *Lactobacillus* species. Antonie van Leeuwenhoek 79: 1-6.

Oberreuter H., Brodbeck A., von Stetten S., Goerges S. and Scherer S. 2003. Fourier-transform infrared (FT-IR) spectroscopy is a promising tool for monitoring the population dynamics of microorganisms in food stuff. Eur. Food Res. Technol. 216: 434-439.

Oberreuter H., Charzinski J. and Scherer S. 2002a. Intraspecific diversity of *Brevibacterium linens*, *Corynebacterium glutamicum* and *Rhodococcus erythropolis* based on partial 16S rDNA sequence analysis and Fourier-transform infrared (FT-IR) spectroscopy. Microbiology 148: 1523-1532.

Oberreuter H., Seiler H. and Scherer S. 2002b. Identification of coryneform bacteria and related taxa by Fourier- transform infrared (FT-IR) spectroscopy. Int. J. Syst. Evol. Microbiol. 52: 91-100.

Pereira-Dias S., Potes M.E., Marinho A., Malfeito-Ferreira M. and Loureiro V. 2000. Characterisation of yeast flora isolated from an artisanal Portuguese ewes' cheese. Int. J. Food Microbiol. 60: 55-63.

Petersen K.M. and Jespersen L. 2004. Genetic diversity of the species *Debaryomyces hansenii* and the use of chromosome polymorphism for typing of strains isolated from surface-ripened cheeses. J. Appl. Microbiol. 97: 205-213.

Petersen K.M., Moller P.L. and Jespersen L. 2001. DNA typing methods for differentiation of *Debaryomyces hansenii* strains and other yeasts related to surface ripened cheeses. Int. J. Food Microbiol. 69: 11-24.

Petersen K.M., Westall S. and Jespersen L. 2002. Microbial succession of *Debaryomyces hansenii* strains during the production of Danish surfaced-ripened cheeses. J. Dairy Sci. 85: 478-486.

Polonelli L. and Morace G. 1986. Reevaluation of the yeast killer phenomenon. J. Clin. Microbiol. 24: 866-869.

Prillinger H., Molnar O., Eliskases-Lechner F. and Lopandic K. 1999. Phenotypic and genotypic identification of yeasts from cheese. Antonie van Leeuwenhoek 75: 267-283.

Provost F., Polonelli L., Conti S., Fisicaro P., Gerloni M. and Boiron P. 1995. Use of yeast killer system to identify species of the *Nocardia asteroides* complex. J. Clin. Microbiol. 33: 8-10.

Pschyrembel W. (Editor), 1994. Pschyrembel Klinisches Wörterbuch. 257. Auflage. de Gruyter, Berlin, New York.

Rademaker J.L.W., Peinhopf M., Rijnen L., Bockelmann W. and Noordman W.H. 2005. The surface microflora dynamics of bacterial smear-ripened Tilsit cheese determined by T-RFLP DNA population fingerprint analysis. Int. Dairy J. 15: 785-794.

Ramani R., Gromadzki S., Pincus D.H., Salkin I.F. and Chaturvedi V. 1998. Efficacy of API 20C and ID 32C systems for identification of common and rare clinical yeast isolates. J. Clin. Microbiol. 36: 3396-3398.

Randazzo C.L., Restuccia C., Romano A.D. and Caggia C. 2004. *Lactobacillus casei*, dominant species in naturally fermented Sicilian green olives. Int. J. Food Microbiol. 90: 9-14.

Randazzo C.L., Torriani S., Akkermans A.D., de Vos W.M. and Vaughan E.E. 2002. Diversity, dynamics, and activity of bacterial communities during production of an artisanal Sicilian cheese as evaluated by 16S rRNA analysis. Appl. Environ. Microbiol. 68: 1882-1892.

Rea M.C., Goerges S., Gelsomino R., Mounier J., Brennan N.M., Vancanneyt M., Scherer S., Swings J. and Cogan T.M. Yeast and bacterial biodiversity on the surface of Gubbeen cheese. Submitted.

Reps A. 1987. Bacterial surface-ripened cheeses. In: Fox PF (ed.), Cheese: chemistry, physics and microbiology. Elsevier Applied Science, London, pp. 151-184.

Robert V., de Bien J.-E., Buyck B. and Hennebert G.L. 1994. "ALLEV", a new program for computer-assisted identification of yeasts. Taxon 43: 433-439.

Robert V., Evrard P. and Hennebert G.L. 1997. BCCM™/Allev 2.00 an automated system for the identification of yeasts. Mycotaxon 64: 455-463.

Rohm H., Eliskases-Lechner F. and Bräuer M. 1992. Diversity of yeasts in selected dairy products. J. Appl. Bacteriol. 72: 370-376.

Rohm H., Lechner F. and Lehner M. 1990. Evaluation of the API ATB 32C system for the rapid identification of foodborne yeasts. Int. J. Food Microbiol. 11: 215-223.

Romano A., Casaregola S., Torre P. and Gaillardin C. 1996. Use of RAPD and mitochondrial DNA RFLP for typing of *Candida zeylanoides* and *Debaryomyces hansenii* yeast strains isolated from cheese. Syst. Appl. Microbiol. 19: 255-264.

Rudolf M. and Scherer S. 2001. High incidence of *Listeria monocytogenes* in European red smear cheese. Int. J. Food Microbiol. 63: 91-98.

