Application of a defined surface culture for ripening of Tilsit cheese

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1. Introduction

Tilsit, Limburg, Chaumes, Appenzell and many other cheese varieties possess a surface microflora called smear. It consists of mostly desirable species of coryneform bacteria, staphylococci and yeasts (1, 2). A certain degree of contamination with enterobacteria, enterococci, pseudomonads, and moulds is quite common (3, 4). The smear is applied to green cheeses by manual or automated brushing or spraying with salt water containing recycled smear from mature cheeses, called old-young cycle. The disadvantages of this approach are apparent: undesirable microorganisms present on mature cheeses are transferred to all new batches. Therefore it is not surprising that contamination of smear cheeses with *Listeria monocytogenes* is reported periodically (5, 6, 7). The last two incidences in Germany were 2006/2007 and concerned Tilsit- and Harzer (acid curd) cheese, with *L. monocytogenes* counts of 400 and 10^5 cfu/g, respectively (Stengel, Lebensmittel-, Veterinaer- und Umweltuntersuchungsamt, Neumuenster, Germany, personal communication). A warning against consumption of acid curd cheese was printed in local newspapers (e.g. Kieler Nachrichten, 30.11.2006). Apart from the economical damage, such cases are accompanied with a considerable image loss, not only for Tilsit and acid curd cheeses.

Due to advances in molecular identification methods, major bacterial groups of several cheese groups were determined, which are essential for surface protection (fast growth) and product quality (sensory and appearance). A typical surface culture for semi-soft cheese (e.g. Tilsit) consists of *Debaryomyces hansenii*, *Staphylococcus equorum*, *Microbacterium gubbeenense*, *Corynebacterium casei* and *Brevibacterium linens* (8). For soft cheese ripening, *Geotrichum candidum* is necessary in addition, whereas the presence of *C. casei* is not essential (9). Different yeasts, *Kluyveromyces marxianus* and *Candida krusei*, dominate ripening of acid curd cheese; *S. equorum* and *B. linens* are the essential bacterial species (10).

A defined surface culture with a composition described above was used successfully for Tilsit cheese ripening at lab scale (8). For each species in the smear liquid a concentration of 10^8 cfu*ml^{-1} was found necessary, which matches recommendations by

starter suppliers. The present paper describes the microbiology of the ripening of experimental semi soft cheeses with defined surface cultures in pilot scale of about 750 kg per batch. Pulsed-field gel electrophoresis (PFGE) of isolated surface bacteria was used to show the presence of the starter strains on the surface of mature cheeses.

2. Materials and methods

2.1 Smear cultures

The smear cultures used for pilot scale cheese ripening are listed in Table 1. The strains were taken from the culture collection of the Institute for Microbiology and Biotechnology, Kiel or from the collection of Arla Food (strains 047-0399, 025-0165). All strains were originally isolated from cheese.

Tab 1: Composition of surface starter cultures used for the experimental cheese batches produced in pilot scale.

Defined cultures	Mix C	Mix D
D. hansenii 6004 (beige)	X	X
B. linens Br5 (light orange)	X	X
B. casei 047-0399 (beige)	X	X
C. casei CA3 (beige-red)	X	X
C. variabile 025-0165 (beige)		X
M. gubbeenense CA12 (yellow)	X	X
S. equorum Staph 2 (orange)	X	X
S. sciuri Staph4 (beige)		X

Production of the strains used for cheese trials was performed in 6 l Erlenmeyer shake flasks in different Danisco production media adapted for *Brevibacterium*, *Microbacterium* and *Corynebacterium*. The coryneforms were grown at 25 °C, *S. equorum* at 30 °C to early stationary phase, determined by measuring the optical density at 650 nm. Growth media allowed yields of $>10^9$ cfu*ml^{-1} for bacteria and $>10^8$ cfu*ml^{-1} for yeasts.

For cheese trials the starters were concentrated and frozen in liquid nitrogen with a production yield of $>10^{13}$ cfu, $>10^{12}$ cfu (*B. linens*) and $>10^{11}$ cfu for yeasts calculated per 6 l flask culture. The preparations were checked for contamination. They were found free of enterobacteria (<1*g^{-1}), staphylococci (<10*g^{-1})), enterococci (<10*g^{-1})), yeasts and moulds (<10/g) and sulphite anaerobes.

