Interactions in Biofilms of *Lactococcus lactis* ssp. *cremoris* and *Pseudomonas fluorescens* Cultured in Cold UHT Milk

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ABSTRACT

Three *Lactococcus lactis* ssp. *cremoris* isolates from refrigerated bulk raw milk were cultured separately and in association with a known psychrotrophic dairy *Pseudomonas fluorescens* strain, in skim UHT milk for 72 h at 7°C, to determine mutual influences in both the planktonic and biofilm phases. Two levels of inoculum of each culture partner were combined. Protocooperation and commensalism cases were found, all of them in the biofilm phase. Type and intensity of the interactions depended on *Lactococcus* strain and on the cell density of each partner. Maximum enhancement of attachment was observed to be approximately 100-fold for *P. fluorescens* and 20,000-fold for one of the *L. lactis* strains. Confocal scanning laser microscopy images show compact masses of *Pseudomonas* trapping lactococci cells in cooperative biofilms.

(Key words: psychrotrophic, biofilm, *Lactococcus*, *Pseudomonas*)

Abbreviation key: CSLM = confocal scanning laser microscopy.

INTRODUCTION

In refrigerated raw milk, *Pseudomonas* spp., specifically *P. fluorescens*, can be considered the specific spoilage organism (Huis in't Veld, 1996). This species is the most numerous and fastest growing bacteria in cold raw milk, and cause off-flavors largely due to extracellular proteinases and lipases. Other psychrotrophs are commonly found in raw milk (Champagne et al., 1994; Sørhaug and Stepaniak, 1997), including gram-positive bacteria such as *Bacillus* and *Listeria*. Some *Lactococcus lactis* strains have been reported to be psychrotrophs (Hamasaki et al., 2003), although they received little attention as such. Raw milk is usually rich in lactococci. In good quality cheese-making milk, lacto-

cocci counts were found to be second only to those of *Pseudomonas* (Desmaures et al., 1997). Both genera have been reported to dominate the microbiota in down-graded Danish bulk tank milk (Holm et al., 2004). Notwithstanding, selected *L. lactis* ssp. *lactis* and *cremoris* are extensively used as mesophilic starters for cheese and butter production. The wild strains also have technological interest.

From an ecological point of view, *P. fluorescens* and *L. lactis* ssp. *cremoris*, when contaminants in raw milk, usually live in different niches; *Pseudomonas* are aerobes, non-lactose utilizing, and frequently psychrotrophic, whereas lactococci are lactose-utilizing anaerobes, and so far considered poor psychrotrophs. However, lactococci can also grow aerobically and some strains grow well at temperatures of 7°C and below.

In raw-milk holding equipment, 2 distinct but connected phases are available for microbial growth: the liquid phase, in which planktonic cells proliferate, and the solid/liquid interface (i.e., liquid-covered equipment walls), where cells can attach and form biofilms (Wong and Cerf, 1995; Somers et al., 2001). Each phase constitutes a unique habitat, and cells can move from one to the other, depending on growth stage, nutrient availability, and flow shear forces (Stoodley et al., 2002). The environment inside the biofilms is anaerobic, with slow diffusion rates for gases, substrates, residues, and secondary metabolite molecules. Ecological relationships between species or strains are thus likely to be different in the liquid and the biofilm phases of raw milk.

As *Pseudomonas* are the specific spoilage organisms of refrigerated raw milk, changes in their growth rate and spoilage activity are useful in predicting shelf life (Gram et al., 2002). Here, we present such a case due to a frequent partner, the lactococci. The outcome of the interaction with *Pseudomonas* on psychrotrophic lactococci could also have an influence on maintaining quality.

To identify the ecological relationships between psychrotrophic *Pseudomonas* and *Lactococcus* in cold milk, batch binary cultures in UHT-sterilized skim milk were used as model systems. *Pseudomonas fluorescens* B52 was combined with 3 *L. lactis* ssp. *cremoris* strains

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at 2 different initial levels. Final planktonic and biofilm counts of binary cultures were divided by those of the same strain and inoculum level at monocultures, to represent either growth enhancement or impairment as a result of the association. Confocal scanning laser microscopy (CSLM) images of representative binary cultures were obtained.

MATERIALS AND METHODS

Strains

Lactococcus strains were isolated from refrigerated bulk raw milk with high microbial load. Serial dilutions of the milk in peptone water (Oxoid, Basingstoke, UK) were plated on M17 agar (Oxoid), to be incubated under anaerobiosis at 32°C for 48 h, for preliminary enrichment in lactococci. Some of the gram-positive, catalase-negative cocci colonies were picked at random from enrichment plates, purified by subculturing, and identified using API 50 CHL strips (BioMérieux, Marcy-l'Étoile, France) and partial 16S rDNA analysis (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) as *L. lactis* ssp. *cremoris*. *Pseudomonas fluorescens* B52 was originally isolated from cold raw milk (Richardson and Te Whaiti, 1978). All strains were stored at -20°C.

