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HPLC analysis of the pigments produced by the microflora isolated from the 'Protected Designation of Origin' French red-smear soft cheeses Munster, Epoisses, Reblochon and Livarot

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Abstract

Red-smear ripened soft cheeses are characterized by their orange-red color which originates from the carotenoids and other pigments produced by ripening bacteria. A total of 114 different bacterial strains, belonging to *Brevibacterium linens* group, *Micrococcaceae* and coryneform bacteria, were isolated from four French red-smear ripened soft cheeses with "protected designation of origin" (i.e., Livarot, Reblochon, Munster, and Epoisses). Among the 114 strains, 67 were selected for their orange or yellow color and their methanolic extracts were analysed by HPLC. All orange bacteria showed the same HPLC profile typical of *B. linens*, which is known to produce the three aromatic carotenoids, isorenieratene, 3-hydroxyisorenieratene, and 3,3′-dihydroxyisorenieratene. Yellow bacteria produced four different chromatographic profiles. Their analysis (retention time and absorption spectrum) revealed strong similarities among three chromatographic profiles and led us to propose that they represent different isomers of the same compound. This study contributes to the characterization of pigments synthesized by the microflora of French red-smear ripened soft cheeses as part of the effort to identify these valuable microorganisms.

Keywords: Cheese ripening; Smear; Bacteria; Carotenoid; Color

1. Introduction

Red-smear ripened soft cheeses are popular dairy products in Europe, characterized by their red to orange-brown coloration. The color is due mainly to carotenoids, as well as other pigments, produced by the cheese microflora during ripening. Studies have shown that the ripening of red-smear ripened soft cheeses progresses through the succession of microbial communities on the cheese surface. Surface ripening begins with the growth of yeasts, which metabolize lactic acid and produce growth factor useful to bacteria. When pH increases to a value higher than 6, bacteria begin to grow and eventually cover the entire cheese surface. The microflora consists of a mixed population predominantly composed of *Brevibacterium linens* group (Gavrish et al., 2004) in addition to *Arthrobacter*, other *Brevibacterium*, *Corynebacterium*, *Microbacterium*, and *Rhodococcus* (Bockelmann, 1997; Valdès-Strauber, Scherer, & Seiler, 1997). Coryneform bacteria are predominant on the surface of ripened cheeses (Eliskases-Lechner & Ginzinger, 1995).

For a long time the coloration of red-smear ripened soft cheeses was only imputed to *B. linens* (Boyaval & Desmazeaud, 1983; Rattray & Fox, 1999). The pigments produced by *B. linens* were first identified as carotenoids (Kohl, Achenbach, & Reichenbach, 1983), and later characterized spectrocolorically (Dufossé, Mabon, & Binet, 2001; Guyomarc'h, Binet, & Dufossé, 2000a). Among microbes, plants, and animals more than 650 different

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carotenoids have been described. Only recently major advances have been made in the genetics, biochemistry, and regulation of carotenoid biosynthesis (Armstrong, 1999; Krubasik & Sandmann, 2000; Sandmann, 2002). However, little is known about the pigments produced by the microflora of red-smear ripened soft cheeses, other than *B. linens*. Considering the small content of *B. linens* in cheese surface microflora, Bockelmann, Fuehr, Martin, and Heller (1997a) expressed reservations that *B. linens* alone would contribute to the rind coloration. Using cheese model system they first reported on the importance of *B. linens* interaction with other bacteria, such as *Arthrobacter* sp., in the development of cheese coloration (Bockelmann & Hoppe-Seyler, 2001). Interactions between the yeast *Debaryomyces hansenii* and coryneform bacteria were shown to influence cheese surface pigmentation (Leclercq-Perlat, Corrieu, & Spinnler, 2004; Masoud & Jakobsen, 2003). Physical and chemical parameters, such as temperature, dissolved oxygen, pH, and culture medium are also important for bacterial development and pigment production (Reps, 1993). Overall, cheese rind coloration is a complex process involving physical, chemical factors, and biotic interaction.