2.2 Cheese production and ripening

Tilsit cheeses with normal characteristics (45 % fat in dry matter) were produced in a pilot plant of Arla Food and brined in freshly prepared brines containing ca. 20% salt and *D. hansenii* and *S. equorum* cultures (10^4 cfu*ml^{-1} and 10^5 cfu*ml^{-1}, respectively). The cheeses were smeared and ripened in a small ripening room separate from production facilities and specially set up for the cheese trials. Temperature and humidity (RH) were

set to 13°C +/- 0.5 °C and 95% RH +/- 1% RH, respectively. The surface cultures used for smearing are listed in Table 1. The concentration of each strain in the smear was set to 10^8 cfu*ml^{-1}. During the two weeks of cheese ripening surface pH was measured on several spots of the cheeses with a surface pH electrode.

2.3 Determination of cell counts

For determination of surface cell counts, a defined area of the cheeses was scraped with cotton wool pads and plated on modified milk agar according to a method described by Hoppe-Seyler et al. (2000; 11). The different species used in starter were identified by colony morphology and cell morphology, as analysed by phase contrast microscopy. Yeasts and moulds were evaluated on YGC agar, staphylococci on SK agar, enterococci on GSP agar, enterobacteria on VRBD agar and pseudomonads on GSP agar (all agars from Merck, Darmstadt).

2.4 Pulsed-field gel electrophoresis

For characterisation of starter strains, PFGE was used according to Hoppe-Seyler et al. (2003; 12). For restriction enzyme digestion of chromosomal DNA, endonuclease AscI was applied, except for staphylococci where SmaI was used. A CHEF-DRII system of Biorad was used for analysis. The voltage was set to 175 V, the pulse ramp was 1-15 s over 24 h, electrophoresis was performed at 14 °C. For size estimation of DNA fragments, the „low molecular range marker“ of New England- Biolabs (Germany) was used (size range: 2-200 kb).

3. Results and Discussion

The first cheese trial (with starter mixes A + B) suffered from mould contamination of the experimental cheeses as well as of the old-young smeared control (reference cheese) due to suboptimal conditions in the ripening room, which had been just setup for the cheese trials (data not shown).

Ripening conditions and starter handling were optimised for the second cheese trial (mix C+D, reference). Total counts in the freshly prepared cheese brines were $3x10^4$ cfu*ml^{-1} for *D. hansenii* 6004 and $9x10^4$ cfu*ml^{-1} for *S. equorum* Staph2. The resulting bacterial surface cell counts after smearing were 5×10^4 cfu*cm^{-2} for mix C and 4×10^4cfu*cm^{-2} for mix D. The values were comparable to the old-young smear (4×10^4 cfu*cm^{-2}). Ripening of the old-young smeared reference cheese proceeded faster than for mix C +D cheeses. Surface cell counts of 10^9 cfu*cm^{-2} were observed after 8d of ripening (14d for mix C + D cheeses, Table 2a-c). Correspondingly, the pH increase (lactate degradation) of the experimental cheeses was slower than for the reference (Fig. 1).

A typical smear developed on all cheeses. The surface microflora of the reference cheese consisted of typical colonies and cells (microscopy) indicating *Corynebacterium*, *Brevibacterium*, *Microbacterium*/ *Arthrobacter* and *Staphylococcus* species (Table 2a-c). Further identification was not performed. Beige pigmented coryneforms (*Corynebacterium* species, 3 colony types) dominated the surface flora in mature cheeses, which is in agreement with results of Rademaker et al. (2005; 13). Staphylococcal counts were highest at the beginning of ripening, dropping over 2 magnitudes in the ripening period. For both experimental cheeses a similar typical smear composition was observed. Only colonies, typical of the starter strains used, were found on modified milk agar. A few

selected isolates (beige, yellow and orange coryneforms, and staphylococci) were identified by ARDRA according to Hoppe-Seyler et al. (12, 14, 15), which confirmed the presence of the expected starter species (data not shown). Classification of strains by pulsed field gel electrophoresis showed the presence of all 7 strains in the surface flora of mature smear cheeses (Fig. 2a-c).