Ryan M.P., Rea M.C., Hill C. and Ross R.P. 1996. An application in cheddar cheese manufacture for a strain of *Lactococcus lactis* producing a novel broad-spectrum bacteriocin, lacticin 3147. Appl. Environ. Microbiol. 62: 612-619.

Ryser E.T., Maisnier-Patin S., Gratadoux J.J. and Richard J. 1994. Isolation and identification of cheese-smear bacteria inhibitory to *Listeria* spp. Int. J. Food Microbiol. 21: 237-246.

Ryser E.T. and Marth E.H. 1991. *Listeria*, listeriosis, and food safety. Marcel Dekker, Inc., New York, USA.

Schlegel H.G. 1992. Allgemeine Mikrobiologie, 7. überarbeitete Auflage. Thieme, Stuttgart.

Schubert K., Ludwig W., Springer N., Kroppenstedt R.M., Accolas J.P. and Fiedler F. 1996. Two coryneform bacteria isolated from the surface of French Gruyere and Beaufort cheeses are new species of the genus *Brachybacterium*: *Brachybacterium alimentarium* sp. nov. and *Brachybacterium tyrofermentans* sp. nov. Int. J. Syst. Bacteriol. 46: 81-87.

Seiler H. 1983. Identification key for coryneform bacteria derived by numerical taxonomic studies. J. Gen. Microbiol. 129: 1433-1471.

Seiler H. 1991. Some additional physiological characteristics for the identification of foodborne yeasts. Neth. Milk Dairy J. 45: 253-258.

Seiler H. 2002. Yeasts in milk and milk products. In: Roginski H, Fuquay JW & Fox PF (eds.), Encyclopedia of Dairy Sciences. Academic Press, London, pp. 2761-2769.

Seiler H. and Busse M. 1988. Identification of yeasts with microtiter plates. Forum Mikrobiologie 11: 505-509.

Siepmann R. and Höhnk W. 1962. Über Hefen und einige Pilze (Fungi imp., Hyphales) aus dem Nordatlantik. Veröffentlichungen des Instituts für Meeresforschung in Bremerhaven 8: 79-98.

St.-Germain G. and Beauchesne D. 1991. Evaluation of the MicroScan Rapid Yeast Identification panel. J. Clin. Microbiol. 29: 2296-2299.

Stackebrandt E., Rainey F.A. and Ward-Rainey N.L. 1997. Proposal for a new hierarchic classification system, *Actinobacteria* classis nov. Int. J.Syst. Bacteriol. 47: 479-491.

Stackebrandt E. and Schumann P. 2000. Description of *Bogoriellaceae* fam. nov., *Dermacoccaceae*, fam. nov. *Rarobacteraceae* fa. nov., and *Sanguibacteraceae*, fam. nov. and emendation of some families of the suborder *Micrococcineae*. Int. J. Syst. Evol. Microbiol. 50: 1279-1285.

Terplan G., Schoen R., Springmeyer W., Degle I. and Becker H. 1986. Vorkommen, Verhalten und Bedeutung von Listerien in Milch und Milchprodukten. Archiv für Lebensmittelhygiene 37: 131-137.

Thomas A., Mathieu B., Bora N., Gelsomino R., Goerges S., Irlinger F., Goodfellow M., Ward A.C., Vancanneyt M., Swings J., Scherer S., Cogan T. and Chamba J.-F. Biodiversity of the surface microflora from Reblochon, a French traditional smear ripened cheese. Submitted.

Tompkin R.B. 2002. Control of *Listeria monocytogenes* in the food-processing environment. J. Food Prot. 65: 709-725.

Török T. and King (Jr.) A.D. 1991. Comparative study on the identification of food-borne yeasts. Appl. Environ. Microbiol. 57: 1207-1212.

Torri Tarelli G., Carminati D. and Giraffa G. 1994. Production of bacteriocins active against *Listeria monocytogenes* and *Listeria innocua* from dairy enterococci. Food Microbiol. 11: 243-252.

Valdés-Stauber N., Götz H. and Busse M. 1991. Antagonistic effect of coryneform bacteria from red smear cheese against *Listeria* species. Int. J. Food Microbiol. 13: 119-130.

Valdés-Stauber N. and Scherer S. 1994. Isolation and characterization of Linocin M18, a bacteriocin produced by *Brevibacterium linens*. Appl. Environ. Microbiol. 60: 3809-3814.

Valdés-Stauber N., Scherer S. and Seiler H. 1997. Identification of yeasts and coryneform bacteria from the surface microflora of brick cheeses. Int. J. Food Microbiol. 34: 115-129.

Vandamme P., Pot B., Gillis M., de Vos P., Kersters K. and Swings J. 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol. Rev. 60: 407-438.

Vaneechoutte M., Riegel P., de Briel D., Monteil H., Verschraegen G., De Rouck A. and Claeys G. 1995. Evaluation of the applicability of amplified rDNA-restriction analysis (ARDRA) to identification of species of the genus *Corynebacterium*. Res. Microbiol. 146: 633-641.

Vasdinyei R. and Deak T. 2003. Characterization of yeast isolates originating from Hungarian dairy products using traditional and molecular identification techniques. Int. J. Food Microbiol. 86: 123-130.

Vazquez-Boland J.A., Kuhn M., Berche P., Chakraborty T., Dominguez-Bernal G., Goebel W., Gonzalez-Zorn B., Wehland J. and Kreft J. 2001. *Listeria* pathogenesis and molecular virulence determinants. Clin. Microbiol. Rev. 14: 584-640.

