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**Abstract**

Pigments were extracted from cheese-ripening bacteria belonging to the species *Arthrobacter arilaitensis*, which plays a role in the colour of cheese. A chromatographic method was applied to characterise the pigment profile. Based on the UV-visible (UV-vis) spectra, the mass spectra, and the elution order, eight different carotenoids were identified from the strains. Four of the pigments, namely, all-*E*-decapreno-anthin, all-*E*-sarcinaxanthin, 9-*Z*-decaprenoanthin and 15-*Z*-decaprenoanthin, which were present as free-form main carotenoids (i.e., non-glycosylated), have been characterised by their UV-vis and mass spectra, both in atmospheric-pressure chemical ionisation negative [APCI (−)] and positive modes. The other minor carotenoids, namely, sarcinaxanthin monoglucoside pentacetate, decaprenoanthxin monoglucoside, decaprenoanthxin diglucoside, decaprenoanthxin-C16:0 (decaprenoanthxin-palmitate), present in very low amounts, were only detected in the APCI (−) ionisation mode. Our results provide the first chemical characterisation of the pigments produced by cheese-ripening *A. arilaitensis* strains, which has not previously been reported.

1. Introduction

Smear-ripened cheeses, also known as surface-ripened cheeses or red-smear ripened cheeses, are economically important dairy products, which involve the activity of various microorganisms during the milk coagulation and ripening stages. The surface of smear-ripened cheeses is a microbial mat, with a yellow-orange-reddish-brown colour, composed of a large diversity of bacteria and yeasts. Several smear-ripened cheeses are characterised by consumers by their characteristic colour and shape, for example, Livarot and Epinette from France, Limburger and Tilsit from Germany, and Taleggio from Italy.

The colour of smear-ripened cheeses is one of the main attributes that impacts upon consumer acceptance of these cheeses, and is related to several cheese qualities, such as maturity, flavour and cleanliness (Dufossé, Mabon, & Binet, 2001). Coloration of smear-ripened cheeses appears to be a complex phenomenon due to interactions amongst microorganisms on the cheese rind (Leclercq-Perlat, Corrieu, & Spinnler, 2004; Mounier et al., 2006, 2008). Pigments generated by the bacteria present at the surface of cheeses are poorly studied, except for bacterial *Brevibacterium* species such as *Brevibacterium linens* and *Brevibacterium aurantiacum*. For a long time, these two species were considered to be the major microorganisms responsible for colour development at

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the surface of cheeses because of the ability to produce orange carotenoids, which have been identified as isorenieratene, 3-hydroxy-isorenieratene, and 3,3′-dihydroxy-isorenieratene (Kohl, Achenbach, & Reichenbach, 1983). Recently, due to the development of improved molecular methods of microbial biodiversity analysis, the presence of many other species possibly involved in the generation of functional properties has been reported in smear-ripened cheeses (Bockelmann & Hoppe-Seyler, 2001; Elkskases-Lechner & Ginzinger, 1995; Goerges et al., 2008; Irlinger, Layec, Hélène, & Dugat-Bony, 2015; Montel et al., 2014). Arthrobacter strains have been known to be part of the cheese smear for a long time, and are now recognised as major microorganisms involved in surface pigmentation of smear-ripened cheeses due to the characteristic overall colour of their biomass and their presence during different stages of cheese production until the end of ripening (Feurer, Valaëys, Corriëu, & Irlinger, 2004; Irlinger & Mounier, 2009; Larpin-Laborde et al., 2011; Mounier et al., 2005). Arthrobacter arilaitensis is frequently present at high levels on smear-ripened cheeses, and produces a yellow pigment (Galaup, Flamin, Carlet, & Dufossé, 2005; Galaup et al., 2007; Irlinger, Bimet, Delettre, Lefèvre, & Grimont, 2005; Leclercq-Perl & Spinolé, 2010; Mounier et al., 2008; Suthiwong et al., 2014). Another Arthrobacter, the psychrophilic bacterium Arthrobacter glacialis, was previously reported to synthesise three C50 carotenoids, i.e., decaprenoxanthin, bisanhydrobacterioruberin, and Ag470 (Arpin, Faisson, Norgard, Borch, & Liaaen-Jensen, 1975). Arthrobacter sp. M3 was also found to produce decaprenoxanthin, and its glucosides (Arpin, Liaaen-Jensen, & Trouilloud, 1972). In nature, C50 carotenoids are synthesised by bacteria which belong to the Actinomyccetes order. Until now, only three different C50 carotenoid biosynthetic pathways have been described: (i) the β-cyclic C50 carotenoid C.p. 450 pathway in Dietzia sp CQ4; (ii) the γ-cyclic C50 carotenoid sarcinaxanthin pathway in Micrococcus luteus NCTC2665; and (iii) the ε-cyclic C50 carotenoid decaprenoxanthin pathway in Corynebacterium glutamicum (Heider, Peters-Wendisch, Netzer, Stafnes, & Brautaset, 2014; Krubasik et al., 2001; Netzer et al., 2010; Tao, Yao, & Cheng, 2007). The whole genome sequencing of the strain originating from cheese, A. arilaitensis Re117, has revealed the presence of a decaprenoxanthin-like carotenoid biosynthetic gene cluster (Monnet et al., 2010).

