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Influence of lactococcal surface properties on cell retention and distribution in cheese curd

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ABSTRACT

During cheese manufacturing, on average 90% of the starter culture cells are believed to be entrapped in the curd, with the remainder lost in whey. This paper shows that plasmid-cured dairy strains of *Lactococcus lactis* show cell retention in the curd of 30–72%, whereas over-expression of pili on the lactococcal cell surface can increase cell retention to 99%. Exopolysaccharide production and cell clumping and chaining do not influence cell retention in cheese curd. *L. lactis* surface alteration also strongly affected the distribution of cells in the cheese matrix: clumping and over-expression of pili led to formation of large cell aggregates embedded in the protein matrix whereas exopolysaccharide expression resulted in cells being surrounding by small serum regions in the protein matrix of the cheese. These results suggest that surface properties of dairy starter cultures strongly determine retention and distribution of the bacteria in cheese curd.

1. Introduction

Approximately 90% of the starter culture cells are typically entrapped in the curd during cheese production, whereas the remaining 10% of cells are “lost” in the whey (Doolan, Nongonierma, Kilcawley, & Wilkinson, 2014; Jeanson et al., 2011). Once entrapped in cheese, *Lactococcus lactis* cells keep on growing to colonies of various sizes, which is a determinant of the pH, flavour profile (Collins, McSweeney, & Wilkinson, 2003; McSweeney & Sousa, 2000; Smit, Smit, & Engels, 2005), taste and texture (Smid & Kleerebezem, 2014) of the final product.

The spatial distribution and specific localisation of bacterial colonies in the curd is suggested to be important for cheese ripening. For instance, it was demonstrated that inoculation density influences the size and spatial distribution of colonies in the cheese matrix (Jeanson et al., 2011). The spatial distribution of cells was shown to influence metabolite production during cheese ripening: in cheeses with roughly the same final cell density, higher amounts of metabolites were detected in cheeses with small bacterial colonies than in cheeses with big colonies (Le Boucher et al., 2016). This phenomenon was related to the differences in surface exchange between the cheese matrix and bacterial colonies (Le Boucher et al., 2016). It was also reported that in cheese with a higher fat content, bacteria tend to locate increasingly in the vicinity of fat droplets or near the fat-protein interphase (Laloy, Vuillemard, El Soda, & Simard, 1996). In the same study a higher cell retention was found in full fat cheese, which was accompanied with increased flavour formation.

Bacterial retention and distribution in the cheese matrix are likely determined by interactions between microbial surface properties, such as surface charge and hydrophobicity, and the properties of, e.g., milk proteins and fat droplets. Surface properties of bacteria are determined by the molecular constituents of their cell walls, such as (lipo-)teichoic acids, proteins, pili or capsular polysaccharides (Delcour, Ferain, Deghorain, Palumbo, & Hols, 1999; Giaouris, Chapot-Chartier, & Briandet, 2009; Meyrand et al., 2013). For example, the presence of pili on lactococcal cell surfaces leads to cell chaining and cell clumping as well as to an increased hydrophobicity and lower negative charge of the cell surface (Tarazanova et al., 2016). Furthermore, milk protein binding seems to be more pronounced in lactococcal dairy isolates than with plant isolates (Tarazanova et al., 2017).

The composition of the bacterial cell wall can affect the textural properties of fermented dairy products through cell chaining,

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clumping, formation of pili (Tarazanova, Huppertz, Kok, & Bachmann, 2018) or of exopolysaccharide (EPS) (Burgain et al., 2014a,b, 2015; Ly-Chatain et al., 2010). *Lb. rhamnosus* EPS-milk protein interactions, for instance, lead to increased water retention and a softer cheese matrix, increased yoghurt viscosity and longer texture (Burgain et al., 2014a). Incidental evidence showed that chaining cells of *L. lactis* were in contact with fat droplets and it was suggested that cell chains even could form bridges between fat droplets (Ly-Chatain et al., 2010).

