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Effect of Alginate Beads on Olfactory Sensory Perception of Paraffin-Coated Cheese

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Abstract

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Flavour encapsulation is now an established technique, but different methods are associated with significant loss of flavour. In this work, we propose a novel alternative: the direct encapsulation of bacteria that produce useful aromatic compounds. The effect of alginate beads containing 3-methylbutanal or *Carnobacterium maltaromaticum* LMA 28 on olfactory sensory perception of paraffin-coated cheese was investigated. The abilities of bacteria to produce 3-methylbutanal and of alginate beads to diffuse this volatile compound in cheese was evaluated. Size, shape and encapsulation efficiency of alginate beads were determined. Moreover, the possible antimicrobial repercussions on the lactic acid flora of cheese were also examined. Alginate beads in both treatments were found to be uniform and spherical with a mean diameter of 1.69 ± 0.15 mm. Entrapped *Carnobacterium maltaromaticum* LMA 28 was able to produce 3-methylbutanal in sufficient amounts to facilitate diffusion through paraffin coating. The results identify bacterial encapsulation as superior to direct encapsulation of volatile compounds for imparting 3-methylbutanal olfactory notes to cheese.

Keywords: aroma; artisanal cheese; bacterial encapsulation

Poro cheese is a fresh raw milk handmade product with the consistency of a soft, slightly pressed paste. It is coated with paraffin wax and packed in yellow cellophane and involuntarily matures in four weeks if its distribution is slow (Villegas de Gante 2004). Cheese coating is a well-established procedure for Poro cheese. Other cheeses are waxed or film-wrapped to create a barrier against mould entry, to reduce the rate of moisture loss and to prevent oiling off thereby making the cheese more attractive and easier to handle (Kampf & Nussinovitch 2000). Encapsulation technology has multiple applications in the food industry, which include controlling oxidative reactions, masking flavours, colours and odours, providing for sustained and controlled release and extending shelf life (Burgain *et al.* 2011). There are different encapsulation techniques and a long list of materials can be used, but none of these can be considered as universally applicable. Calcium alginate hydrogels are frequently used for the entrapment of microbial cells. It is well known that microorganisms play a positive role in cheese ripening as they provide specific cheese flavours (Irlinger & Mounier 2009). Among lactic acid bacteria (LAB), *Carnobacterium*

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maltaromaticum is a bacterium frequently found in food (meat, fish and dairy products) and is well-known for the production of the flavour compound 3-methylbutanal from the catabolism of leucine and for the inhibition of pathogenic microorganisms due to its bacteriocin-producing ability (Afzal *et al.* 2010, 2012, 2013a,b). In cheese, odour descriptors used for 3-methylbutanal are malty, chocolate and caramel (Ayad *et al.* 2003; Smit *et al.* 2004) with detection and recognitions thresholds of 0.5 and 1.2 µg/l of water (Czerny *et al.* 2008). The aim of this work was to improve the sensory characteristics of Poro cheese by incorporating calcium alginate beads into paraffin coating containing either direct encapsulation of 3-methylbutanal or strains of *C. maltaromaticum* LMA 28. The impact on cheese sensorial perception was tested with a cheese model by a panel of experts who evaluated aroma.

MATERIALS AND METHODS

Preparation of encapsulation solutions. A 10^4 µM 3-methylbutanal (Sigma-Aldrich, France) solution was prepared and mixed with a 2.6% (w/v) sodium alginate (Sigma-Aldrich, France) solution to obtain 5000 µM 3-methylbutanal with 1.3% (w/v) final concentration of alginate encapsulation mix (Mix 1). *Carnobacterium maltaromaticum* LMA 28 was cultivated at 30°C for 24 h in tryptic soy broth-yeast extract (TSB-YE) (Biomerieux and Becton Dickinson, France) adjusted to pH 6.5. Culture was added to 1.3% (w/v) alginate in TSB-YE media enriched with 2.5 g/l of supplementary glucose (Merck Millipore, Germany) to obtain a second encapsulation mix (Mix 2) with a cell concentration of 10^8 CFU/g. Beads were produced using an extrusion system coupled to a peristaltic pump. The solutions were pumped through a 0.3-mm-diameter needle into 200 ml of a 1.5% (w/v) $CaCl_2$ (Sigma-Aldrich, France) sterile solution. Beads remained in the solution for 10 min for hardening, and then they were recovered by filtration and rinsed with sterile distilled water. The overall experimental diagram is summarised in Figure 1.