Vermeiren L., Devlieghere F. and Debevere J. 2004. Evaluation of meat born lactic acid bacteria as protective cultures for the biopreservation of cooked meat products. Int. J. Food Microbiol. 96: 149-164.

Walker S.J., Archer P. and Banks J.G. 1990. Growth of *Listeria monocytogenes* at refrigeration temperatures. J. Appl. Bacteriol.157-162.

Wallin J. and Gnarpe H. 1975. Possible inhibition of *N. gonnorrhoeae* by *C. albicans*. A clinical study. Br. J. Vener. Dis. 51: 174-175.

Ward J.H., Jr. 1963. Hierarchical grouping to optimize an objective function. J. Am. Stat. Assoc. 58: 236-244.

Welthagen J.J. and Viljoen B.C. 1998. Yeast profile in Gouda cheese during processing and ripening. Int. J. Food Microbiol. 41: 185-194.

Wenning M., Seiler H. and Scherer S. 2002. Fourier-transform infrared microspectroscopy, a novel and rapid tool for identification of yeasts. Appl. Environ. Microbiol. 68: 4717-4721.

Zhao T., Doyle M.P. and Zhao P. 2004. Control of *Listeria monocytogenes* in a biofilm by competitive-exclusion microorganisms. Appl. Environ. Microbiol. 70: 3996-4003.

Appendix

Table 1: Yeasts selected from the Weihenstephan yeast collection for preliminary experiment PE1 in the agar-membrane screening assay. Yeasts were screened for their anti-listerial activity against two *L. monocytogenes* indicator strains (WSLC 1001 and 1364).

No.	Strain	Species	Source
1	WSYC 1	*Kluyveromyces marxianus*	milk product
2	WSYC 2	*Pichia membranifaciens*	kummis
3	WSYC 10	*Trichosporon ovoides*	unknown
4	WSYC 21	*Candida zeylanoides*	beef
5	WSYC 22	*Candida zeylanoides*	throat
6	WSYC 24	*Clavispora lusitaniae*	unknown
7	WSYC 30	*Debaryomyces hansenii*	cheese
8	WSYC 33	*Debaryomyces hansenii*	cheese
9	WSYC 39	*Pichia anomala*	unknown
10	WSYC 44	*Kluyveromyces lactis*	unknown
11	WSYC 45	*Kluyveromyces lactis*	cream
12	WSYC 46	*Kluyveromyces lactis*	creamery
13	WSYC 48	*Kluyveromyces lactis*	cheese
14	WSYC 51	*Candida parapsilosis*	yoghurt
15	WSYC 53	*Kluyveromyces marxianus*	kummis
16	WSYC 66	*Saccharomyces castellii*	buttermilk
17	WSYC 78	*Yarrowia lipolytica*	maize-processing plant
18	WSYC 79	*Yarrowia lipolytica*	maize-processing plant
19	WSYC 93	*Saccharomyces cerevisiae*	distillery
20	WSYC 97	*Kluyveromyces marxianus*	milk
21	WSYC 115	*Kluyveromyces lactis*	winery installation
22	WSYC 122	*Galactomyces geotrichum*	unknown
23	WSYC 126	*Debaryomyces hansenii*	cheese brine
24	WSYC 128	*Kluyveromyces lactis*	cheese brine
25	WSYC 130	*Candida parapsilosis*	cheese brine
26	WSYC 131	*Debaryomyces hansenii*	cheese brine
27	WSYC 132	*Pichia jadinii*	cheese brine
28	WSYC 134	*Candida intermedia*	cheese brine
29	WSYC 135	*Clavispora lusitaniae*	cheese brine
30	WSYC 136	*Trichosporon beigelii*	cheese brine
31	WSYC 138	*Candida zeylanoides*	cheese brine
32	WSYC 139	*Kluyveromyces lactis*	cheese brine
33	WSYC 140	*Pichia jadinii*	cheese brine
34	WSYC 141	*Kluyveromyces marxianus*	cheese brine
35	WSYC 145	*Candida maltosa*	cheese brine
36	WSYC 148	*Kluyveromyces lactis*	cheese brine
37	WSYC 149	*Candida maltosa*	cheese brine
38	WSYC 150	*Debaryomyces hansenii*	cheese brine
39	WSYC 151	*Torulaspora delbrueckii*	cheese brine
40	WSYC 152	*Issatchenkia orientalis*	cheese brine
41	WSYC 153	*Candida maltosa*	cheese brine
42	WSYC 156	*Trichosporon beigelii*	cheese brine
43	WSYC 159	*Trichosporon beigelii*	cheese brine
44	WSYC 161	*Trichosporon beigelii*	cheese brine
45	WSYC 162	*Pichia jadinii*	cheese brine
46	WSYC 164	*Debaryomyces hansenii*	cheese brine
47	WSYC 165	*Torulaspora delbrueckii*	feta