Here the impact of *L. lactis* cell morphology, resulting from alterations of surface properties such as decoration with pili, cell chaining and/or cell clumping, on the retention of cells in Gouda-type cheese and on their distribution patterns in the cheese matrix was studied. To this end 10 isogenic *L. lactis* strains were used that only differed in known cell surface properties and/or morphology. The results demonstrate that the presence of pili on the bacterial cell surface significantly increases immobilisation of cells in the cheese curd. Furthermore, cell clumping and overexpression of pili lead to the formation of large cell aggregates in the cheese matrix.

2. Materials and methods

2.1. Bacterial strains, growth and enumeration

The strains used as starter cultures for the manufacturing of Gouda-type cheese are listed in Table 1. They were pre-cultured overnight at 30 °C in 20 mL sterilised (115 °C, 10 min) full-fat bovine milk (3.5% fat, 3.3% protein). For lactose-(Lac$^-$) and protease-negative strains, glucose (50% stock solution dissolved in sterilised full-fat milk) and Bacto™ casitone (BD Biosciences, Breda, The Netherlands; 20% stock solution in demi-water) were added to final concentrations of 1% and 0.2%, respectively. When required, erythromycin (Ery; 10 μg mL^{-1}), chloramphenicol (Cm; 5 μg mL^{-1}), rifampicin (Rif; 50 μg mL^{-1}), or streptomycin (Str; 100 μg mL^{-1}) were added to the indicated final concentrations.

2.2. Gouda-type cheese production

Gouda-type cheese was made from pasteurised (72 °C for 15 s) non-homogenised full-fat bovine milk (3.5% fat, 3.3% protein, 4.5% lactose) supplied by the NIZO pilot plant. To ensure growth of all strains, the milk was supplemented with 0.2% casitone, and with 4% glucose for lactose-deficient strains. The milk (2.0 L) was inoculated with 20 mL of an overnight culture (~10^9 cfu mL^{-1}) of the strains listed in Table 1. In contrast to the preculture, for the actual cheese making no antibiotics were added. Further, 400 μL L^{-1} of a 35% $CaCl_2$ solution and 230 μL L^{-1} rennet (Kalase 150 IMCU mL^{-1}, CSK Food Enrichment, Ede, The Netherlands) were added and the milk was allowed to coagulate for 45 min at 30.5 °C. Subsequently, the curd was cut into cubes with an edge length of 0.3–0.5 cm by slowly moving a cutting device through the curds for 10 min. When the curd volume had been reduced to ~30% of the initial milk volume, and 70% of the original volume had thus been released as whey, an amount of whey equivalent to 40% of the total volume (the 1st whey) was removed and replaced with an equivalent volume of sterile tap water of 45 °C. This washing step is typical for Gouda cheese manufacture, with the purpose to wash out lactose (and thus limit final acidity) and to raise the temperature to increase syneresis. The curd was then stirred for 1 min with 10 min intervals over 1.5 h at 36 °C. The curd was transferred into cheese moulds and pressed at 27 g cm^{-2} for 1.5 h at room temperature. After 45 min, the cheese was turned upside down in the mould and pressed for another 45 min. The cheese was subsequently incubated for 18 h at 30 °C, after which the pH was measured and it was salted for 1 h in brine solution containing 23% salt. After brining, the cheese was dried in a sterile flow cabinet for 1 h at room temperature and vacuum packed under 1% nitrogen, and ripened at 13 °C for 12 wk.

2.3. Distribution of starter cells between curd and whey

Colony forming units in curd and the 1st whey were determined at the point of removal of the 1st whey, as outlined in section 2.2. For the determination of cell counts, 3 g of curd were mixed with 27 mL of sterile 2% sodium citrate (t = 40 °C) and homogenised for 8 min in a Stomacher (Model no. BA 60201, Seward Medical UAC House, London, UK). For each sample of either the 1st whey or the curd, three replicate serial dilutions were prepared and subsequently plated on M17 (Oxoid Ltd, Basingstroke, UK) agar medium containing 1% glucose. Colony forming units were quantified after 48 h of incubation at 30 °C. The cell retention in curd, taking into account the amount of curd and whey at the time of sampling, was calculated as the fraction of cells in curd as given in Eq. (1). Alternatively the volume corrected fraction of cells retained in the 30% curd was calculated as outlined in Eq. (2). This correction gives a clearer picture of cell retention in curd as the final curd/whey ratio changes throughout the process and this calculation gives a volume independent measure. Throughout the paper - the volume corrected fractions were used for comparisons.

$$\textit{Cell fraction in curd} = 100 \times \frac{\textit{cfu per g curd}}{(\textit{cfu per g curd} + \textit{cfu per mL whey})} \quad (1)$$

Table 1
List of strains used for Gouda-type cheese manufacturing in this study.