In the face of the expanding cheese processing industry, traditional cheeses and the unique microflora they contain may be lost. In an attempt to save this biodiversity, to identify the bacteria and make these unique bacterial strains available to cheese makers, in relation to cheese-making technology, Association de Coordination Technique pour l'Industrie Agro-alimentaire (ACTIA) is conducting the screening of traditional French cheeses for their orange-red pigment producing microflora. To this end bacteria were isolated from four French red-smear ripened soft cheeses with 'Protected Designation of Origin' (PDO), and methanolic extracts prepared for HPLC analysis. Chromatograms of pigmented extracts obtained from the cheese rind gave some indication of the nature of the molecules responsible for the color. However, the bacterial strains producing these pigments have yet to be identified and characterized. As part of the database being assembled to identify traditional cheese bacterial microflora, this study has produced the first pigment fingerprints of bacteria strains found in French red-smear ripened soft cheeses Livarot, Reblochon, Munster, and Epoisses.

2. Materials and methods

2.1. Cheeses used in this study and isolation of bacterial strains

Four red-smear ripened soft cheeses with PDO, i.e., Livarot, Reblochon, Munster, and Epoisses, were selected by traditional cheese makers based on their organoleptic properties (texture, color and flavor). Bacteria strains were isolated and grown using FP and MSC culture media on Petri dishes. A fraction of the numerous yellow and orange strains were selected whereas all bacteria presenting red, pink, cream, or beige hues were screened. From hundreds of strains, 114 were selected based on their ability to grow under salty and acidic conditions. Sixty seven strains out of the 114 were finally selected, based on their pigment content and further used for HPLC analysis. These bacterial strains fell into four groups, i.e., *B. linens*, *Micrococcus* sp., *Staphylococcus sp.* and coryneform bacteria.

2.2. Culture media for bacteria screening, numeration and isolation

The composition of the FP medium used for the total pigmented microflora was as follows: 25 g/L Tryptone soy broth (BK 028) (bacto-tryptone 15 g/L, bacto-soytone, i.e., papainic digest of soy 5 g/L, NaCl 5 g/L), 33.4 g/L Na lactate, 35 g/L NaCl, 6 g/L yeast extract, 15 g/L agar, 40 mg/L nalidixic acid, 80 mg/L amphotericin B, adjusted to pH 6.5. Incubation took place at 20 °C for up to 10 days. MCM medium used for coryneform bacterial strains (i.e., *Micrococcaceae*, *Brevibacterium*, *Corynebacterium*) was composed of FP medium supplemented with furazolidone (10 mg/L). MSC medium used for *Staphylococcaceae* was made of FP medium incubated in anaerobiosis.

2.3. Culture medium for HPLC analysis of bacterial pigments

Cultures were grown in 250 mL Erlenmeyer flasks containing 50 mL of a medium composed of 20 g/L D-glucose (Carlo Erba), 5 g/L casamino acids (Difco), 1 g/L yeast extract (Biokar), 5 g/L NaCl and 1 g/L KH_2PO_4. The pH was adjusted to 6.9 and the medium was heat-sterilized 121 °C, 15 min. Flasks were inoculated with 1% of 72 h-old preculture (v/v), and incubated at 25 °C for 4 days with stirring (150 rpm).

2.4. Extraction of pigments

Extracts were obtained from 20 mL of culture. Cells were first centrifuged at 6000*g* for 15 min. The supernatant was discarded and the pellet rinsed with 5 mL deionized water, vortexed and centrifuged at 6000*g* for 15 min. The pellet was mixed with 8 mL methanol, blended to prevent clotting, and extracted with constant agitation (50 rpm), protected from direct light with aluminium foil, until cells were bleached (within 2 h). The sample was then centrifuged (6000*g*, 15 min), the pellet was discarded and the supernatant further centrifuged (10,000*g* for 15 min). The resulting supernatant, i.e., methanol extract, was then injected in the HPLC system.

2.5. HPLC analysis

Methanol extracts were evaporated to dryness under vacuum at 75 °C in a Büchi rotavapor, within minutes. Dry pigments were dissolved in 1 mL methanol, filtered through Millex-GV 0.2-μm hydrophilic membrane (Millipore), and injected (20 μL) onto a LichroCART 250-4 RP-18 (250 × 4 mm, 5-μm particle size) column (Merck). The HPLC apparatus consisted of Waters 600 constant flow pump and controller, and Waters 996 photodiode array detector. Separation was achieved using reverse phase HPLC at a flow rate of 0.5 mL/min. Solvents and conditions used for separation were as follows: 0 to 45 min, 100% methanol; 45 to 80 min, 100% methanol to 80% methanol/20% chloroform; 80 to 130 min, 80% methanol/20% chloroform.