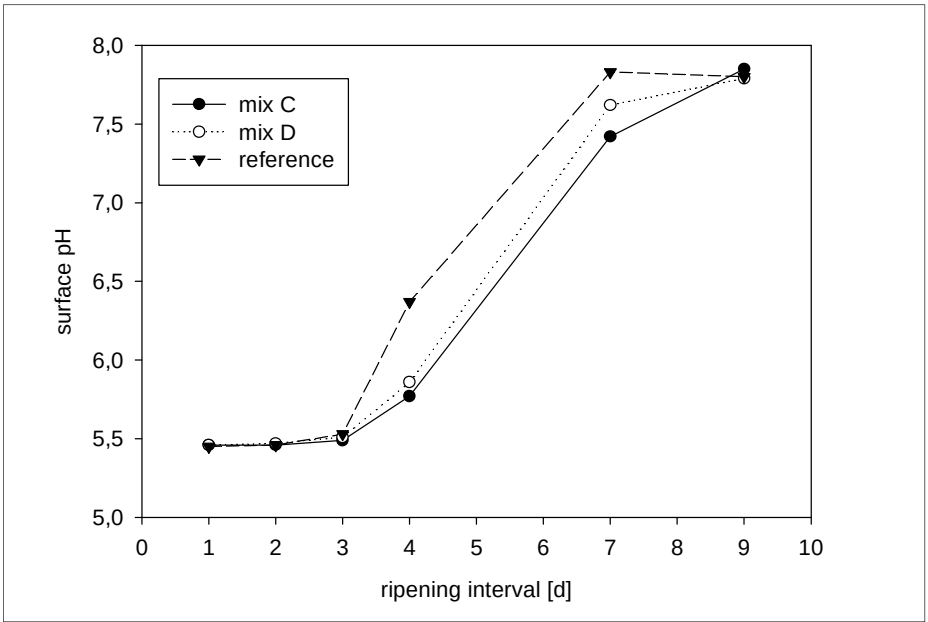

Fig. 1: Deacidification of the cheese surface measured with a flat surface pH electrode. Mix C & D were defined starters, the reference was typically old-young smeared.

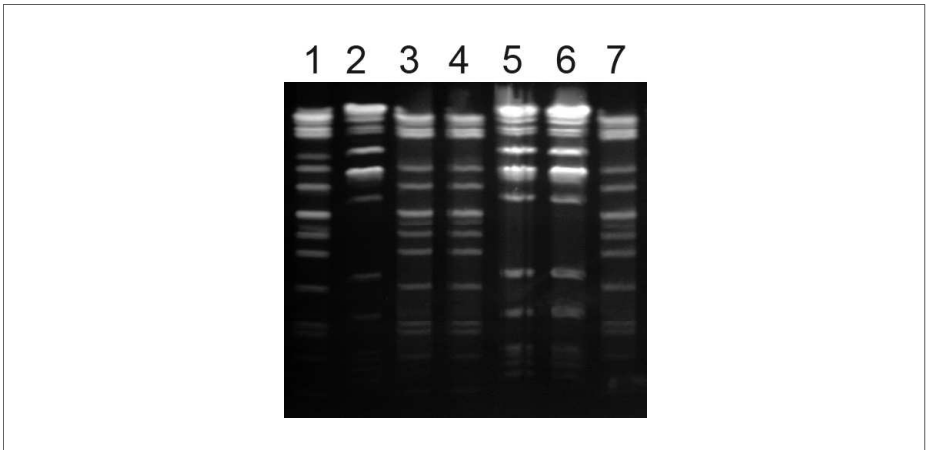

Fig. 2a: PFGE restriction analysis (SmaI digest) of *Staphylococcus* strains applied with smearing. Lane 1: reference strain *S. equorum* Staph2; lane 2: reference strain *S. sciuri* Staph4; lane 3 *S. equorum* Staph2 (preparation used for smearing); lane 4+7 *S. equorum* Staph2 isolated from mix C+D cheeses; lane 5+6 *S. sciuri* Staph4 isolated from mix D cheeses.