Cultures

Frozen *Lactococcus* strains were transferred to de Man, Rogosa, and Sharpe broth (Oxoid) and incubated at 30°C for 12 h. *Pseudomonas* were precultured in mineral medium (McKellar and Cholette, 1984) at 21°C for 24 h. Cells were harvested by centrifugation at 4000 × *g* for 10 min, and were washed twice with the mineral medium. Dilution with the original medium adjusted the 2 inoculum levels, referred to here as low and high, to obtain approximately 10³ and 10⁵ cfu/mL, respectively, in UHT skim milk. Single strain cultures at each level of inoculum were used as controls. Four types of binary cultures were run for each *Lactococcus* strain in association with *Pseudomonas*, combining the 2 levels of inoculum for each organism. Biofilm formation was studied on single-use 22-mm² thin, borosilicate glass coverslips; 16 coverslips were held vertically by marginal insertion into the narrow radial slits of a Teflon carousel platform (6.6-cm diameter) and its lid, both assembled to an axial metallic rod, for handling. Coverslips, carousel, and the covered 600-mL beaker were heat-sterilized as a unit, before aseptically introducing 55 mL of UHT milk with the corresponding inocula. The system, exposing about 4 cm² of available attachment surface for each milliliter of culture liquid, was incubated at 7°C in a rotating shaker. Final pH in all cul-

tures remained naturally above 6. After 72 h, duplicate samples were taken of both planktonic and coverslip-attached cells to be plated. Before removal of the biofilm using a swab dipped in sterile peptone water, weakly attached cells were removed from the coverslips by carefully rinsing in sterile 0.9% NaCl. *Pseudomonas fluorescens* counts were obtained after plating in *Pseudomonas* selective agar (Oxoid), incubating for 48 h at 32°C. Those of *Lactococcus* were obtained from M17 agar plates under anaerobiosis, also after 48 h at 32°C. All cultures were reproduced 3 times.

Statistical Analyses

Analysis of variance of the association effect was performed on planktonic and biofilm counts of *P. fluorescens* and *L. lactis*, using SPSS Win 9.0 software (SPSS Inc., Chicago, IL). Significant differences were assessed by Tukey's test at *P* < 0.01 using the same program.

CSLM Biofilm Images

For microscopic observation, coverslips were rinsed in 0.9% NaCl and stained in the dark, at room temperature, first with 100 µg/mL Texas Red-X conjugate of wheatgerm agglutinin (Molecular Probes, Eugene, OR) for 10 min (selectively binding to gram-positive bacteria, and appearing red) and then with 0.25% 4',6-diamidino-2-phenylindole dihydrochloride solution (Molecular Probes, Eugene, OR) for 10 min; 4',6-diamidino-2-phenylindole dihydrochloride is an unspecific nucleic acid dye, giving blue stain to both gram-negative and gram-positive bacteria.

Confocal scanning laser microscope images were obtained using a TCSSP 2 model (Leica Lasertechnik, Heidelberg, Germany), using a 63× lens. Displayed images were processed with IMARIS software (Bitplane AG, Zürich, Switzerland).

RESULTS AND DISCUSSION

Monocultures

Comparison of planktonic counts of *P. fluorescens* at low (10³ cfu/mL) single inoculum cultures (Figure 1A, white bars) showed a faster growth rate than those starting with a high inoculum level (10⁵ cfu/mL; Figure 1C, white bars), suggesting that at 72 h and 7°C, cells were entering stationary phase. In biofilms, low inoculum cultures yielded 10⁴ cfu/cm², whereas high inoculum cultures yielded 10⁶ cfu/cm². A 100-fold higher proportion of the total cells were located in biofilms in advanced growth cultures.

Figure 1. *Pseudomonas fluorescens* B52 counts (in UHT milk 7°C for 72 h) in pure cultures (white bars), or cocultured with *Lactococcus lactis* (strains 34, 41, or 47), with either low (gray bars) or high (black bars) inoculum levels. A) Planktonic counts and B) biofilm counts using low inoculum of *P. fluorescens*; C) planktonic counts and D) biofilm counts using high inoculum of *P. fluorescens*. Error bars represent the confidence interval for the mean (n = 3). ^{a,b}Values within the same bar group with different letters are significantly ($P < 0.01$) different.