Characterisation of bead morphology and encapsulation efficiency. Bead size distribution was measured using optical microscopy (Olympus AX70 PROVIS, objective 40). Images were taken with a camera (Olympus DP70) coupled with a microscope. Diameter and shape factor (ratio between the largest and smallest capsule diameters) were obtained by image analysis (Visilog 7.1; Noesis, France) on 20 randomly selected beads from each solution.

The encapsulation efficiencies for 3-methylbutanal and *C. maltaromaticum* LMA 28 beads were calculated using Equations 1 and 2 respectively:

$$EY_{3\text{–Mal}} = (C/C_i) \times 100 \quad (1)$$

where: C_i – initial concentration of 3-methylbutanal in encapsulation solution (Mix 1); C – concentration of 3-methylbutanal containing Mix 1 beads

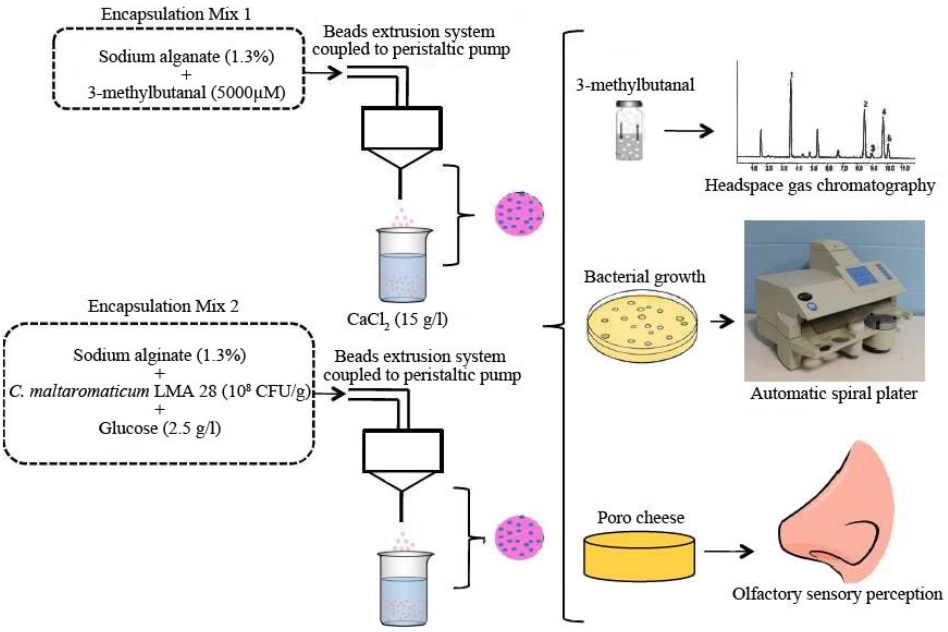

Figure 1. Overall scheme of the experiment

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As 3-methylbutanal is a highly volatile compound, it was not possible to use a control showing the remaining concentration of 3-methylbutanal in the $CaCl_2$ solution.

$$EY_{Carno} = (N/N_i) \times 100 \quad (2)$$

where: N_i – number of CFU of *C. maltaromaticum* LMA 28 in encapsulation solution (Mix 2); N – number of viable CFU in fresh beads

Survival of C. maltaromaticum LMA 28 and 3-methylbutanal production assays. The amount of 3-methylbutanal released from beads was measured using headspace gas chromatography according to a method described previously (Afzal *et al.* 2012). Survival of *C. maltaromaticum* LMA 28 and 3-methylbutanal production/release kinetics of Mix 2 beads were followed in two different conditions: beads without paraffin coating and beads with paraffin coating. For survival tests, beads were conditioned in vials containing 1 g of beads without paraffin coating or beads with paraffin coating and incubated at 30°C. Cell concentrations were expressed as CFU/g of capsule mass. For 3-methylbutanal production/release, 10-ml vials containing 1 g of beads without paraffin coating or beads with paraffin coating were prepared and stored at 30°C. Three vials of each treatment were taken at different time intervals over the course of 72 h and analysed.