No	*L. lactis* strains	Characteristic	Reference
1	NCDO712	*L. lactis* ssp. *cremoris* wild type dairy isolate. Lac$^+$; Contains 6 plasmids − pLP712, pSH71, pSH72, pSH73, pSH74, pNZ712	Gasson, 1983; Tarazanova et al., 2016
2	MG1363	Plasmid-cured derivative of *L. lactis* NCDO712; Lac$^-$	Gasson, 1983
3	MG1363(pIL253*pil*)	*L .lactis* MG1363 harboring pIL253*pil* encoding the pilin operon *spaCB-spaA-srtC1-srtC2* from NCDO712; Ery^R; Lac$^-$	Tarazanova et al., 2016
4	MG1363(pNZ4120)	*L. lactis* MG1363 harboring EPS gene cluster from *L. lactis* subsp. *cremoris* B40 on pNZ4120; Ery^R; Lac$^-$	Boels et al., 2003
5	MG1614	*L. lactis* MG1363; Str^R; Rif^R; Lac$^-$;	Gasson, 1983
6	MG1614_clu$^+$	*L. lactis* MG1614 transconjugant harboring pLP712 and showing a clumping phenotype. Str^R; Rif^R; Lac$^+$	Tarazanova et al., 2018
7	MG1614_clu$^-$	*L. lactis* MG1614 transconjugant carrying pLP712 and showing a non-clumping phenotype. Str^R, Rif^R, Lac$^+$	Tarazanova et al., 2018
8	IL1403	Plasmid-free derivative of *L. lactis* ssp. *lactis* IL594; Lac$^-$	Bolotin et al., 2001
9	IL1403Δ*acmAacmD*	IL1403 in which the *acmA* and *acmD* genes were removed by double cross-over recombination, resulting in a chaining phenotype; Lac$^-$	Visweswaran et al., 2013
10	IL1403(pIL253*pil*)	IL1403 harboring the pilin operon *spaCB-spaA-srtC1-srtC2* from strain NCDO712 on pIL253; shows chaining phenotype and high hydrophobicity; Ery^R; Lac$^-$	Tarazanova et al., 2016

$$\text{Volume corrected cell fraction in curd} = 100 \times \frac{30 \times \text{cfu per g curd}}{(30 \times \text{cfu per g curd} + 70 \times \text{cfu per mL whey})} \quad (2)$$

2.4. Localisation of cells in the cheese matrix

Confocal laser scanning microscopy (CLSM) was applied on cheese samples after 12 wk of ripening. From the centre of a cheese sample a slice of 2–3 mm thickness and 4–5 mm in length was cut with a sterile scalpel blade. A mixture of 0.5% Acridine Orange (AO) (Sigma–Aldrich, Schnelldorf, Germany) and 0.025% Rhodamine B (Sigma–Aldrich) in water was placed on top of the cheese slice to stain bacteria and the protein matrix, respectively. Surplus dye was removed after 1–2 min, and the specimen was placed on a 25 × 50 mm glass slide such that firm contact was formed between the cheese and the glass slide. Confocal images were taken using a Leica TCS SP 5 confocal laser-scanning microscope (Leica, Mannheim, Germany) with Leica application Suite Advanced Fluorescence software v. 2.7.3. build 9723. The Argon laser was used to visualise the bacteria stained with AO, while the DPSS 561 laser was used to visualise the cheese protein matrix stained by Rhodamine B. Fat droplets remained unstained.