Single low inoculum cultures of *L. lactis* (Figure 2A and B, white bars) showed different biofilm-forming rates for the 3 strains tested. This finding may be due to the quality, quantity, or timing of their adhesin, or extracellular polysaccharide production (Ruas-Madiedo et al., 2005; Vuong et al., 2005).

Effects of Association on *Pseudomonas*

Figure 1 shows that coculture had no significant effect on the planktonic count of *Pseudomonas*, independently of the size of its own inoculum or that of the strain of *L. lactis*. Biofilm counts indicated that 2 of the 3 *Lactococcus* strains enhanced the attachment of *Pseudomonas* cells by 15- to 100-fold in the low inoculum cultures (Figure 1A). *Pseudomonas* enhancement was not proportional to the size of *Lactococcus* inocula

and was highest with the slowest biofilm former, strain 41, of the *L. lactis* strains tested.

Images from CSLM (Figure 3) show that in cooperative cocultures of *Pseudomonas* and *L. lactis* strain 41, biofilms appeared more compact, and both cell types were close together. Spatial arrangement of consortia partners in biofilms (as seen by CSLM) relates to the type of ecological relationship existing between them. Shorter distances between different microcolonies or mixed-cell patterns usually correspond to positive interactions (Cowan et al., 2000; Nielsen et al., 2000) as in Figure 3 (strain 41).

Pseudomonas in cold milk are known to produce extracellular proteinases after they reach 10^5 to 10^6 cfu/mL (Matta et al., 1997). Below this level, they are short of utilizable C and N sources, but proximity to *Lactococcus* (as in biofilms) could potentially relieve that short-

Figure 2. *Lactococcus lactis* (strains 34, 41, or 47) counts (in UHT milk 7°C for 72 h) in pure cultures (white bars), or cocultured with *Pseudomonas fluorescens*, with either low (gray bars) or high (black bars) inoculum levels. A) Planktonic counts and B) biofilm counts using low inoculum of *L. lactis*; C) planktonic counts and D) biofilm counts using high inoculum of *L. lactis*. Error bars represent the confidence interval for the mean ($n = 3$). ^{a,b}Values within the same bar group with different letter are significantly ($P < 0.01$) different.

age. *Pseudomonas* can use lactic acid as a source of C and energy (SanJose et al., 1987). In biofilms, it is possible that the rates of lactic acid production (by *L. lactis*) and consumption (by *Pseudomonas*) are compensated, preventing pH decrease. *Pseudomonas* can also benefit by obtaining extra nutrients from autolysis of the lactococci (Garde et al., 2002). Phillips et al. (1983) found that nisin added to pasteurized double cream increased proteolysis due to fluorescent pseudomonads, thereby improving their growth rate. Jaspe et al. (1995a) reported an improvement in planktonic *Pseudomonas* growth due to a cheese-making starter containing *L. lactis*.

Most reports on relationships between lactic acid bacteria and *Pseudomonas*, or other psychrotrophic gram-negative bacteria in milk and dairy products, deal with attempts to antagonize the psychrotrophs for biopreser-

vation purposes (Champagne et al., 1994). Required levels of lactic acid bacteria needed to obtain that interference, however, are usually above 10^6 cfu/mL. On the contrary, the cooperative effect reported herein relies on both low initial populations and simultaneous growth.

Effects of Association on *Lactococcus*

Coculture did not have a significant quantitative effect on planktonic growth of the lactococci (Figure 2A and C) and the outcome on biofilm cell density depended on both inocula size and *Lactococcus* strain. In binary cultures with a large (10^5 cfu/mL) inoculum of strain 47 (Figure 2D), attached lactococci increased by 15-fold (with low *Pseudomonas* inoculum) and 40-fold (with high *Pseudomonas* inoculum), compared with the corresponding monocultures. The more outstanding stimu-

Figure 3. Confocal scanning laser microscopy images of horizontal (left) and vertical (right) sections of mixed biofilms of *Pseudomonas fluorescens* (blue) and *Lactococcus lactis* (red). Counts were for *L. lactis* strain 34, 4.0×10^3 cfu/cm² with 2.5×10^6 cfu/cm² of *Pseudomonas*; for strain 41, 6.3×10^3 cfu/cm² with 1.0×10^6 cfu/cm² of *Pseudomonas*; and for strain 47, 4.0×10^5 cfu/cm² with 7.9×10^6 cfu/cm² of *Pseudomonas*.