The aim of this study was to identify the pigments biosynthesised by A. arilaitensis strains originating from cheese to provide new information useful to elucidate the nature of the smear-ripened cheeses colour, and to understand the importance of selecting this bacterium as a part of ripening flora.

2. Materials and methods

2.1. Bacterial strains, medium and growth conditions

Two yellow-pigmented A. arilaitensis strains, namely A. arilaitensis Poi102 isolated from Pont-l’Évêque cheese, and A. arilaitensis Stp101 isolated from Saint-Paulin cheese, were used in this study. These were obtained from the GMPA (Génie et Microbiologie des Procédés Alimentaires) culture collection (Unité Mixte de Recherche 782, Institut National de la Recherche Agronomique, Thiverval-Grignon, France) and maintained during this study on milk ingredient-based agar, stored at 4 °C and subcultured monthly. The milk ingredient-based agar medium contained 5 g casein hydrolysate (Difco, Le Pont de Claix, France), 1 g yeast extract (BD Bacto, Le Pont de Claix, France), 5 g NaCl (Fisher Scientific, Illkirch, France), 20 g glucose (Fisher Scientific), 1 g KH₂PO₄ (Fisher Scientific) and 15 g agar granulated (BD Difco) per litre of deionised water. Before sterilising at 121 °C for 15 min, the pH of the medium was adjusted to 7.0 ± 0.2. The choice of these two strains was due to a strong yellow colour amongst the whole collection of A. arilaitensis strains of our laboratory. It was previously shown that all the strains presented the same HPLC pigment profile, including the strain Re117, the genome sequence of which is available (Sutthiwong & Dufossé, 2014).

After inoculation of 10 mL of milk ingredient-based liquid medium (same composition as described previously, without agar) in a 50 mL conical flask with a colony of A. arilaitensis, the pre-cultures were incubated for 72 h at 25 °C on a rotary shaker at 150 rpm. The same growth medium was then inoculated at 1% (v/v) with the pre-culture and incubated under the same conditions.

2.2. Pigment extraction

After 7 d of growth, A. arilaitensis cells were harvested by centrifugation at 6000 × g (25 °C) for 15 min. The cell pellets were washed with deionised water, frozen at −80 °C for 48 h, and then lyophilised to dryness (COSMOS-80 manufactured by CRYOTEC, vacuum below 200 mtorr, temperature of the shelf increased to −20 °C for overnight primary drying then to 20 °C for 2 h for the secondary drying phase). Lyophilised cells (1 g) were extracted with 8 mL of 95% methanol (Carlo Erba, Pepsin, France) including a sonication step (Transsonic T420™ homogeniser, Elma, Singen, Germany, HF-frequency 35 kHz, 10 min, on ice), at room temperature for 90 min. The mixture was centrifuged at 6000 × g (25 °C) for 15 min to separate the cell debris from the carotenoid-containing supernatant. The cell debris were re-extracted two times with 8 mL of methanol and twice with 8 mL of methyl tert-butyl ether (MTBE; Carlo Erba) until complete bleaching of the biomass was obtained. The extracts were combined and subsequently evaporated to dryness under vacuum at 55 °C using a Büchi Rotavapor (BUCHI Laborteknik AG, Flawil, Switzerland). The residue was dissolved in 1 mL methanol/MTBE (1:1, v/v), filtered through Milllex-GV 0.2-μm hydrophilic membrane filter (Millipore, Molsheim, France), and stored at −20 °C in an amber vial prior to HPLC analysis.

2.3. Chromatographic analysis

Analyses were carried out using a Nexera liquid chromatography (LC) system (Shimadzu, Milan, Italy), consisting of a CBM-20A™ controller, two LC-30AD™ dual-plunger parallel-flow pumps, a DGU-20 A5™ degasser, a CTO-30A™ column oven, and an SIL-30A™ autosampler. A 0.1 mm internal diameter stainless steel tubing (zero dead volume) was employed for column connection. The LC system was coupled to a liquid chromatography-mass spectrometry ion trap time-of-flight (LCMS-IT-TOF) mass spectrometer (MS) through an atmospheric-pressure chemical ionisation (APCI) source operating in both positive and negative mode (Shimadzu, Kyoto, Japan). Data acquisition was carried out by means of the LCMS solution software (Version 3.50.346; Shimadzu).