2.5. Statistical analysis

Results were analysed using Microsoft Excel. Pairwise comparisons of cell distribution between surface altered and their parental strains were analysed with a two-tailed t-test and considered significant if *p*-values of were smaller than 0.01.

3. Results and discussion

3.1. Distribution of cells between curd and whey

To investigate the effect of *L. lactis* surface properties on bacterial cell retention in cheese curd and the patterns of cell localisation in the cheese matrix, 10 strains of surface-engineered isogenic *L. lactis* strains were used. The surface morphology of the strains was modified in terms of cell chaining, clumping, EPS formation and pili expression. EPS formation was achieved by introducing the EPS gene cluster from *L. lactis* subsp. *cremoris* B40 (Boels et al., 2003) into strain MG1363. The production of pili was achieved through expression of the Spa-pilin gene cluster *spaCB-spaA-srtC1-srtC2*, from plasmid pSH74 of strain NCDO712, and cloned in the multicopy plasmid pIL253 (Tarazanova et al., 2016). To our knowledge, this is the first study where surface altered lactococci were used to study cell retention in curd.

The results showed that 89% of the starter culture was retained in the curds when the wild-type *L. lactis* subsp. *cremoris* dairy isolate NCDO712 was used, but this decreased to 30% for its plasmid-free derivative MG1363. The plasmid-cured *L. lactis* subsp. *lactis* strain IL1403 showed 53% cell retention. Over-expression of the EPS cluster in MG1363(pNZ4120) also resulted in a low retention of cells in the curd (33.7 ± 4.6%). The results of cell retention in curd for the wild type strain NCDO712 are consistent with literature findings (Doolan et al., 2014). However, cell retention of plasmid cured NCDO712 and IL594 derivatives MG1363, MG1363(pNZ4120) and IL1403 were much lower. The plasmid cured strain MG1614 was selected for spontaneous resistance to rifampicin and streptomycin and shows significantly increased cell retention in curd compared with its parent strain MG1363. While this increased retention in cured coincides with decreased zeta potential and increased hydrophobicity (Tarazanova et al., 2018) the molecular link to this phenotype is not clear.

The loss of cells in the whey fraction could arise from several processes. It could be caused by release of cells from the surface of curd granules after cutting of the curd, similar to the loss of some of the fat globules to whey (Heino, Uusi-Rauva, & Outinen, 2010). However, assuming an even cell distribution and the loss of all cells in the outer 10 μm surface layer of a curd particle with the dimensions of 5 × 5 × 5 mm, this would account for <1% of cell loss. Additionally, higher losses could possibly take place if large clusters of cells are present in weak conglomerates in the matrix at the position where cutting of curd occurs. However, even those losses are unlikely to explain the loss of 10% or more of cells into the whey. Hence, there appears to be a mechanism of 'active removal' of cells from the cheese curd. This is most likely related to the syneresis process where whey is drained from the curd and any materials unattached to the matrix can be removed with the whey if their size is smaller than the pores in the curd matrix.

Table 2
Distribution (%) of cells between whey and curd.[a]