3. Results and discussion

Using the protocol illustrated in Fig. 1, 1421 bacterial strains were isolated from the four selected French red-smear ripened soft cheeses with PDO. Further screening based on NaCl concentration (3 g/L), temperature (12 °C), optimum pH (6.5–7.0), and strain-specific pH for minimum growth in synthetic medium (*Brevibacterium* sp., pH 5.8; *Staphylococcaceae* and main other *Corynebacterium*, pH 5.4) narrowed down the number of pigmented strains to 114. Twenty four strains were isolated from Livarot (10 *B. linens*, 8 *Corynebacterium*, 4 *Staphylococcaceae*, and 2 *Micrococcaceae*), thirty from Reblochon (6 *B. linens*, 20 coryneform bacteria, and 4 *Micrococcaceae*), thirty from Munster (13 *B. linens*, 9 unidentified rods, 5 beige unidentified rods, and 3 unidentified cocci), and thirty from Epoisses (8 *B. linens*, 12 coryneform bacteria, 6 *Micrococcaceae*, and 4 *Staphylococcaceae*). This microbial diversity has been previously described on the surface of smear cheeses, such as Livarot and Pont l'Evêque (Denis, Gueguen, Henry, & Levert, 2001), Tilsit (Bockelmann et al., 1997b; Eliskases-Lechner & Ginzinger, 1995), and Gubbeen (Brennan et al., 2002). Because some of the 114 strains isolated lost color intensity during repeated growths, only 67 strains were selected for their orange or yellow color and their methanolic extracts analysed by HPLC.

Despite the diverse geographical origins of the cheeses selected for this study and the large number of bacterial strains isolated, only seven distinct chromatographic profiles were found. This observation suggested that the range of carotenoids and other pigments produced by the bacterial microflora is quite limited. Moreover, some red-smear ripened soft cheeses possessed a higher microbial diversity than others (Fig. 2).

For instance, Reblochon cheeses presented five different bacterial pigment HPLC profiles, whereas Livarot and Munster had four and Epoisses had three. HPLC Profile A was present in all red-smear ripened soft cheeses and accounted for the majority of bacterial strains in Epoisses, Munster, and Livarot. HPLC Profile B was only found in Munster, while Profile D was specifically associated with Reblochon. Profile C was present in Munster, Livarot and Reblochon. Among the seven types of pigmented bacteria only five yielded sufficient material for further HPLC analysis (retention time, UV–Vis spectra) (Fig. 3). Based on the elution profile of these pigments and the hydrophobic packing of the C_{18} column it was reasonable to assume that most of the extracted pigments were polar.

All orange bacteria had the same HPLC profile (Profile A). This profile was found in 41 bacterial strains. It was characterized by two groups of peaks; the first group was composed of one major peak at 12.80 min and smaller

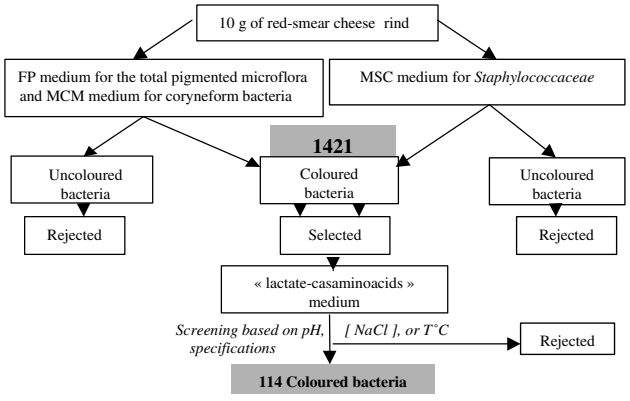

Fig. 1. Selection of colored bacteria for HPLC analysis.