No.	Strain	Species	Source
48	WSYC 172	*Dekkera anomala*	feta
49	WSYC 175	*Pichia anomala*	feta
50	WSYC 179	*Torulaspora delbrueckii*	feta
51	WSYC 180	*Yarrowia lipolytica*	feta
52	WSYC 181	*Torulaspora delbrueckii*	feta
53	WSYC 182	*Pichia membranifaciens*	feta
54	WSYC 184	*Debaryomyces hansenii*	feta
55	WSYC 185	*Candida zeylanoides*	feta
56	WSYC 186	*Torulaspora delbrueckii*	feta
57	WSYC 193	*Candida zeylanoides*	feta
58	WSYC 195	*Yarrowia lipolytica*	feta
59	WSYC 199	*Pichia membranifaciens*	feta
60	WSYC 204	*Debaryomyces hansenii*	feta
61	WSYC 209	*Kluyveromyces marxianus*	feta
62	WSYC 215	*Kluyveromyces marxianus*	feta
63	WSYC 216	*Kluyveromyces marxianus*	feta
64	WSYC 221	*Hanseniaspora uvarum*	yoghurt with fruit
65	WSYC 223	*Pichia membranifaciens*	yoghurt with fruit
66	WSYC 226	*Pichia cactophila*	yoghurt with fruit
67	WSYC 244	*Candida catenulata*	hyperkeratinic food
68	WSYC 247	*Pichia membranifaciens*	brewery
69	WSYC 248	*Candida intermedia*	pilsener beer
70	WSYC 249	*Candida magnoliae*	magnolia flower
71	WSYC 263	*Issatchenkia orientalis*	fruit juice or berries
72	WSYC 270	*Pichia jadinii*	unknown
73	WSYC 281	*Williopsis californica*	soil
74	WSYC 289	*Candida glabrata*	feces
75	WSYC 293	*Pichia guilliermondii*	butter
76	WSYC 294	*Candida parapsilosis*	sausage
77	WSYC 295	*Pichia anomala*	brewery
78	WSYC 298	*Candida ethanolica*	industrial fodder
79	WSYC 300	*Candida intermedia*	grape
80	WSYC 301	*Candida parapsilosis*	olives
81	WSYC 312	*Issatchenkia occidentalis*	soil
82	WSYC 313	*Pichia anomala*	berries
83	WSYC 314	*Pichia anomala*	sourdough
84	WSYC 316	*Pichia triangularis*	soya
85	WSYC 328	*Issatchenkia occidentalis*	unknown
86	WSYC 343	*Saccharomyces dairenensis*	dry fruit of Diospyros sp.
87	WSYC 435	*Galactomyces geotrichum*	solvent contaminated soil
88	WSYC 437	*Candida catenulata*	unknown
89	WSYC 438	*Candida catenulata*	grape must
90	WSYC 439	*Issatchenkia orientalis*	beer
91	WSYC 443	*Yarrowia lipolytica*	unknown
92	WSYC 451	*Yarrowia lipolytica*	rancid margarine
93	WSYC 455	*Clavispora lusitaniae*	milk of mastitic cow
94	WSYC 456	*Pichia jadinii*	cow with mastitis
95	WSYC 459	*Galactomyces geotrichum*	milk
96	WSYC 472	*Clavispora lusitaniae*	citrus essence
97	WSYC 496	*Pichia triangularis*	unknown
98	G 1077	*Pichia triangularis*	unknown
99	G 1085	*Pichia triangularis*	unknown
100	G 1086	*Pichia triangularis*	unknown

Table 2: Yeasts from different European smear-ripened cheeses screened for their anti-listerial activity. Yeasts were screened for their anti-listerial activity against six *L. monocytogenes* indicator strains (WSLC 1001, 1039, 1211, 1364, 1416 and 1685). *Cheeses are listed in Table 3.

Species	Almkäse	Austrian red smear cheese	Graukäse	Gubbeen	Limburger	Livarot	Tilsit	19 European red smear cheeses*
C. anglica								3
C. catenulata				1		1		
C. intermedia								17
C. tropicalis								1
C. zeylanoides								1
Cl. lusitaniae	36			1				3
D. hansenii	57			1	73			68
G. geotrichum					14	1		6
I. occidentalis			1					
K. marxianus								1
P. triangularis							1	
S. unisporus								3
Tr. ovoides	5	1						
Y. lipolytica	2					1		4
not identified								10
Total number of isolates	100	1	1	3	87	3	1	117

Table 3: 19 European red smear cheeses investigated for the presence of anti-listerial yeast in the cheese smear.

No.	Cheese	Country	Cheese type	Used milk
1	Romadur	Germany	soft cheese	unknown
2	Romadur	Germany	soft cheese	unknown
3	Romadur	Germany	soft cheese	unknown
4	Chaumes	France	soft cheese	unknown
5	Reblochon	France	semi-hard cheese	raw milk
6	Bergkäse	Italy	hard cheese	raw milk
7	Appenzeller	Switzerland	hard cheese	raw milk
8	Tilsit - Havarti	Denmark	semi-hard cheese	pasteurized milk
9	Limburger	Germany	soft cheese	unknown
10	Raclette	France	semi-hard cheese	unknown
11	Tilsit – Alter Mommark	Denmark	semi-hard cheese	unknown
12	regional cheese delicacy	Germany	semi-hard cheese	unknown
13	Bergkäse	Austria	hard cheese	raw milk
14	Exquis Herve	Belgium	soft cheese	raw milk
15	Petit Munster	France	soft cheese	pasteurized milk
16	Rouy	France	soft cheese	unknown
17	Epoisses AOC	France	soft cheese	raw milk
18	Petit Munster	France	soft cheese	pasteurized milk
19	Elsässer Winzerkäse	France	soft cheese	unknown

Table 4: Physiological tests on representative Limburger yeasts: Ability to use carbon substrates for growth. Lane 1 to 5: representatives of *D. hansenii*, type 1 colony morphology; lane 6 and 7: representatives of *D. hansenii*, type 2 colony morphology; lane 8 to 10: representatives of *G. geotrichum*.