Strain	cfu mL^{-1} whey	cfu g^{-1} curd	Cell fraction in curd (%)	Volume corrected cell fraction in curd (%)
Wild type dairy isolate				
NCDO712	1.73E + 06 ± 1.29E + 06	1.94E + 08 ± 1.86E + 08	94.6 ± 5.7	89 ± 11.7
Pili over-expression				
IL1403(pIL253*pil*)	4.23E + 06 ± 3.46E + 06	1.03E + 07 ± 6.66E + 06	65.4 ± 31.2	53.4 ± 36.4
IL1403	2.72E + 05 ± 1.17E + 04	5.98E + 07 ± 9.28E + 06	99.5 ± 0.1*	98.9 ± 0.2*
MG1363	1.54E + 07 ± 1.28E + 07	1.42E + 07 ± 1.13E + 07	49.7 ± 8.2	30.2 ± 7.7
MG1363(pIL253*pil*) (colony 1)	6.62E + 05 ± 3.48E + 05	1.73E + 07 ± 1.18E + 07	95.7 ± 1.3*	90.6 ± 2.6*
MG1363(pIL253*pil*) (colony 2)	1.76E + 06 ± 1.43E + 06	8.08E + 07 ± 7.29E + 07	97.4 ± 0.8*	94.2 ± 1.8*
Mixture of EPS and pili producing strains				
MG1363(pNZ4120)	5.62E + 06 ± 1.18E + 06	6.77E + 06 ± 1.99E + 06	54.1 ± 5.2	33.7 ± 4.6
MG1363(pIL253*pil*) + MG1363(pNZ4120)	5.23E + 05 ± 3.73E + 05	4.48E + 07 ± 3.45E + 07	98.8 ± 0.3*	97.1 ± 0.7*
Clumping phenotype				
MG1614	6.56E + 06 ± 2.18E + 06	3.83E + 07 ± 6.22E + 06	85.1 ± 5.5	71.5 ± 8.7
MG1614_clu$^-$	7.40E + 06 ± 1.21E + 06	6.35E + 07 ± 1.70E + 07	89.0 ± 3.4	77.8 ± 6
MG1614_clu$^+$	4.32E + 06 ± 1.21E + 06	2.23E + 07 ± 3.03E + 06	83.7 ± 4.8	69 ± 7.6
Chaining phenotype				
IL1403	4.23E + 06 ± 3.46E + 06	1.03E + 07 ± 6.66E + 06	65.4 ± 31.2	53.4 ± 36.4
IL1403Δ*acmAacmD*	7.60E + 06 ± 6.78E + 06	1.13E + 07 ± 1.16E + 06	64.5 ± 17.2	45.6 ± 17.1

[a] For comparisons surface altered strains are grouped with their isogenic parent. Values are means ± standard deviation; significance levels (* indicates $p < 0.01$) are in comparison with the parental strain. Fractions of cell retention were calculated per sample and subsequently the mean and SD were determined.