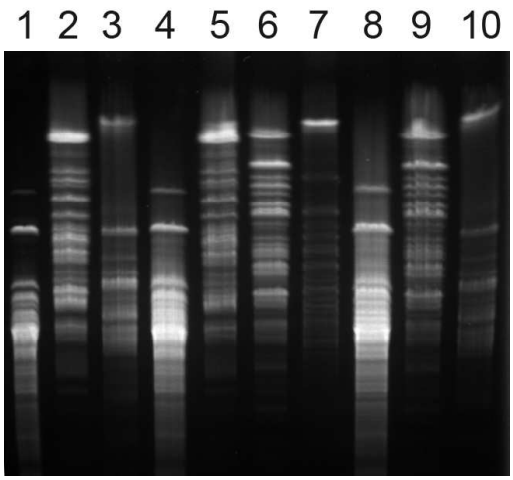

Fig. 2b: PFGE restriction analysis (AscI) of coryneform strains applied to mix C+D with smearing. Lane 1: reference strain *M. gubbeenense* CA12; lane 2: reference strain *B. linens* Br5; lane 3 reference strain *C. variabile* 025-0165; lane 6: reference strain *B. casei* 047-0399; lane 4+8: *M. gubbeenense* isolates matching strain CA12; lane 5: *B. linens* isolate matching strain Br5; lane 7: unknown coryneform isolate; lane 9: *B. casei* isolate matching strain 047-0399; lane 10: *C. variabile* isolate matching strain 025-0165.

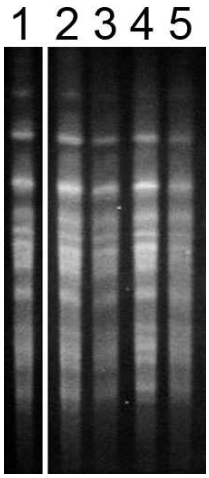

Fig. 2c: PFGE restriction analysis of *Corynebacterium* strains applied to mix C+D with smearing (AscI digest). Lane 1: reference strain *C. casei* CA3; lane 2-5: isolates matching strain CA3.

Tab. 2a: Surface microflora of reference cheeses (old-young smear)

Reference	Cell counts [cfu*cm^{-2}]	48h	8d	14d	28d	56d
mould		n.d.*	-	5.1x10^{4}	-	2.5x10^{4}
yeast		n.d.	5.7x10^{6}	4.6x10^{5}	2.6x10^{7}	3.9x10^{4}
bacteria		n.d.	1.2x10^{9}	1.3x10^{9}	4.2x10^{8}	1.4 x10^{8}
coryneforms (beige)		n.d.	10.4%	79.9%	81.8%	52.0%
coryneforms (beige-red)		n.d.	5.8%	8.8%	3.9%	45.5%
coryneforms (beige)		n.d.	-	5.8%	5.3%	0.7%
rods (beige)		n.d.	-	3.6%	1.0%	-
Microbacterium / Arthrobacter (yellow)		n.d.	-	-	3.9%	-
brevibacteria (light orange)		n.d.	-	1.3%	3.9%	1.7%
staphylococci (white-beige)		n.d.	10.4%	0.6%	0.2%	0.1%
staphylococci (light-orange)		n.d.	73.5%	-	-	<0.1%
pseudomonads (GSP)		n.d.	-	-	-	-
enterococci (KAA)		n.d.	-	-	-	<0.1%
enterobacteria (VRBD)		n.d.	-	-	-	-

*n.d. = not determined, „-“ = not detected, detection limit: 100 cfu*cm^{-2}

Tab. 2b: Surface microflora of reference cheeses (old-young smear)