lus, however, was observed in binary cultures with low (10^3 cfu/mL) inoculum of strain 41, in which their attached cells increased by 20,000-fold due to the association with a low *Pseudomonas* inoculum (Figure 2B). Because *L. lactis* strain 41 is a poor biofilm former by itself, one possibility is that *P. fluorescens* enhanced lactococcal strain attachment by providing a quickly developed biofilm matrix to host it. Meanwhile, the fast growth of *P. fluorescens* likely consumed much of the available oxygen inside the biofilms. The resulting anaerobic conditions may stimulate the growth of the lactococcal strain. The lactic acid produced by the lactococcal strain, on the other hand, could be used as a C source by the *Pseudomonas* located at the biofilm's surface. However, strains 34 and 47 are also poor biofilm formers and anaerobes, but do not benefit from *P. fluorescens* association, suggesting factors other than the anaerobic condition may be involved in the observed growth enhancement.

Besides lactococci, cases of *Pseudomonas* favoring the growth of other types of bacteria in the same biofilm have been reported for *Listeria monocytogenes* (Sasahara and Zottola, 1993), *Lactobacillus amylophilus* and *Lactobacillus casei* (Demirci et al., 1993), *Bacillus cereus* (Lindsay et al., 2002), and *Acinetobacter* (Christensen et al., 2002).

Ecological Interactions and Relevance for Milk Quality

Different types of interactions were observed in these binary cultures, depending on planktonic or biofilm phase, initial population of each partner, and *L. lactis* strain. Protocooperation was the most remarkable of all, occurring only in biofilms of cocultures with low inocula levels and strain 41. The other detected interactions, all in biofilms, corresponded to cases of commensalism, in which growth rates of either *Lactococcus* or *Pseudomonas* (but not both simultaneously) benefited from the association.

Low population levels of *Pseudomonas* and lactococci are common in good quality raw milk (Desmasures et al., 1997), but it is unknown how prevalent are the strains behaving as strain number 41 are among *L. lactis* ssp. *cremoris* present in raw milk samples. If they were in relatively high proportion, biofilms at the milking and storage equipment walls should be carefully controlled to avoid proteolytic and lipolytic *Pseudomonas*-induced defects. In addition, competition could take place between starter strains and wild lactococci established in the biofilms.

As expected, most of the positive interactions observed in this study took place in biofilms from cultures with low inoculum levels of both types of bacteria. An

exception was *L. lactis* strain 47, which obtained a better outcome from the association with *Pseudomonas* when its own inoculum was high (Figure 2D). This finding may suggest that, at low cell densities of both partners, *Pseudomonas* would be the favored commensal (as in Figures 1 and 2B), whereas at more advanced growth stages, strain 47 would take over that role (as in Figure 2D).

Although positive interactions probably have a higher chance to occur in young biofilms, other types of relationships could take place, at least transiently. Early settlers on a surface might select their incoming neighbors, checking compatibility on arrival. Carpentier and Chassaing (2004) assessed 29 bacterial isolates from food industry premises as possible biofilm hosts for *Listeria monocytogenes* incorporated later, finding both positive and negative interactions at early attachment stages.

Mixed cultures with high population densities are more likely to engage in interference competition. Both partners studied here are well known for their antagonistic resources. *Pseudomonas fluorescens* are exploited as biocontrol agents against plant pathogens, and lactic acid bacteria are used as probiotics, starters for making fermented products, or food biopreservation agents. The presence of some of the antagonistic or virulence-related exoproducts of *Pseudomonas* seems to be associated with transition to stationary phase (Heeb et al., 2005) or quorum sensing (Wagner et al., 2004). An apparently neutral growth outcome (e.g., Figures 1A, C, and D and 2A and C) could correspond to a balanced situation with both cooperative and competitive aspects.

It is important to note that these results were obtained at 7°C. *Pseudomonas* expresses a different phenotype at refrigeration temperatures (Regeard et al., 2000). Its production of extracellular gluconate (Lynch and Franklin, 1978), proteinase (Jaspe et al., 1995b), and cytotoxic agents (Picot et al., 2004) has been found to be greater at 7 to 8°C than at higher temperatures. Interference of planktonic *Listeria monocytogenes* (Farrag and Marth, 1989) and *Escherichia coli* (Samelis and Sofos, 2002) by *Pseudomonas* strains has also been reported to be temperature-dependent.

Low counts of wild psychrotrophic strains of *L. lactis* ssp. *cremoris* and *P. fluorescens* in milk can positively interact during cold storage, causing an increase in the cell numbers of both species at biofilms without significant effect on planktonic counts or milk pH. As these counts and strains are common in raw milk, such rich biofilms could add spoiling organisms into subsequent raw milk batches that come in contact with them, if not adequately removed.

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