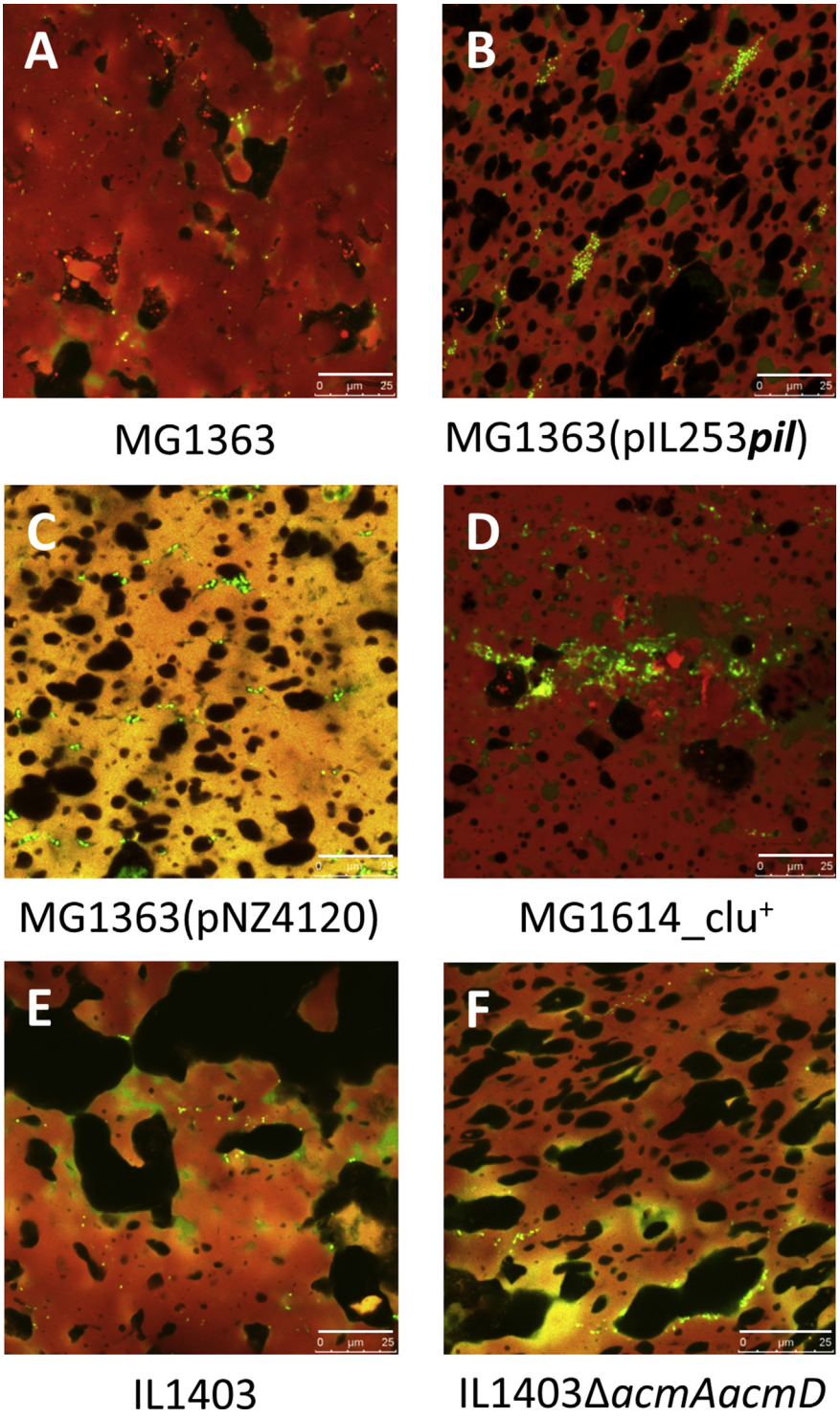

Fig. 1. Microstructure of Gouda-type cheese made with *L. lactis* strains with altered surface properties. Bacterial cells are green, the protein matrix appears orange/red; the black areas represent oil droplets, air pockets or serum regions. White bars: 25 μm. (A) *L. lactis* MG1363, (B) *L. lactis* MG1363(pIL253*pil*) over-expressing Spa pili and leading to a chaining, clumping and hydrophobic phenotype, (C) EPS-producing *L. lactis* MG1363(pNZ4120), (D) clumping and hydrophobic transconjugant MG1614_clu^{+}, (E) *L. lactis* IL1403, and (F) *L. lactis* IL1403Δ*acmAacmD*, exhibiting a chaining phenotype.

After rennet-induced hydrolysis of κ-casein in milk, the volume fraction of para-casein micelles is ~8–10%, with fat globules and bacterial cells suspended in the serum phase. At this point, the pores are sufficiently large for bacterial cells and fat globules to be removed with the whey (Fox, McSweeney, Cogan, & Guinee, 2004). However, as this pore size gradually decreases during syneresis, the expulsion of cells is ultimately hindered, leading to their entrapment as well as that of fat globules in the curd, as previously described by Laloy et al. (1996).

Taken together, it appears that bacterial cell surface properties can affect retention of the cells in the cheese curd. This could either be due to interactions of the bacteria with the matrix, or to cell

clustering, which would lead to an increased size and more restricted movement through the curd matrix. Clumping of bacteria, however, did not significantly affect cell retention in the cheese curd (Table 2). Interestingly, over-expression of the Spa-pilus gene cluster in strains MG1363 and IL1403 increased cell retention in the curd, to about 90.6–98.9% (Table 2). The co-culturing of the EPS-expressing MG1363(pNZ4120) with Spa-pili over-expressing MG1363(pIL253*pil*) increases cell retention in the curd up to 97.1 ± 0.7%.

The fact that the expression of pili in only a part of the starter culture cells in the curd is sufficient for cell retention suggests that interactions take also place between the pili producers and non-producers. Pili over-expression also alters properties such as cell surface hydrophobicity and zeta potential (Tarazanova et al., 2018). To distinguish between the direct effects of pili and putative other surface properties on cell retention in curd, two derivatives of MG1363 were used, in which the lactose/protease plasmid pLP712 had been transferred via conjugation (Tarazanova et al., 2018). One of these transconjugants, MG1614_clu$^+$, has a clumping phenotype and a significantly higher surface hydrophobicity and slightly lower net-negative charge than its parent, MG1363. The other transconjugant, MG1614_clu$^-$, does not clump and has surface properties similar to those of MG1363, except for hydrophobicity, which remained ~70–80% (Tarazanova et al., 2018). Using these strains for cheese-making showed that surface alterations except for pili over-expression did not affect cellular distribution in curd and whey.