Mix C	Cell counts [cfu*cm^{-2}]	48h	8d	14d	28d	56d
mould		<1x10^3	-	-	-	2.0 x10^5
yeast		2.6 x10^3	6.6x10^7	5.2x10^7	2.6x10^7	3.0x10^7
bacteria		5.3 x10^4	2.3x10^8	2.1 x10^9	5.7x10^9	2.1 x10^9
C. casei CA3 (beige)		-	14.7%	40.1%	87.9%	79.4%
M. gubbeenense CA12 (yellow)		-	25.5%	5.3%	5.8%	7.9%
B. linens Br5 or B. casei 047-0399		-	24.7%	43.0%	2.8%	2.7%
S. equorum (beige)		71.2%	-	-	-	<0.1%
S. equorum Staph2 (orange)		-	35.1%	11.6%	3.5%	8.4%
rods (beige)		28.8%	-	-	-	-
pseudomonads (GSP)		-	-	-	-	-
enterococci (KAA)		0.18%	-	-	-	-
enterobacteria (VRBD)		-	-	-	-	-

*n.d. = not determined, „-“ = not detected, detection limit: 100 cfu*cm^{-2}

Tab. 2c: Surface microflora of reference cheeses (old-young smear)

Mix D	Cell counts [cfu*cm^{-2}]	48h	8d	14d	28d	56d
mould		-	-	-	-	8.1x10^3
yeast		-	6.5x10^6	1.2x10^7	4.0x10^6	3.7x10^6
bacteria		1.6 x10^3	5.0x10^8	9.6x10^8	1.3x10^4	8.3x10^8
C. casei CA3 (beige)		-	29.9%	1.9%	1.9%	25.2%
C. variabile 025-0165 (white)		-	8.2%	71.6%	41.9%	19.2%
M. gubbeenense CA12 (yellow)		-	0.7%	3.4%	9.3%	10.7%
B. linens Br5 or B. casei 047-0399		-	37.8%	15.6%	44.2%	37.2%
S. sciuri Staph4 (white)		-	21.9%	7.9%	2.5%	5.9%
S. equorum Staph2 (orange)		-	1.6%	0.4%	0.3%	1.9%
rods (beige)		100%	-	-	-	-
pseudomonads (GSP)		-	-	-	-	-
enterococci (KAA)		-	-	-	-	-
enterobacteria (VRBD)		-	-	-	-	-

*n.d. = not determined, „-“ = not detected, detection limit: 100 cfu*cm^{-2}

On all cheeses mould contamination was detected, however, mould spots were only visible on mix C + D cheeses (Table 2a-c). This corresponded to a dryer surface and lower surface smear counts of mix C+D cheeses in the first week of ripening than the reference. To achieve mould-free ripening the smear counts of Tilsit cheese should develop to approx. 10^9 cfu*cm^{-2} after one week of ripening (16, 17). The overall flavour of all cheeses was typical (evaluation by Arla Food, data not shown). This indicates that it might be possible to replace an undefined old-young smear with a defined starter consisting of only a few strains and keep the original aromatic properties. Compositional analysis of the experimental cheeses (mix C, D, reference) by Hannon et al. (2004; 18) indicated that the surface of cheeses differed from the core due to the action of the smear but that the composition of the cheeses made with the defined strain smear were similar to those made with the reference smears. By principal component analysis of proteolysis products, cheeses made with the reference smear (R) were separated from those made with defined strains (mix C/D) at the end of ripening, reflecting differences in proteolysis. At the time of the cheese trial, the use of defined starters would probably not have been

necessary, because no contamination of the reference smear (old-young type) with enterobacteria, enterococci or pseudomonads was detected indicating no potential negative effect of the old-young smearing. However, bacterial contamination of commercial smear cheeses is rather common and not an exception (16).

Tab. 3: Identification of smear strains on the surface of ripened Mix C + D cheeses by PFGE. Bacteria were isolated after 2 + 8 weeks of ripening.

Bacteria used in starter	Colony colour	No. of isolates	PFGE-identification
B. linens, B. casei	beige-orange	14	12 x *B. linens* Br5 1 x *B. casei* 047-399 1 x unknown pattern
M. gubbeenense	yellow	9	9 x *M. gubbeenense* CA12
C. casei, C. variabile	beige	18	10 x *C. variabile* 025-165 8 x *C. casei* CA3
S. equorum	orange	7	7 x *S. equorum* Staph2*
S. sciuri	beige	6	5 x *S. sciuri* Staph4 1 x unknown pattern

* The pattern of the isolates did not match the original pattern of *S. equorum* Staph2 from the strain collection of the institute. It matched, however, the DNA pattern of the lyophilised preparation of the *S. equorum* produced in bulk quantities for the experimental cheese trials (see Fig. 2a).

