The language of cheese-ripening cultures

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Abstract

Microbial interactions are of importance for the establishment and growth of cheese ripening cultures. An interesting aspect of microbial interactions is cell-cell communication, often referred to as quorum sensing; the process in which micro-organisms communicate with signalling molecules and co-ordinate gene expression in a cell density dependent manner. Little is known about quorum sensing in foods. However, as quorum sensing is expected to be a general phenomenon in micro-organisms, it is likely to be of importance for micro-organisms in foods. An example of a food product where quorum sensing could be of importance is surface ripened cheeses. The present review focuses on our findings on quorum sensing systems in cheese ripening cultures. The main focus is on the group of bacterial non-species-specific signalling molecules referred to as autoinducer-2 (AI-2) in smear bacteria as well as alcohol-based and ammonia signalling in the dairy-relevant yeast *Debaryomyces hansenii*. Furthermore, the influence of cheese matrices on quorum sensing systems is briefly mentioned. Finally, we discuss how knowledge on quorum sensing systems in cheese ripening cultures may be used for optimisation of the ripening processes.

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For many years, micro-organisms were considered individual organisms that primarily search for nutrients and multiply. Today we know that micro-organisms, which in nature often create complex communities, interact with each other. Cheese ripening is highly dependent on microbial interactions (Irlinger and Mounier 2009). However, only a few microbial interactions important for cheese ripening have been described, and the mechanisms underlying those are not well understood. The positive effects of microbial interactions not only assist in inhibition of undesired micro-organisms including spoilage micro-organisms and food-borne pathogens, but also serve in the development of desired micro-organisms, including starter cultures.

An interesting aspect of microbial interactions is cell-cell communication, often referred to as quorum sensing (for extended reviews, see Ryan and Dow 2008; Ng and Bassler 2009; Raina *et al*. 2009). The general features of quorum sensing involve production, secretion and detection of signalling molecules. When micro-organisms grow, they produce signalling molecules which are secreted to the extracellular environment. The extracellular concentration of signalling molecules increases proportionally to cell density. When a critical threshold level of signalling molecules corresponding to a certain cell number is achieved, receptors bind the signalling molecules and trigger signal transduction cascades that result in synchronised changes in gene expression. Thus, quorum sensing allows a community of unicellular organisms to turn on group behaviours in a manner similar to multicellular organisms. For intraspecies communication (within the same species), Gram-negative bacteria produce acyl homoserine lactones (AHLs) as signalling molecules. AHLs are composed of homoserine lactone (HSL) rings carrying acyl chains of various numbers of carbon and modifications. In contrast, Gram-positive bacteria produce modified oligopeptides as signalling molecules for intraspecies communication. However, most of the time, micro-organisms do not live by themselves but in a mixture of different species. So in parallel, both Gram-negative and Gram-positive bacteria produce the group of signalling molecules referred to as autoinducer-2 (AI-2), which they use for interspecies communication (between different species). This allows bacteria to sense not only how many of its own, but also how many of others are present in the environment. In general, it is anticipated that more than 10% of the genes in the genome are controlled by quorum sensing (Schuster *et al*. 2003; Wagner *et al*. 2003). Processes such as bioluminescence, sporulation, virulence, conjugation, biofilm formation and bacteriocin production are among those reported to be under quorum sensing control. Until now, quorum sensing systems have primarily been investigated in pathogenic bacteria, as blockage of these systems might be an alternative to antibiotics. However, as quorum sensing is expected to be a general phenomenon in micro-organisms, it is likely to be of importance in micro-organisms found in foods.

An example of a food product, where quorum sensing could be of importance, is surface ripened cheeses. Surface ripened cheeses including limburger, tilsiter, romadour, brick and the danish danbo are characterised by development of a viscous, red-orange smear consisting of both yeasts and bacteria on the surface during ripening (Brennan *et al*. 2004). Yeasts, primarily *Debaryomyces hansenii*, are present during the initial period of ripening, and initiate the ripening process by raising pH at the cheese surface which allows the growth of less acid tolerant bacterial flora, consisting of primarily of Gram-positive coryneforms and staphyloccocci. The smear on the surface plays an important role in the final aroma and texture of the cheeses, and is often responsible for the unique characteristics of cheeses.

Traditionally, yeasts and bacteria are introduced to the cheese surface by a slurry containing smear from previously produced cheeses. However, slurries may also introduce undesirable micro-organisms, such as *Listeria monocytogenes*. Therefore, the use of well-defined ripening cultures has been paid attention with the purpose of ensuring the best quality control (Bockelmann *et al*. 2005). Several starter cultures have been chosen due to properties such as aroma formation and anti-microbial activity.