Together, these data indicate that over-expression of Spa-pili in different lactococcal strains consistently leads to the retention of a higher fraction of cells in cheese curd while cell hydrophobicity and zeta potential alterations, due to pLP712 plasmid transfer into MG1363 did not have such effects. Cell retention is significantly lowered as a consequence of curing of the six plasmids from strain NCDO712 (compare the results of NCDO712 with those of its plasmid-free derivative MG1363). The plasmids of NCDO712 code for several dairy-related properties such as lactose utilisation, an extracellular protease, an endopeptidase, peptide transport and others (Tarazanova et al., 2016; Wegmann, Overweg, Jeanson, Gasson, & Shearman, 2012). Plasmid pSH74 of NCDO712 encodes the pilin gene cluster *spaCB-spaA-srtC1-srtC2* that was over-expressed in several of the strains used in this study. The relatively high cell curd-retention reported here for strain NCDO712 and previously for other dairy strains suggests that this might be a property of starter strains that has been selected for.

3.2. Distribution of cells with altered surface properties in the cheese matrix

To investigate whether bacterial cell surface alterations can affect the distribution of cells in the cheese matrix, 12 week-old cheese samples were examined by CLSM imaging. All strains described above were used in these examinations. Three main phenomena with respect to bacterial distribution in the cheese matrix were observed: (1) small groups of cocci are randomly embedded throughout the matrix, (2) cells are present as aggregates, (3) EPS producing cells seem to be surrounded by small serum regions of the protein network.

The first of these distribution patterns was seen for the plasmid-free strains MG1363 and IL1403. The cells of these strains were predominantly present as small groups of cocci entrapped throughout the protein matrix of the cheese (Fig. 1A and E). The second apparent distribution pattern was in form of cell aggregates that occurred upon over-expression of the pilin operon *spaCB-spaA-srtC1-srtC2* in MG1363 (Fig. 1B). A similar trend was seen for Spa pili over-expressing *L. lactis* IL1403 (data not shown). Interestingly, the chaining phenotype observed in IL1403Δ*acmAacmD* and the clumping transconjugant *L. lactis* MG1614_clu$^+$ also led to formation of cell aggregates in cheese (Fig. 1F and D). The third distribution pattern was seen for the EPS-producing MG1363(pNZ4120): cells of this strain seem to be surrounded by small serum regions (Fig. 1, C). Overall, the CLSM results indicate that alterations in cell chaining, clumping, EPS production or Spa-pili over-expression influence the distribution of lactococcal cells in the cheese matrix.

Based on the described findings we propose that cells with altered surface properties may have altered functionalities in cheese. This is in line with an earlier study showing that the alteration of cell surface morphology (chaining, clumping, EPS formation, pili expression) not only affects cell surface charge, hydrophobicity and the attachment of cells to proteins, but it can also lead to differences in gel hardness and viscosity of milk fermented with the engineered strains (Tarazanova et al., 2018). The current study indicates that by altering surface properties of dairy starter cultures it is possible to minimise the loss of cells in whey during cheese manufacturing, which might be applied to create a cleaner whey or to alter textural and, ultimately also, sensory qualities of cheese.

4. Conclusions

L. lactis cell surface properties play an important role in the distribution and retention of starter culture cells in curd during cheese making. While the curing of plasmids from a wild type dairy isolate, *L. lactis* NCDO712, leads to a decrease in cell retention, over-expression of the pilus gene cluster *spaCB-spaA-srtC1-srtC2* results in a significant increase in cell-retention in the model strains of the two *L. lactis* subsp. *cremoris* MG1363 and *L. lactis* subsp. *lactis* IL1403. The alteration of cell retention in curd might open possibilities to modify starter culture functionality as well as whey and cheese quality.

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