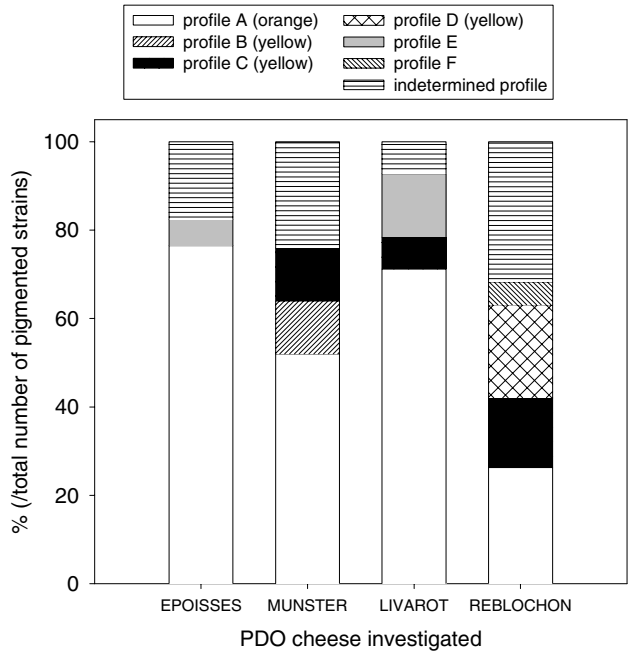

Fig. 2. Occurrence of HPLC profiles from pigmented bacteria among PDO cheeses.