1	2	3	4	5	6	7	8	9	10	
1M19	2E6	2E21	3E44	3M46	1E36	1L9	1M24	1M49	2M32	**Carbon substrates**
+	+	+	+	+	+	+	+	+	+	D-Glucose
+	+	+	+	+	+	+	+	+	+	D-Galactose
+	+	+	+	+	+	+	+	+	+	L-Sorbose
-	-	-	-	-	+	+	-	-	-	D-Glucosamine
-	-	-	-	-	-	-	-	-	-	D-Ribose
+	+	+	+	+	+	+	+	+	+	D-Xylose
+	+	+	+	+	+	+	-	-	-	L-Arabinose
-	-	-	-	-	-	-	-	-	-	D-Arabinose
+	+	+	+	+	+	+	-	-	-	L-Rhamnose
+	+	+	+	+	+	+	-	-	-	Sucrose
+	+	+	+	+	+	+	-	-	-	Maltose
+	+	+	+	+	+	+	-	-	-	α,α-Trehalose
+	+	+	+	+	+	+	-	-	-	α-D-Methyl-D-glucoside
+	+	+	+	+	+	+	-	-	-	Cellobiose
+	+	+	+	+	+	+	-	-	-	Salicin
+	+	+	+	+	+	+	-	-	-	Arbutin
-	-	-	-	-	-	-	-	-	-	Melibiose
+	+	+	+	+	+	+	-	-	-	Lactose
+	+	+	+	+	+	+	-	-	-	Raffinose
+	+	+	+	+	+	+	-	-	-	Melizitose
-	-	-	-	-	-	-	-	-	-	Erythritol
+	+	+	+	+	+	+	-	-	-	Ribitol
+	+	+	+	+	+	+	-	-	-	Xylitol
-	-	-	-	-	+	+	-	-	-	L-Arabinitol
+	+	+	+	+	+	+	+	+	+	D-Glucitol
+	+	+	+	+	+	+	+	+	+	D-Mannitol
-	-	-	-	-	-	-	-	-	-	Galactitol
-	-	-	-	-	-	-	-	-	-	myo-Inositol
+	+	+	+	+	+	+	+	+	+	D-Glucono-1,5-lactone
-	-	-	-	-	+	+	-	-	-	2-Keto-D-gluconate
-	-	-	-	-	-	-	-	-	-	5-Keto-D-gluconate
+	+	+	+	+	+	+	-	-	-	D-Gluconate
+	+	+	+	+	+	+	-	-	-	D-Glucuronate
+	+	+	+	+	+	+	+	+	+	DL-Lactat
+	+	+	+	+	+	+	+	+	+	Succinate
+	+	+	+	+	+	+	-	-	-	Citrate
+	+	+	+	+	+	+	+	+	+	Glycerol

Table 5: Physiological tests on representative Limburger yeasts: Ability to use nitrogen substrates for growth. Lane 1 to 5: representatives of *D. hansenii*, type 1 colony morphology; lane 6 and 7: representatives of *D. hansenii*, type 2 colony morphology; lane 8 to 10: representatives of *G. geotrichum*.

1	2	3	4	5	6	7	8	9	10	
1M19	2E6	2E21	3E44	3M46	1E36	1L9	1M24	1M49	2M32	**Nitrogen substrates**
-	-	-	-	-	+	+	-	-	-	Nitrite
-	-	-	-	-	+	+	+	+	+	Ethylamine
+	+	+	+	+	+	+	+	+	+	L-Lysine
+	+	+	+	+	+	+	+	+	+	Cadaverine
+	+	+	+	+	+	+	+	+	+	Glucosamine

Table 6: Physiological tests on representative Limburger yeasts: Ability to grow without vitamins or without thiamine and test for cycloheximide resistance. Lane 1 to 5: representatives of *D. hansenii*, type 1 colony morphology; lane 6 and 7: representatives of *D. hansenii*, type 2 colony morphology; lane 8 to 10: representatives of *G. geotrichum*.

1	2	3	4	5	6	7	8	9	10	
1M19	2E6	2E21	3E44	3M46	1E36	1L9	1M24	1M49	2M32	
+	+	+	+	+	+	+	+	+	+	w/o vitamins
+	+	+	+	+	+	+	+	+	+	w/o Thiamine
-	-	-	-	-	-	-	+	+	+	0,01% Cycloheximide
-	-	-	-	-	-	-	+	+	+	0,1% Cycloheximide

Table 7: Physiological tests on representative Limburger yeasts: Ability for urea hydrolysis. Lane 1 to 5: representatives of *D. hansenii*, type 1 colony morphology; lane 6 and 7: representatives of *D. hansenii*, type 2 colony morphology; lane 8 to 10: representatives of *G. geotrichum*.

1	2	3	4	5	6	7	8	9	10
1M19	2E6	2E21	3E44	3M46	1E36	1L9	1M24	1M49	2M32
-	-	-	-	-	-	-	-	-	-

Table 8: Physiological tests on representative Limburger yeasts: Ability to ferment D-glucose. Lane 1 to 5: representatives of *D. hansenii*, type 1 colony morphology; lane 6 and 7: representatives of *D. hansenii*, type 2 colony morphology; lane 8 to 10: representatives of *G. geotrichum*.