Chromatographic separation was achieved on Ascentis Express Fused-core C18 columns, 150 × 4.6 mm i.d., 2.7 μm d.p., kindly donated by Supelco/Sigma–Aldrich (Bellevonte, PA, USA). The mobile phases consisted of methanol (elucent A) and methyl tert-butyl ether (elucent B). Elution from the column was isocratically carried out from 100% (A) for 25 min then a gradient was established over the next 45 min with (B) to a final ratio of 80% (A:B), followed by a further elution for 15 min with 70:30 (A:B). The column was then returned to the initial conditions and equilibrated over 20 min. The flow rate was 1 mL min⁻¹ and the injection volume was 20 μL. The UV–visible (UV–vis) spectra were
acquired in the range of 250–600 nm, while the chromatograms were extracted at 440 nm.

LCMS-IT-TOF MS detection parameters were as follows: detector voltage, 1.50 kV; interface temperature, 400 °C; curved desolvation line temperature, 250 °C; block heater temperature, 230 °C; nebulising gas flow (N₂), 2.5 L min⁻¹; ion accumulation time, 30 msec; full scan range, 300–800 m/z; event time, 300 ms; repeat, 3; automatic sensitivity control, 70%. For MS/MS, full scan range, 50–800 m/z; ion accumulation time, 30 ms; collision induced dissociation energy: 50%. Samples were analysed in triplicate. Carotenoids were identified by their UV-vis spectra, including spectral fine structure, and MS spectra recorded in both positive and negative APCI ionisation modes, considering the respective elution order.

3. Results

The carotenoid extracts obtained from the cheese ripening bacteria *A. arilaitensis*, Po102 and Stp101, had chromatographic profiles which were similar to those of 14 other *A. arilaitensis* strains previously investigated (Sutthiwong & Dufossé, 2014). A typical chromatogram (Fig. 1A) recorded at 440 nm displayed two major groups of peaks according to the elution time. The first group contains peak 1, eluting at 9.79 min, then peak 2 eluting approximately 1 min later (Fig. 1A). Peaks 3 and 4 (Fig. 1A) represent the second group, eluted between 16.5 and 17.5 min.

The main carotenoids were identified as all-*E*-decaprenoxanthin (peak 1, Fig. 1A), all-*E*-sarcinaxanthin (peak 2, Fig. 1A), 9-*Z*-decaprenoxanthin (peak 3, Fig. 1A) and 15-*Z*-decaprenoxanthin (peak 4, Fig. 1A). These molecules have been characterised by both their UV-vis spectra and MS spectra, both in APCI (−) and (+) ionisation modes. Fig. 1 also shows the photodiode array detector (PDA) UV-vis spectra of the identified peaks (Fig. 1B,C,D,E). Interestingly, there is a small hypsochromic shift in λ max of 3–7 nm for the cis isomers compared with the trans isomer for decaprenoxanthin. The appearance of a typical cis absorption band at around 142 nm below the longest-wavelength absorption maximum was also observed for the 9-*Z*-decaprenoxanthin (at 318 nm) and for 15-*Z*-decaprenoxanthin (at 320 nm), which correspond to peaks 3 and 4 respectively. Moreover, the cis band for the 15-*Z*-decaprenoxanthin isomer showed a greater intensity being nearer to the centre of the molecule and also showed a double cis-peak appearance, i.e., with two maxima, characteristic of a chromophore bearing a long aliphatic moiety.

The mass spectra of the two principal types of carotenoids biosynthesised by *A. arilaitensis*, i.e., decaprenoxanthin and sarcinaxanthin (shown as 1 and 2, respectively, in Table 1) in APCI (−) and APCI (+) ionisation modes, are reported in Fig. 2.
Another interesting result of the present study is the concomitant presence of decaprenoxanthin and derivatives (carotenoids with an \( \varepsilon \) ring) and sarcinaxanthin and derivative (carotenoids with a \( \gamma \) ring). To the best of our knowledge, this is the first time that the simultaneous presence of these two C50 cycles is described in a non-genetically engineered bacterium. Up to now, sarcinaxanthin biosynthesis was described in \textit{M. luteus} (Netzer et al., 2010) and decaprenoxanthin biosynthesis in \textit{C. glutamicum} (Heider, Peters-Wendisch, & Wendisch, 2012; Krubasik et al., 2001).