Bacteria inhibition test. Bacterial inhibition assays were conducted to test the impact of *C. maltaromaticum* LMA 28 on the lactic acid flora of Poro cheese. Beads of *C. maltaromaticum* LMA 28 were deposited on TSA-YE (TSB-YE added to bacteriological agar type A; Biokar, France) plates and incubated for 24 h at 30°C. Eight strains including a positive control *Listeria monocytogenes* EGDe lux (Table 1) were tested by inoculating with and without beads of *C. maltaromaticum* LMA 28. Each culture strain in an amount of 150 µl was inoculated in tubes containing 15 ml of TSA-YE, carefully mixed and poured into plates containing *C. maltaromaticum* LMA 28 beads. After solidification, plates were stored for 24 h at 4°C and subsequently incubated for 48 h at 30°C. Tests were performed in duplicate. The Presence or absence of inhibition was monitored. All culture strains were obtained from laboratory (LIBio) collections.

Sensorial effect. In order to test if beads have a real sensorial effect on cheese, mini-babybel® cheeses were coated with paraffin with or without *C. maltaromaticum* LMA 28 beads (1 g of beads per cheese).

Table 1. Antimicrobial activity of *C. maltaromaticum* LMA 28 against the tested strains

Tested lactic acid bacteria	Antimicrobial activity
Lactobacillus fermentum F4D10	–
Lactobacillus plantarum F3C2	–
Lactobacillus farciminis F2C1	–
Lactobacillus rhamnosus F2D2	–
Lactobacillus brevis F1C3	–
Lactobacillus pentosus F4D8	–
Enterococcus faecium F1D2	+
Listeria monocytogenes EGDe lux*	+

*Positive control

Paraffin temperature was 65 ± 2°C to minimise the effect of temperature on the viability of the encapsulated bacteria. Thirty-six coated cheeses were incubated in olfactory test flasks (Thermo Fisher Scientific, France) for 48 h at 30°C to promote bacterial growth and 3-methylbutanal production. Half of these cheeses were immediately used and others were stored at 30°C for one week. A trained olfactory panel of ten members performed sensory analysis. A paired-comparison difference test was applied to determine which of the coated cheeses with or without *C. maltaromaticum* LMA 28 beads had more 3-methylbutanal (malty or chocolate-like) aroma. Before evaluation, sample coatings were fractured and cheeses were equilibrated in their flask at 40°C for 1 hour. Two different samples were simultaneously presented to each panel assessor. A judge was asked to determine if samples were the same or not.

Statistical analysis. Values are expressed as means ± standard deviation of triplicate determinations for 3-methylbutanal concentrations of 20 capsules for size and shape determinations and of duplicates for cells concentrations. The significance of differences between samples was evaluated using a one-tailed binomial test for a ten-member panel. The difference between samples was significant if the number of correct answers was nine ($P < 0.05$) or ten ($P < 0.01$) (NF V 09-012, 1983 ISO 5495).

RESULTS AND DISCUSSION

Encapsulation rate and release of 3-methylbutanal. The initial 3-methylbutanal concentration of Mix1 was 5000 µM. The t_0 concentration of 3-methyl-

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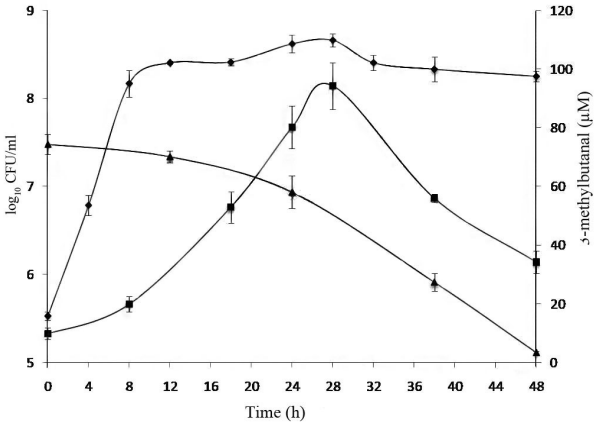

Figure 2. Growth of *C. maltaromaticum* LMA 28 and production kinetics of 3-methylbutanal. Curves with symbol (♦) represent growth, (■) 3-methylbutanal production, and (▲) direct encapsulated 3-methylbutanal, respectively