However, studies have shown that these starter cultures do not necessarily establish well in the cheesemaking environment and are outcompeted by the indigenous flora in the dairy (Petersen *et al*. 2002; Mounier *et al*. 2005). This shows that even though a single culture showes good properties on a laboratory scale, it does not among other cultures. Several factors could be of importance in the lack of establishment of starter cultures, such as adhesion properties and NaCl tolerance (Gori *et al*. 2005; Mortensen *et al*. 2005). However, other yet unknown factors such as quorum sensing may also be involved.

Autoinducer-2 (AI-2) activity in dairy-relevant bacteria

Among bacterial signalling molecules, the group called autoinducer-2 (AI-2) plays a unique role as it consists of the only presently known signalling molecules produced by both Gram-negative and Gram-positive bacteria, and presumably allows bacteria to respond to both endogenously produced AI-2 as well as AI-2 produced by other bacterial species in the vicinity. AI-2s are all derived from the same precursor, 4,5-dihydroxy-2,3-pentanedione (DPD), a by-product of the activated methyl cycle in a reaction catalysed by the LuxS enzyme (Schauder *et al*. 2001). Through cyclisation, hydration and borate ester formation, if enough borate is present, DPD rearranges spontaneously into a mixture of compounds, of which some function as signalling molecules. Bacterial species recognise chemically distinct forms of AI-2. The LuxP receptor protein of *Vibrio harveyi* binds (*2S*,*4S*)-2-methyl-2,3,3,4-tetrahydroxytetrafuran-borate (*S*-THMF-borate) (Chen *et al*. 2002), whereas the receptor protein LsrB of *Salmonella enterica* serovar Typhimurium binds (*2R*,*4S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF) (Miller *et al*. 2004). Due to instability, detection of AI-2s is difficult. Typically AI-2 activity is detected indirectly by the *Vibrio harveyi* assay, which relies on the stimulation of bioluminescence in the reporter strains *V. harveyi* BB170 (Surette and Bassler 1998). However, recently a promising method involving gas chromatography-mass spectrometry for direct detection of AI-2s was reported (Thiel *et al*. 2009).

For the moment, the LuxS-AI-2 quorum sensing system has been found in hundreds of bacterial species. Among dairy-relevant pathogenic bacteria, AI-2 has been found to control biofilm formation in *Listeria monocytogenes* and virulence in both *Staphylococcus aureus* and *Escherichia coli* (Surette and Bassler 1998; Winzer and Williams 2001; Barrios *et al*. 2006). However, AI-2 controlled behaviours are not only restricted to pathogenic bacteria, but are also found among dairy-relevant starter cultures and probiotic bacteria such as acid stress regulation in *Lactococcus lactis* and *Lactobacillus* spp., respectively (Frees *et al*. 2003; Moslehi-Jenabian *et al*. 2009). Finally, we have found that several smear bacteria on surface ripened cheeses produce AI-2 activity (Gori *et al*. 2010). AI-2 activity was for the first time determined in the supernatants of strains belonging to *Arthrobacter nicotianae*, *Corynebacterium ammoniagenes*, *Corynebacterium casei*, *Microbacterium barkeri*, *Microbacterium gubbeenense* and *Staphylococcus equorum*. Conversely, no AI-2 could be determined in supernatants of strains belonging to *Brevibacterium linens*. For all the bacterial strains producing AI-2 activity, AI-2 activity was generally found to increase during the exponential growth period. Maximum AI-2 activity levels were observed at the transition from the exponential phase to the stationary phase, after which the AI-2 activity rapidly disappeared. Finally, in some cases, dairy-relevant stress conditions including low pH and high NaCl conditions increased the AI-2 activity of the smear bacteria. However, the exact phenotypic changes affected by AI-2 in smear bacteria from surface ripened cheese still have to be elucidated.

Various foods are known to either repress or enhance quorum sensing systems, e.g. AI-2 signalling (Lu *et al*. 2004). Such inhibition or stimulation of quorum sensing activity could originate from either the microbial flora or compounds present in the foods. Except for the study by Lu *et al*. (2004) indicating that mozzarella and goat milk cheeses inhibit AI-2 signalling, the influence of cheese matrices on quorum sensing systems is unknown. For determination of AI-2 activity of smear bacteria in their natural environment, we prepared a solid cheese model substrate containing Danish Danbo cheese. Preliminary results showed that extracts of pure Danish Danbo cheese and solid cheese model substrate, respectively, decreased bioluminescence in reporter strain Vibrio harveyi BB170 indicating an inhibitory effect on AI-2 signalling (unpublished results). Furthermore, a similar inhibitory effect was observed, when supernatants containing AI-2 were mixed with the cheese extracts. Finally, only minor levels of AI-2 activity were determined for smear bacteria growing on solid cheese model substrate probably due to the shown inhibitory effect of cheese matrices.