4. Conclusions

The main advantage of defined starters is that recycling of undesirable microorganisms (moulds, enterobacteria, pathogens etc.) via smearing can be avoided. Results show that it is possible to start surface ripening using defined smear starters. The cheeses, were more sensitive to mould growth than the old-young smeared reference cheeses which indicates that probably shorter smearing intervals are necessary, when defined starters are used. More trials with different species and strains are necessary to show if aromatic properties of defined smeared cheeses can compete with old-young smeared cheeses. The aroma of defined smeared cheeses might be less intense, however, this may not be a disadvantage, since many, especially young consumers, seem to prefer milder products.

5. References

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7. Summary

Bockelmann, W., Jaeger, B., Hoppe-Seyler, T.S., Lillevang, S.K., Sepulchre, A., Heller, K.J.: **Application of a defined surface culture for ripening of Tilsit cheese**. Kieler Milchwirtschaftliche Forschungsberichte **59** (3) 191-202 (2007)

26 Microbiology (old-young smearing, smear cheeses, Listeria, Brevibacterium, Corynebacterium, Microbacterium, Staphylococcus, defined surface culture)

Three batches of Tilsit cheeses with normal characteristics (45 % fat in dry matter) were produced in a pilot plant of Arla Food (700 kg scale) and salted in freshly prepared cheese brines containing *Debaryomyces hansenii* and *Staphylococcus equorum* cultures (10^4 cfu*ml^{-1} and 10^5 cfu*ml^{-1}, respectively). Experimental cheeses were smeared with 2 different defined surface cultures (ca. 10^8 cfu*ml^{-1}) containing *D. hansenii*, *S. equorum*, *Corynebacterium casei*, *Brevibacterium linens* and *Microbacterium gubbeenense*. One surface culture contained *C. variabile* and *Staphylococcus sciuri* in addition. The

reference cheese was typically old-young smeared as control. The fastest smear development and deacidification of the cheese surface was observed for the old-young smeared reference cheese. In spite of slower smear development, the two experimental cheeses developed typical aroma and colour. Bacteriological analysis showed mould contamination for all cheeses including the control. The contamination (mould spots) was visible on the experimental cheeses, only. By pulsed-field gel electrophoresis it could be shown that strains used in the surface starter were present on mature cheeses.

It was concluded that it is possible to start surface ripening using defined smear starters. The cheeses were more sensitive to mould growth than the old-young smeared reference cheeses, which indicates that probably shorter smearing intervals are necessary at ripening start when defined surface cultures are used. The main advantage of defined starters is that recycling of undesirable microorganisms (moulds, enterobacteria, pathogens etc.) via old-young smearing can be avoided.

Zusammenfassung

Bockelmann, W., Jaeger, B., Hoppe-Seyler, T.S., Lillevang, S.K., Sepulchre, A., Heller, K.J.: **Einsatz einer definierten Oberflächenkultur zur Reifung von Tilsiter Käse.** Kieler Milchwirtschaftliche Forschungsberichte **59** (3) 191-202 (2007)

26 Mikrobiologie (old-young smearing, smear cheeses, Listeria, Brevibacterium, Corynebacterium, Microbacterium, Staphylococcus, defined surface culture)