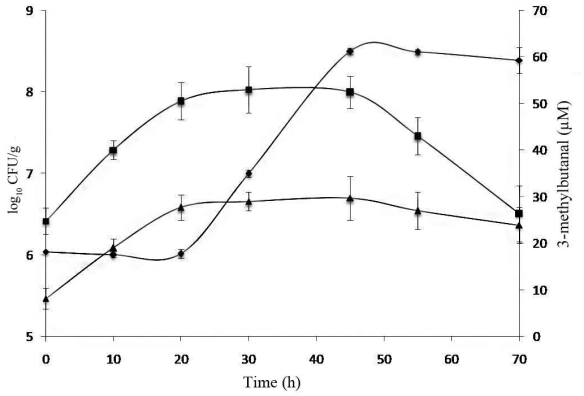

Figure 3. Growth of entrapped *C. maltaromaticum* LMA 28 and 3-methylbutanal production/release kinetics. Curves with symbol (♦) represent growth, (■) 3-methylbutanal beads release, and (▲) 3-methylbutanal paraffin release, respectively

butanal containing Mix1 beads was detected to be 75 µM, which slightly decreased to 58 µM after 24 h and after 48 h, a concentration of only 3 µM was measured (Figure 2). Flavour encapsulation by extrusion has been used previously and our results are consistent with previous finding where a loss of aromatic molecules was reported (Manojlovic *et al.* 2008). Compounds with a higher affinity for polysaccharide might be better retained (Voilley & Etievant 2006). Retention seemed to be related to polarity, such that more polar compounds have lower retention rates; furthermore, compared with other chemical groups, aldehydes were retained to a lower extent by polysaccharides (Goubet *et al.* 1998).

Carnobacterium maltaromaticum LMA 28 growth and 3-methylbutanal production kinetics. The growth kinetics of *C. maltaromaticum* LMA 28 was characterised by a stationary phase starting after 8 h that persisted for at least 40 h, while 3-methylbutanal production was never stable. It reached a maximal point at 28 h (94 µM) and decreased to 34 µM after 48 h (Figure 2). Our findings are consistent with previous results shown by Afzal *et al.* (2012, 2013), where, in batch culture with 90% of oxygen saturation, *C. maltaromaticum* LMA 28 produced 180 µM of 3-methylbutanal after 15 h, while production also decreased to 56 µM after 48 hours. The growth kinetics of encapsulated bacterial showed a 2.5 log increment after 45 h followed by a stationary phase of at least 25 hours. Initial 3-methylbutanal bead concentration/release was 24.7 µM, during which time bacteria were in the exponential phase. After 20–45 h of kinetic initiation, 3-methylbutanal production/release reached its maximum level of 52 µM and remained almost constant. After 45 h, production/release decreased and after 70 h was only 26 µM (Figure 3). The initial concentration of 3-methylbutanal was 8 µM and after 8 h, increased to almost 28 µM, attaining its maximal level (29.7 µM) at around 45 h of monitoring. After 70 h, the concentration decreased slightly to 23 µM (Figure 3). Similar to other polymers, alginate is known to allow flowthrough of nutrients and metabolite diffusion (Martin *et al.* 2013). When mixed with cell culture media, it could be considered as an active packaging system providing a stimulatory enviroment for cell development. Paraffin is one of the best additives to reduce water permeability.

Bead characterization and encapsulation efficiency. The concentration of 3-methylbutanal in fresh Mix1 beads was 90 µM/g, while the initial concentration was 5000 µM. At this point, the encapsulation efficiency of 3-methylbutanal beads was only 1.5%. These losses might be due to high compound volatility combined with the encapsulation technique. Concerning *C. maltaromaticum* LMA 28 encapsulation efficiency, cell concentration in Mix 2 was 108 CFU/ml and cell concentration in fresh beads was 106 CFU/g. At that point, encapsulation efficiency was 70.9%. There was no significant difference between beads ($P < 0.05$) as revealed by optical microscopy (Figure 4). Both were almost spherical and uniform. The average diameter and shape factor of the beads were 1.69 ± 0.15 and 0.86 ± 0.01 mm, respectively. Contrary to what was reported by (Krasaekoopt