Alcohol-based quorum sensing in *Debaryomyces hansenii*

Quorum sensing has also been described for yeasts. However, this subject has not been investigated as well as for bacteria. The best studied quorum sensing system in yeasts involves alcohols as signalling molecules. The dimorphic yeast *Candida albicans* has especially been investigated with respect to alcohol-based quorum sensing. The sesquiterpene farnesol has been found to be a signalling molecule inhibiting both the yeast-to-hyphal shift and biofilm formation of *C. albicans* (Hornby *et al*. 2001; Ramage *et al*. 2002). Furthermore, Chen *et al*. (2004) showed that the aromatic alcohol tyrosol, due to its promotion of hyphal development, shortened the lag phase time of *C. albicans*.

Furthermore, the aromatic alcohols phenylethanol and tryptophol have been found to be signalling molecules in *Saccharomyces cerevisiae*, where they stimulate pseudohyphal growth (Chen and Fink 2006). Furthermore, phenylethanol was found to stimulate invasive growth. Addition of tryptophol resulted in an even greater invasive growth, whereas tryptophol alone did not have an effect on invasive growth. *C. albicans* also produce phenylethanol and tryptophol, but the function is different from what is observed in *S. cerevisiae*. However, in both *C. albicans* and *S. cerevisiae*, alcohol production is highly affected by growth conditions, including the availability of aromatic amino acids, presence of ammonium, pH and oxygen levels (Chen and Fink 2006; Ghosh *et al*. 2008).

Presently, we are investigating phenylethanol, tyrosol, tryptophol and farnesol as potential quorum sensing molecules in the dairy-relevant yeast *Debaryomyces hansenii*. The alcohol production and the growth conditions regulating the alcohol production in *D. hansenii* strains were examined. Furthermore, the involvement of the alcohols in different aspects of adhesion of *D. hansenii* is being elucidated.

Ammonia signalling in *Debaryomyces hansenii*

When grown on agar plates, neighbouring colonies of various yeasts, including dairy-relevant yeasts such as *Saccharomyces* *cerevisiae* and *Kluyveromyces lactis* among others, have been shown to communicate with each other using pulses of ammonia (Palkova *et al*. 1997). The ammonia pulses result in growth inhibition of the parts of the colonies facing other colonies, allowing for overall coordinated growth of colonies away from one another and toward potential fresh nutrient sources instead. Similarly, we reported ammonia as a signalling molecule involved in the co-ordination of the growth on agar plates for three strains of *D. hansenii* (Gori *et al*. 2007). Thus, ammonia pulses appear to be an example of interspecies communication among yeasts. Oriented ammonia production of *D. hansenii* was both determined on a model substrate previously used for the detection of ammonia as a signalling molecule as well as a cheese substrate mimicking the cheese surface. Furthermore, on the model substrate, increases of ammonia production for double colonies compared to single colonies were observed for all three strains investigated, whereas on the cheese agar only minor increases in ammonia production for double colonies compared to single colonies were observed. The lack of increases in ammonia production on cheese agar might be correlated with the ability of the yeast strains to release amino acids from the cheese agar, as ammonia production involved in signalling has previously been shown to be highly dependent on free amino acids (Zikanova *et al*. 2002).

Perspectives

Increasing research effort is being made in understanding the knowledge of interactions between micro-organisms in microbial communities. The promising results on the potential use of quorum sensing for inhibition of pathogenic bacteria in a clinical context may have the potential to be transferred to the food industry. Increased knowledge of how starter cultures, as well as spoilage and pathogenic micro-organisms, in foods such as dairy products use quorum sensing to control growth may be of particular importance to obtain both better quality and safety. Although spoilage and pathogenic micro-organism growth might be prevented by blockage of their quorum sensing systems, starter cultures might be stimulated by the addition of the so-called pro-quorum sensing compounds, resulting in increased microbial communication which could be used to ensure the establishment and growth of starter cultures and thus stimulate important technological properties such as aroma formation and anti-microbial activity.

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