Drei Chargen Tilsiter Käse mit normalen Eigenschaften (Fett 45% in Trockenmasse) wurden in einer Versuchsanlage der Firma Arla Food (Arhus, Dänemark) im 700 kg-Maßstab hergestellt. Sie wurden in frisch angesetzten Salzbädern gesalzen, die *Staphylococcus equorum* und *Debaryomyces hansenii* Kulturen enthielten (10^5 KbE*ml^{-1} bzw. 10^4 KbE*ml^{-1}). Die Versuchskäse wurden mit 2 verschiedenen definierten Oberflächenkulturen geschmiert, die *D. hansenii*, *S. equorum*, *Corynebacterium casei*, *Brevibacterium linens* und *Microbacterium gubbeenense* enthielten (ca. 10^8 KbE*ml^{-1}). Eine der Oberflächenkulturen enthielt zusätzlich *Corynebacterium variabile* und *Staphylo-coccus sciuri*. Als Kontrolle wurden die Referenzkäse traditionell alt-jung-geschmiert. Die schnellste Schmiereentwicklung und Entsäuerung der Käseoberfläche wurde beim Referenzkäse mit der Alt-Jung-Schmiere beobachtet. Trotz langsamerer Schmierebildung entwickelten die beiden Versuchkäse typisches Aroma und Farbe. Die bakteriologische Analyse der Käseoberfläche zeigte Kontaminationen mit Schimmelpilzen für alle Käse einschließlich der Kontrolle auf. Schimmelpilzstellen waren nur auf den Versuchskäsen sichtbar. Durch Pulsfeld-Gelelektrophorese konnte nachgewiesen werden, dass die in der Oberflächenstarterkultur eingesetzten Stämme auch auf den reifen Versuchskäsen vorhanden waren.

Die Ergebnisse zeigen, dass Rotschmierekäse auch mit definierten Schmierekulturen erfolgreich gereift werden können. Die Versuchskäse waren anfälliger für Schimmelpilzbildung als die alt-jung geschmierten Referenzkäse, was darauf hinweist, dass wohl kürzere Pflegeintervalle zu Beginn der Reifung notwendig sind, wenn definierte Oberflächenkulturen eingesetzt werden. Der Hauptvorteil definierter Starterkulturen ist, dass das Recycling unerwünschter Mikroorganismen (Schimmelpilze, Enterobakterien, Krankheitserreger usw.) über das alt-jung Schmieren vermieden werden kann.

Résumé

Bockelmann, W., Jaeger, B., Hoppe-Seyler, T.S., Lillevang, S.K., Sepulchre, A., Heller, K.J.: **Application d'une culture de surface définie pour la maturation des fromages Tilsit**. Kieler Milchwirtschaftliche Forschungsberichte **59** (3) 191-202 (2007)

26 Microbiologie (old-young smearing, smear cheeses, Listeria, Brevibacterium, Corynebacterium, Microbacterium, Staphylococcus, defined surface culture)

Trois charges de fromage Tilsit avec des caractéristiques normales (matière grasse 45% dans la matière sèche) ont été fabriquées dans une installation pilote d'Arla Food (Arhus, Danemark) à une échelle de 700 kg. Elles ont été salées dans des bains de saumure fraîchement préparés qui contenaient des cultures de *Staphylococcus equorum* et *Debaryomyces hansenii* (10^5 ufc*ml^{-1}, voire 10^4 ufc*ml^{-1}). Les fromages d'essai ont été enduits de deux différentes cultures de surface définies qui contenaient *D. hansenii, S. equorum, Corynebacterium casei, Brevibacterium linens* et *Microbacterium gubbeenense* (environ. 10^8 ufc*ml^{-1}). Une des cultures de surface contenait en plus C*orynebacterium variabile* et *Staphylococcus sciuri.* Comme le fromage de contrôle, les fromages de référence ont été enduits traditionnellement de morge "old-young". La formation de morge et l'acidification la plus rapide de la surface du fromage était observée pour le fromage de référence avec la morge "old-young". Malgré la formation plus lente de la morge, les deux fromages d'essai développaient un arome et une couleur typiques. L'analyse bactériologique de la surface du fromage montrait une contamination avec des moisissures pour tous les fromages, les fromages de contrôle inclus. Les moisissures étaient seulement visibles sur les fromages de contrôle. L'électrophorèse en champ pulsé permettait de détecter que les souches utilisées dans la culture starter de surface étaient également présentes sur les fromages de contrôle affinés.

Les résultats révèlent que le fromage avec de la morge rouge peuvent également être affinés avec des cultures de morge définies. Les fromages de contrôle étaient plus susceptibles à la formation de moisissure que les fromages de référence "old-young", ce qui indique que des intervalles de traitement plus courts au début de la maturation sont nécessaires quand on applique des cultures de surface définies. L'avantage principal des cultures starter définies est que le recyclage d'organismes non-désirables (moisissure, entérobactéries, pathogènes, etc.) peut être évité par l'application de morge "old-young".