The whole genome sequencing of the strain originating from cheese, \textit{Arthrobacter arilaitensis} Re117, reveals the presence of genes encoding the following proteins: Idi, CrtE, CrtB, CrtI, CrtEb, CrtYe and CrtYf, whose counterparts in \textit{C. glutamicum} (from 44 to 54% identity) catalyse the production of decaprenoxanthin from lycopene (Monnet et al., 2010). Cyclases, such as CrtYeYf, are key enzymes of the C50 carotenoid biosynthesis pathway and have been investigated using molecular biology (Netzer et al., 2010). The \textit{M. luteus} CrtYgYh polyketides constitute a \( \gamma \)-cyclase, which specifically converts flavoxanthin into sarcinaxanthin. Interestingly, it was shown that the \textit{C. glutamicum} CrtYeYf cyclase not only converts flavoxanthin into decaprenoxanthin. Indeed, when expressed in \textit{Escherichia coli}, CrtYeYf also catalyses the synthesis of sarcinaxanthin and sarprenoxanthin (Netzer et al., 2010). Whether these additional reactions may also occur during the growth of \textit{C. glutamicum} has not been established. However, we may hypothesise that the simultaneous production of decaprenoxanthin and sarcinaxanthin in \textit{A. arilaitensis} cells could be due to multiple catabolic activities of its CrtYeYf cyclase.

**4. Discussion**

In this study, the pigments extracted from the biomass of \textit{A. arilaitensis} strains originating from cheese were characterised by an HPLC-PDA-APCI-MS methodology. According to the data of the UV–vis spectra, the mass spectra both in APCI (–) and APCI (+) ionisation modes, and the elution order, \textit{A. arilaitensis} produces eight \( C_{50} \) carotenoids. It produces mainly free-form (non-glycosylated) all-\( E \)-decaprenoxanthin (carotenoid with \( \varepsilon \) ring), and two of the \textit{cis} isomers, 9-\( Z \)-decaprenoxanthin and 15-\( Z \)-decaprenoxanthin, in minor amounts. Decaprenoxanthin is also produced as mono- and diglucosides, and as an ester with palmitic acid as the acid moiety.

9-\( Z \)-decaprenoxanthin and 15-\( Z \)-decaprenoxanthin isomers are always in the same proportions within these bacterial pigmented extracts, as analysed in our laboratory over many years, representing over 100 liquid chromatography injections, even before the official description of the new species \textit{A. arilaitensis} was made in the literature by Irlinger et al. (2005), or the in-depth analysis of the chemical structures of the pigments carried out in the present study. Contrary to the many conclusions found in the literature, \textit{Z} isomers of carotenoids (as observed in this study) are not artefacts. Indeed, these isomers are naturally produced, as shown by Melendez-Martinez, Stinco, Liu, and Wang (2013).
Fig. 2. The mass spectra of decaprenoxanthin and sarcinaxanthin in atmospheric-pressure chemical ionisation (APCI) negative (−) and APCI positive (+) ionisation modes. (A) decaprenoxanthin in APCI negative (−) mode, (B) decaprenoxanthin in APCI positive (+) mode, (C) sarcinaxanthin in APCI negative (−) mode, (D) sarcinaxanthin in APCI positive (+) mode.
species, *A. arilaitensis*, may promote greater acceptability for ingredients extracted from food-grade bacteria, which may lead to an increase in the panel of carotenoids available as food colorants, as well as for other purposes, such as the application of C50 carotenoids in light protecting cosmetics and sunscreens, as these pigments were previously described to be effective UV and visible light filters.

**5. Conclusions**

The pigments extracted from the biomass of cheese-originating *A. arilaitensis* strains were characterised by an HPLC-PDA-APCI-MS methodology. According to the data of the UV–vis spectra, the mass spectra both in APCI (−) and APCI (+) ionisation modes, and the elution order, *A. arilaitensis* produces eight C50 carotenoids. It
produces mainly free-form (non-glycosylated) all-ß-decaprenoxanthin (carotenoid with an ß ring), and in minor amounts, two of the cis isomers, 9-Z-decaprenoxanthin and 15-Z-decaprenoxanthin. Decaprenoxanthin is also produced as mono- and diglucosides, and as an ester with palmitic acid as the acid moiety. One interesting result of the present study is the concomitant presence of decaprenoxanthin and derivatives (carotenoids with an ß ring) and sarnicaxanthin and derivative (carotenoids with a γ ring). To the best of our knowledge, this is the first time that the simultaneous presence of these two C50 cycles is described in a non-genetically engineered bacterium.

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References


Bockelmann, W., & Hoppe-Seyler, T. (2001). The surface flora of bacteria smear-


tation to the surface cheese. PLoS ONE, 5, e15489.


Mounier, J., Goerges, S., Gelsomino, R., Vancanneyt, M., Vandeneulverbroecke, K., Hoste, B., et al. (2006). Sources of the adventitious microflora of a smear-