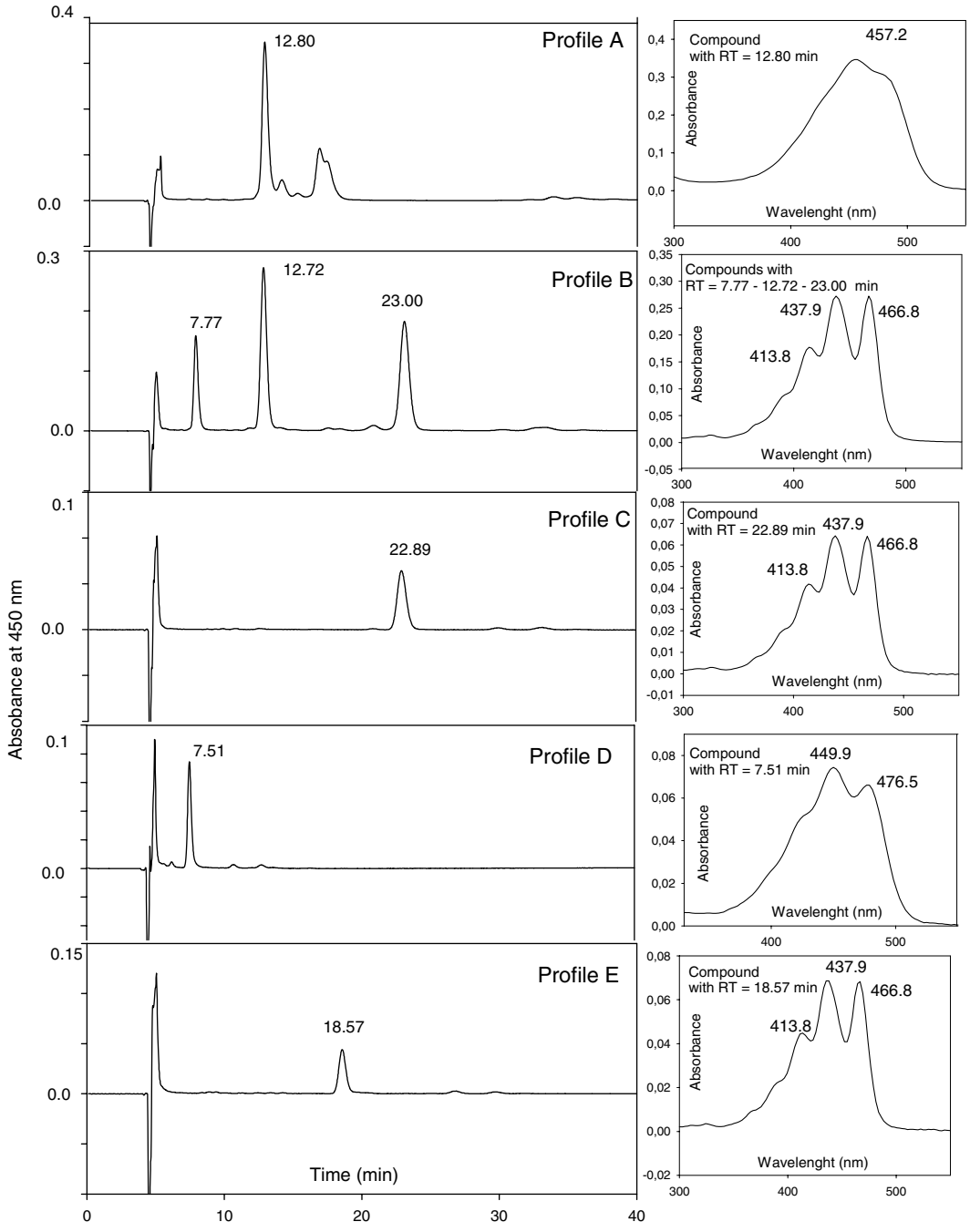

Fig. 3. Chromatographic profiles of pigments extracted from bacteria isolated from the rind of French red-smear soft cheeses.

accompanying peaks. The second group, with very weak intensity, came out between 32 and 39 min. These orange bacteria were identified as different strains of *B. linens* group, which is known to produce three aromatic carotenoids, i.e., isorenieratene, 3-hydroxyisorenieratene, and 3,3′-dihydroxyisorenieratene (Kohl et al., 1983). However, isorenieratene, an apolar compound, was not found in any of the strains tested confirming previous studies from our laboratory (Guyomarc'h et al., 2000a, Guyomarc'h, Binet, & Dufossé, 2000b). Among the isolated bacteria, *B. linens* group was the most abundant, representing 75% of the total number of pigmented strains isolated from Epoisses, 70% from Livarot, and 50% from Munster.

The four remaining chromatographic profiles (Profiles B, C, D and E) corresponded to yellow bacteria. These bacteria belonged to different taxonomic groups of unidentified rods and cocci. Profile B was common to four bacterial strains isolated from Munster. This profile is characterized by three major peaks at 7.77, 12.72, and 23.00 min. The absorption spectra of these three compounds showed the same close maxima (413.8, 437.9 and 466.8 nm) suggesting that it might

correspond to isomers of the same molecule. Profile C was found in 9 bacterial strains isolated from Reblochon, Munster and Livarot. It showed one major peak at 22.89 min characterized by three absorption maxima at 413.8, 437.9, and 466.8 nm. Profile C showed strong similarities with the third peak of Profile B based on retention time and absorption spectrum. Profile D was unique to 4 bacterial strains isolated only from Reblochon and characterized by one major peak at 7.51 min and absorption maxima at 449.9 and 476.5 nm. This pigment was only produced by Reblochon bacteria. Profile E was found in 4 bacterial strains isolated from Epoisses and Livarot, and characterized by one major peak at 18.57 min and absorption maxima at 413.8, 437.9 and 466.8 nm. Although the 18.57 min peak was unique to Profile E, its absorption spectrum was identical to that of Profiles B and C. Owing to the strong similarities in retention time and absorption spectrum between Profiles B, C and E, we assumed that yellow bacteria may synthesize several isomers of the same compound. Additional bacterial extracts could not be interpreted because they did not contain sufficient material for HPLC analysis (Profile F and indetermined profile).

For a long time, the coloration of red-smear ripened soft cheeses was solely attributed to *B. linens* group or to its interactions with other bacterial or yeast strains. Our work shows that the color results at least from two families of pigments, one produced by *B. linens* group and another produced by different microorganisms, including *Micrococcus sp.*, unidentified cocci and coryneform bacteria. While the pigments produced by *B. linens* could be present on all four red-smear ripened soft cheeses, the second family of pigments was made of isomers of the same compound, and at least one of these isomers could be found on the surface of all four red-smear ripened soft cheeses. Another pigment was only produced by Reblochon bacteria.

Further research using LC-MS and NMR is needed to investigate the structure of these pigments and to confirm the view that these bacteria synthesized a variety of isomers. However, one should keep in mind that the color of the rind may also result from interactions among bacteria (Brennan et al., 2002), as well as between bacteria and yeast (Leclercq-Perlat et al., 2004). Additionally, comparative studies on pigment production in mixed cultures and isolated bacteria should be conducted on dairy models and cheese rinds themselves in order to elucidate the significance of microbe interaction.

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