1	2	3	4	5	6	7	8	9	10
1M19	2E6	2E21	3E44	3M46	1E36	1L9	1M24	1M49	2M32
-	-	-	-	-	-	-	-	-	-

Table 9: Selected yeast for preliminary experiments PE2 and PE3 in the agar-membrane screening assay. Yeasts were selected according to their anti-listerial activity detected in preliminary experiment PE1. In experiments PE2 and PE3 yeasts were screened for their anti-listerial activity against six *L. monocytogenes* indicator strains (WSLC 1001, 1039, 1211, 1364, 1416 and 1685).

No.	Strain	Species	Source	Anti-listerial activity in experiment PE1
1	WSYC 1	*Kluyveromyces marxianus*	milk products	strong
2	WSYC 51	*Candida parapsilosis*	yoghurt	strong
3	WSYC 223	*Pichia membranifaciens*	yoghurt with fruit	strong
4	WSYC 263	*Issatchenkia orientalis*	fruit juice or berries	strong
5	WSYC 312	*Issatchenkia occidentalis*	soil	strong
6	WSYC 455	*Clavispora lusitaniae*	milk of mastitic cow	strong
7	WSYC 24	*Clavispora lusitaniae*	unknown	moderate
8	WSYC 39	*Pichia anomala*	unknown	moderate
9	WSYC 122	*Galactomyces geotrichum*	unknown	moderate
10	WSYC 141	*Kluyveromyces marxianus*	cheese brine	moderate
11	WSYC 186	*Torulaspora delbrueckii*	feta	moderate
12	WSYC 472	*Clavispora lusitaniae*	citrus essence	moderate
13	WSYC 22	*Candida zeylanoides*	throat	no / poor
14	WSYC 164	*Debaryomyces hansenii*	cheese brine	no / poor
15	WSYC 184	*Debaryomyces hansenii*	feta	no / poor
16	WSYC 215	*Kluyveromyces marxianus*	feta	no / poor
17	WSYC 343	*Saccharomyces dairenensis*	dry fruit of Diospyros sp.	no / poor
18	G1077	*Pichia triangularis*	unknown	no / poor

Table 10: Selected yeast for the reproducibility test of the agar-membrane screening assay. Yeasts were screened for their anti-listerial activity against six *L. monocytogenes* indicator strains (WSLC 1001, 1039, 1211, 1364, 1416 and 1685).

No.	Strain	Species	Source
1	WSYC 1	*Kluyveromyces marxianus*	milk products
2	WSYC 22	*Candida zeylanoides*	throat
3	WSYC 51	*Candida parapsilosis*	yoghurt
4	WSYC 122	*Galactomyces geotrichum*	unknown
5	WSYC 184	*Debaryomyces hansenii*	feta
6	WSYC 215	*Kluyveromyces marxianus*	feta
7	WSYC 223	*Pichia membranifaciens*	yoghurt with fruit
8	WSYC 263	*Issatchenkia orientalis*	fruit juice or berries
9	WSYC 312	*Issatchenkia occidentalis*	soil
10	G1077	*Pichia triangularis*	unknown
11	H-2a21	*Trichosporon ovoides*	Austrian red smear cheese
12	H-AG43	*Issatchenkia occidentalis*	Graukäse
13	TS3M4	*Pichia triangularis*	Tilsit
14	IV1L8	*Yarrowia lipolytica*	Livarot
15	IV1M1	*Galactomyces geotrichum*	Livarot
16	IV2M20	*Candida catenulata*	Livarot
17	LM1E36	*Debaryomyces hansenii*	Limburger
18	LM1M24	*Galactomyces geotrichum*	Limburger
19	LM2E21	*Debaryomyces hansenii*	Limburger
20	1.37.10Y	*Clavispora lusitaniae*	Gubbeen
21	2.23S2Y	*Candida catenulata*	Gubbeen
22	3.4S22Y	*Debaryomyces hansenii*	Gubbeen

Table 11: Anti-listerial activity of yeast against *L. monocytogenes* indicator strains WSLC 1001, 1039 and 1211 in the reproducibility test of the agar-membrane screening assay. Anti-listerial activity was evaluated according to the score based evaluation system (Figure 25). Total anti-listerial activity of each yeast strain against the individual *L. monocytogenes* indicator strain is given as a sum of scores (Σ) obtained in four independent experiments (I to IV). Maximum value: 20; minimum value: 0.