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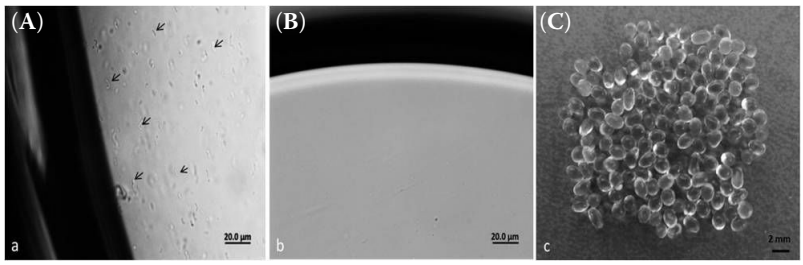

Figure 4. Microscopic images of beads: (**A**) with bacteria, (**B**) without bacteria, and (**C**) whole beads

et al. 2004), who obtained an efficiency of 99.9% using a pre-existing extrusion technique to entrap *Lactobacillus acidophilus* in a 2% alginate matrix, *C. maltaromaticum* LMA 28 was less efficient (70.9%). Other techniques such as emulsification have also been used for bacteria encapsulation with good efficiency rates (Mokarram *et al.* 2009). Alginate has high biocompatibility, low costs and is extensively used for cell entrapment (Burgain *et al.* 2011).

Effect of C. maltaromaticum LMA 28 beads on Poro cheese flora and sensorial effect. Due to the typicity of Poro cheese, *C. maltaromaticum* LMA 28 incorporation into Poro cheese coating could not have an adverse effect on its microflora. However, as *C. maltaromaticum* LMA 28 is known to produce bacteriocins effective against various pathogenic and spoilage microorganisms (Afzal *et al.* 2010, 2013), it was important to verify its effect against the principal lactic acid bacteria of Poro cheese which were responsible for most of its characteristics. Table 1 shows the main LAB isolated from Poro cheese and the antibacterial effect of *C. maltaromaticum* LMA 28 beads on each strain. From seven strains evaluated, only *Enterococcus faecium* F1D2 was found to be inhibited by the antimicrobial effect of *C. maltaromaticum* LMA 28. The antibacterial effect of *C. maltaromaticum* was due to its bacteriocin production, including carnobacteriocins (Cbn) B2 and CP5, which were found to be active against *E. faecium.* However, these bacteriocins as well as Cbn BM1 were not active against other LAB such as *Lactobacillus fermentum* and *Lactobacillus plantarum* (Jasniewski *et al.* 2009). Poro cheese is made exclusively from raw cow's milk. Whey obtained from the previous day's cheese production was added to the milk to ensure the presence of a similar microbial population that determines the characteristics of Poro cheese (Pogacic *et al.* 2013). Lactic acid bacteria in Poro cheese might come from raw milk, whey culture and the cheese factory environment. The strains used in this study integrate Poro cheese non-starter lactic acid flora. These types of bacteria generally play a significant role during cheese ripening, influencing cheese quality, flavour characteristics, peculiarity and authenticity (Settanni & Moschetti 2010; Steele *et al.* 2013); thus, it is important to minimisee the effect of *C. maltaromaticum* LMA 28 and its bacteriocins against the technological flora of Poro cheese. *Carnobacterium maltaromaticum* LMA 28 beads were able to inhibit *Listeria monocytogenes* used as a positive control. This is an important advantage since *L. monocytogenes* is one of the most important pathogenic bacteria affecting raw milk fresh cheeses (Almeida *et al.* 2013).

CONCLUSIONS

In this study, we proposed a new alternative method for incorporation of volatile compounds into paraffin coating using the encapsulation of *C. maltaromaticum* LMA 28, a strain well-known in the research field, and observed production of 3-methylbutanal (Afzal et al. 2010, 2012, 2013a,b, 2017). Our results also showed that *C. maltaromaticum* LMA 28 entrapment is a better alternative to control 3-methylbutanal release over time in comparison with direct encapsulation of 3-methylbutanal. Besides the advantages provided to Poro cheese producers, the originality of this research finding lies in the bacterial encapsulation strategy. This novel strategy for aromatic compound production is thus more significant than any probiotic or antibacterial properties.

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