		Listeria monocytogenes indicator strain WSLC														
		1001					1039					1211				
		Score in					Score in					Score in				
No.	Strain	I	II	III	IV	**Σ**	I	II	III	IV	**Σ**	I	II	III	IV	**Σ**
1	WSYC 1	4	3	3	3	**13**	3	2	2	3	**10**	2	2	4	4	**12**
2	WSYC 22	2	2	2	2	**8**	n.e.	n.e.	n.e.	n.e.	**-**	0	0	n.e.	n.e.	**0**
3	WSYC 51	3	4	3	2	**12**	4	3	3	2	**12**	3	3	3	4	**13**
4	WSYC 122	3	3	n.e.	2	**8**	1	2	1	1	**5**	2	3	1	1	**7**
5	WSYC 184	1	0	1	1	**3**	0	0	1	2	**3**	0	0	0	2	**2**
6	WSYC 215	0	1	1	0	**2**	0	0	1	1	**2**	0	0	1	1	**2**
7	WSYC 223	5	4	4	4	**17**	5	4	4	4	**17**	4	3	4	4	**15**
8	WSYC 263	5	4	4	4	**17**	5	4	4	4	**17**	3	4	3	3	**13**
9	WSYC 312	5	4	4	4	**17**	5	4	4	4	**17**	4	3	4	4	**15**
10	G1077	1	2	1	1	**5**	0	0	1	n.e.	**1**	1	0	1	1	**3**
11	H-2a21	n.e.	n.e.	n.e.	n.e.	**-**	n.e.	n.e.	n.e.	n.e.	**-**	3	n.e.	1	n.e.	**4**
12	H-AG43	4	4	4	4	**16**	4	3	1	4	**12**	4	2	1	4	**11**
13	TS3M4	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**
14	IV1M1	n.e.	1	n.e.	1	**2**	0	1	0	0	**1**	1	1	0	0	**2**
15	IV1L8	2	4	2	3	**11**	3	3	1	3	**10**	3	4	2	3	**12**
16	IV2M20	3	4	3	3	**13**	2	1	n.e.	2	**5**	2	3	1	2	**8**
17	LM1E36	3	3	1	2	**9**	2	3	1	2	**8**	0	1	1	2	**4**
18	LM1M24	1	2	1	1	**5**	2	0	1	2	**5**	2	0	1	2	**5**
19	LM2E21	2	2	1	1	**6**	1	1	1	1	**4**	0	0	0	1	**1**
20	1.37.10Y	n.e.	1	n.e.	1	**2**	n.e.	n.e.	n.e.	n.e.	**-**	n.e.	n.e.	n.e.	n.e.	**-**
21	2.23S2Y	1	2	3	2	**8**	2	1	1	1	**5**	2	2	2	2	**8**
22	3.4S22Y	1	1	0	0	**2**	1	1	0	0	**2**	1	0	0	0	**1**

Table 12: Anti-listerial activity of yeast against *L. monocytogenes* indicator strains WSLC 1364, 1416 and 1685 in the reproducibility test of the agar-membrane screening assay. Anti-listerial activity was evaluated according to the score based evaluation system (Figure 25). Total anti-listerial activity of each yeast strain against the individual *L. monocytogenes* indicator strain is given as a sum of scores (Σ) obtained in four independent experiments (I to IV). Maximum value: 20; minimum value: 0.

		Listeria monocytogenes indicator strain WSLC														
		1364					1416					1685				
		Score in					Score in					Score in				
No.	Strain	I	II	III	IV	**Σ**	I	II	III	IV	**Σ**	I	II	III	IV	**Σ**
1	WSYC 1	3	3	4	3	**13**	3	3	4	4	**14**	4	5	5	5	**19**
2	WSYC 22	n.e.	n.e.	n.e.	1	**1**	0	0	1	n.e.	**1**	1	2	2	1	**6**
3	WSYC 51	4	4	4	3	**15**	3	5	4	5	**17**	5	4	5	5	**19**
4	WSYC 122	3	4	1	2	**10**	2	1	2	2	**7**	3	3	1	2	**9**
5	WSYC 184	0	0	1	3	**4**	0	0	1	2	**3**	1	0	2	3	**6**
6	WSYC 215	0	0	n.e.	1	**1**	0	0	1	1	**2**	2	1	1	2	**6**
7	WSYC 223	5	4	4	4	**17**	4	2	4	4	**14**	5	5	5	5	**20**
8	WSYC 263	5	5	3	4	**17**	4	3	3	4	**14**	5	5	5	5	**20**
9	WSYC 312	4	4	4	4	**16**	4	2	4	5	**15**	5	5	5	5	**20**
10	G1077	1	n.e.	0	n.e.	**1**	0	0	0	1	**1**	3	1	1	1	**6**
11	H-2a21	n.e.	n.e.	n.e.	n.e.	**-**	n.e.	n.e.	n.e.	n.e.	**-**	3	2	2	n.e.	**7**
12	H-AG43	4	3	2	4	**13**	4	1	2	4	**11**	5	4	4	4	**17**
13	TS3M4	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**
14	IV1M1	1	1	0	3	**5**	1	0	0	0	**1**	3	2	0	1	**6**
15	IV1L8	3	4	3	3	**13**	3	2	3	4	**12**	4	4	4	3	**15**
16	IV2M20	2	4	1	3	**10**	3	1	1	3	**8**	4	4	3	3	**14**
17	LM1E36	1	1	1	2	**5**	1	1	3	2	**7**	2	3	3	3	**11**
18	LM1M24	2	1	1	2	**6**	1	0	1	2	**4**	3	1	2	3	**9**
19	LM2E21	1	n.e.	1	1	**3**	0	0	1	1	**2**	2	2	2	2	**8**
20	1.37.10Y	n.e.	n.e.	n.e.	n.e.	**-**	1	n.e.	n.e.	1	**2**	1	2	1	2	**6**
21	2.23S2Y	2	2	1	3	**8**	2	2	2	2	**8**	3	3	4	3	**13**
22	3.4S22Y	1	1	0	0	**2**	1	1	0	0	**2**	2	1	0	0	**3**

Table 13: Anti-listerial activity of yeast against six different *L. monocytogenes* indicator strains, WSLC 1001, 1039, 1211, 1364, 1416 and 1685, in the independent experiments of the reproducibility test of the agar-membrane screening assay. Total anti-listerial activity given as a sum of inhibition values obtained in the individual experiments (I to IV). Maximum value in each experiment: 30; minimum value: 0

No.	Strain	Values in experiment no.				Score of total anti-listerial activity
		I	II	III	IV	
1	WSYC 223	28	22	25	25	100
2	WSYC 312	27	22	25	26	100
3	WSYC 263	27	25	22	24	98
4	WSYC 51	22	23	22	21	88
5	WSYC 1	19	18	22	22	81
6	H-AG43	25	17	14	24	80
7	IV1L8	18	21	15	19	73
8	IV2M20	16	17	9	16	58
9	2.23S2Y	12	12	13	13	50
10	WSYC 122	14	16	6	10	46
11	LM1E36	5	12	10	13	40
12	LM1M24	11	4	7	12	34
13	LM2E21	6	5	6	7	24
14	WSYC 184	2	0	6	13	21
15	G1077	6	3	4	4	17
16	IV1M1	6	6	0	5	17
17	WSYC 22	3	4	5	4	16
18	WSYC 215	2	2	5	6	15
19	3.4S22Y	7	5	0	0	12
20	H-2a21	6	2	3	0	11
21	1.37.10Y	2	3	1	4	10
22	TS3M4	0	0	0	0	0

Publications

Goerges S., Aigner, U., Silakowski, B. and Scherer, S. Food-borne yeast inhibit *Listeria monocytogenes* in a co-cultivation assay. Submitted.

Goerges S., Bora N., Gelsomino R., Rea M., Irlinger F., Beduhn R., Cogan T.M., Goodfellow M., Swings J., Vancanneyt M., Ward A.C. and Scherer S. Biodiversity of surface microbial consortia from Limburger cheese. Submitted.

Goerges S., Mounier J., Rea M., Gelsomino R., Cogan T.M., Swings J., Vancanneyt M. and Scherer S. Incidence of commercial surface starter microorganisms during ripening of a German red smear cheese: Competition with resident microbes of the dairy. In preparation.

Hohenegger M., Bora N., Gelsomino R., Goerges S., Goodfellow M., Ward A.C., Swings J., Vancanneyt M., Scherer S. and Sebastiani H. Surface microflora of Tilsit cheese. Submitted.

Larpin S., Bonaïti C., Bora N., Gelsomino R., Goerges S., Irlinger F., Goodfellow M., Ward A.C., Vancanneyt M., Swings J., Scherer S., Guéguen M. and Desmasures N. Biodiversity of surface microbial consortia from Livarot, a French traditional smear ripened cheese. Submitted.

Mounier J., Gelsomino R., Goerges S., Vancanneyt M., Vandemeulebroecke K., Hoste B., Scherer S., Swings J., Fitzgerald G.F. and Cogan T.M. 2005. Surface microflora of four smear-ripened cheeses. Appl. Environ. Microbiol., in press.

Mounier J., Goerges S., Gelsomino R., Vancanneyt M., Vandemeulebroecke K., Hoste B., Brennan N.M., Scherer S., Swings J., Fitzgerald G.F. and Cogan T.M. Sources of the adventitious microflora of a smear-ripened cheese. Submitted.

Oberreuter H., Brodbeck A., von Stetten S., Goerges S. and Scherer S. 2003. Fourier-transform infrared (FT-IR) spectroscopy is a promising tool for monitoring the population dynamics of microorganisms in food stuff. Eur. Food Res. Technol. 216: 434-439.

Rea M.C., Goerges S., Gelsomino R., Mounier J., Brennan N.M., Vancanneyt M., Scherer S., Swings J. and Cogan T.M. Yeast and bacterial biodiversity on the surface of Gubbeen cheese. Submitted.

Thomas A., Mathieu B., Bora N., Gelsomino R., Goerges S., Irlinger F., Goodfellow M., Ward A.C., Vancanneyt M., Swings J., Scherer S., Cogan T. and Chamba J.-F. Biodiversity of the surface microflora from Reblochon, a French traditional smear ripened cheese. Submitted.

CURRICULUM VITAE

STEFANIE GOERGES

PERSONAL DATA

Date of birth	1977-02-14
Place of birth	Augsburg
Nationality	German
Marital status	unmarried

EDUCATION

Jan. 2002 – Aug. 2005	Ph.D. in Food Microbiology (Dr. oec. troph.) Abteilung Mikrobiologie, Zentralinstitut für Ernährungs- und Lebensmittelforschung Weihenstephan (ZIEL, formerly FML), TU München
1996 - 2001	Diploma studies of Nutritional Science and Home Economics (Ökotrophologie) TU München - Weihenstephan
May 1996	Abitur (general qualification for university entrance) Gymnasium Maria Stern, Augsburg

WORK EXPERIENCE

Since 2002	Providing expert opinions on microbial analyses for clients from the food industry Abteilung Mikrobiologie, Zentralinstitut für Ernährungs- und Lebensmittelforschung Weihenstephan (ZIEL), TU München
June – Nov. 2001	Diploma thesis: Extension of the existing coryneform spectra database for identifying coryneforms from red smear cheese by FTIR spectroscopy Institut für Mikrobiologie, Forschungszentrum für Milch und Lebensmittel (FML) Weihenstephan, TU München
1999 (eight weeks)	Practical training in the microbiological laboratory Allgäuer Alpenmilch GmbH, Nestlé Deutschland AG, Biessenhofen