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1 **First isolation of *Brevibacterium* sp. pigments in the rind of an industrial**
2 **red-smear ripened soft cheese**

3

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26

1

2 **Abstract**

3

4 The smear ripened soft cheeses are characterized by a surface orange-red-brown color which
5 has a microbial origin. For a long time this coloration was mainly imputed to *Brevibacterium*
6 *linens*. However the latest published works, based on molecular biology, have shown a minor
7 role for this bacterium. The present study shows the results obtained with an industrial cheese
8 named Vieux-Pané which is characteristic due to the presence of carotenoids from
9 *Brevibacterium linens* group at its surface. This demonstrates that, under certain conditions,
10 *Brevibacterium linens* group (*Brevibacterium linens* and *Brevibacterium aurantiacum* sp.
11 nov.) is able to produce pigments and to colour effectively cheeses.

12

13 **Keywords** *Brevibacterium linens*, Carotenoid, Isorenieratene, Cheese rind, Color.

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1 INTRODUCTION

2

3 A previous study from our laboratory presented an extraction method for the analysis of
4 pigments in the cheese rinds (Galaup *et al.* 2005). This method was then applied to high
5 quality red-smear soft cheeses such as those under the “Protected Designation of Origin
6 (PDO)” legislation and the first pigment fingerprints of such cheeses were obtained (Galaup
7 *et al.* 2005). It has also shown that the origin of pigments seems essentially related to the
8 presence of yellow bacteria such as *Arthrobacter* or *Microbacterium* species (Galaup *et al.*
9 2007), the pigments of *Brevibacterium linens* group being only present at trace amounts on
10 Maroilles (Guyomarc’h *et al.* 2000a) [**Supplementary materials 1&2**]. All these studies
11 seem to confirm the minor role of *Brevibacterium linens* group in cheese colouring.

12 We have recently applied this method to different industrial cheeses, and this paper shows the
13 pigment fingerprint of the rind of Vieux-Pané produced in France, which is characterized by
14 the predominant presence of pigments from *Brevibacterim linens* group.

15

16 MATERIALS AND METHODS

17 Bacterial strains, culture media, cheeses, extraction of pigments and HPLC conditions were
18 described in previous publications (Guyomarc’h *et al.* 2000b; Galaup *et al.* 2005).
19 [**Supplementary material 3**].

20

21 RESULTS AND DISCUSSION

22

23 **HPLC profiles of the *Brevibacterium linens* extracts**

24 For all strains investigated (*Brevibacterium linens* and *Brevibacterium aurantiacum* sp. nov.),
25 the color reaction of biomass or methanol extracts in the presence of alkali is positive. It is

1 due to the ionization of phenols into phenolates in both mono- and di-hydroxylated
2 isorenieratene derivatives (Britton *et al.* 1995). This reaction is characteristic of the pigments
3 of *Brevibacterium linens* group. Moreover, all strains (over one hundred strains tested up to
4 now in all of our experiments –including the 30 strains of the present work) have shown the
5 same typical chromatogram, even if some of these strains (e.g. ATCC 9175) are reclassified
6 as *Brevibacterium aurantiacum* (Gavrish *et al.* 2004). Even if it is possible to differentiate
7 these two species by molecular biology methods, they show the same pigment fingerprint and
8 develop a carmine-red color in contact with strong alkali.

9 The chromatographic profile of *Brevibacterium linens* or *Brevibacterium aurantiacum*
10 (Figure 1A) consisted of a series of three groups of peaks (Guyomarc'h *et al.* 2000b). The
11 first group (I) was eluted between 15 and 22 minutes. A main peak elutes between (15-17
12 min) followed at 1.5 min by a smaller one. Between 17 and 18 minutes, 2 other peaks were
13 eluted. The second group (II) consisted of a series of small peaks eluted between 30 and 45
14 minutes. The last group of peaks (III), slightly detected was eluted between 70 and 80 min.
15 The three groups of peaks were identified as respectively related to 3,3'-dihydroxy-
16 isorenieratene (group I), 3-hydroxy-isorenieratene (group II) and isorenieratene (group III).
17 On synthetic medium, *Brevibacterium linens* produced mainly the 3,3'-dihydroxy-
18 isorenieratene. The groups representing the 3-monohydroxy and isorenieratene were present
19 only as traces.

20

21 (insert Figure 1)

22

23 By the study of UV/visible spectrum, more information could be obtained such as *cis/trans*
24 isomerisation (Schieber and Carle 2005) or hydroxylation. The identification of the *cis*
25 compounds was possible due to their characteristic 'cis-peak' (320-380 nm, see

1 **Supplementary material 4**). Moreover, when a compound is hydroxylated, it becomes more
2 polar and presents a more rounded UV/visible spectrum.

3 The compounds which are eluted in each group seem to be isomers of the same molecule.
4 Indeed, like in the case of lycopene (Chasse *et al.* 2001; Ishida *et al.* 2001), a quite high
5 number of *cis/trans* isomers of isorenieratene, mono-hydroxy-isorenieratene and di-hydroxy-
6 isorenieratene could be obtained, based on the position of the isomerisation (Figure 2, *cis*
7 isomers of 3-3'-dihydroxy-isorenieratene).

8

9 (insert Figure 2)

10

11 **HPLC profiles of the cheese extracts**

12 The profile presents the three distinct groups of pigments very characteristic of
13 *Brevibacterium linens* group (Figure 1B). A first group of pigments was eluted between 12
14 and 18 minutes (group I); including dihydroxy-isorenieratene and its isomers (*all-trans*
15 molecule and various *cis* isomers). A second group (II) was characterized by a very important
16 set of compounds eluted between 30 and 45 minutes and corresponded to 3-mono-hydroxy-
17 isorenieratene and its isomers. The third group consisted of some isorenieratene isomers. Due
18 to this molecule which is not commercially available, the identification was made with
19 isorenieratene chemically synthesized in our laboratory (Valla *et al.* 2007).

20

21 **Comparison between the two profiles**

22 The profiles obtained in the present study on cheeses showed a strong similarity with pigment
23 profiles of *Brevibacterium linens* extracts (Figure 1), with three groups of pigments (groups I,
24 II and III), of variable polarity (Guyomarc'h *et al.* 2000a). That was confirmed by the analysis
25 of the absorption spectra in UV/visible and of times of retention. The similarity between the

1 group of 3,3'-dihydroxy-isorenieratene (RT from 12 to 18 min) present in synthetic medium
2 and cheese matrix was clear. Even if the concentrations in pigments of groups II and III were
3 very different among the 2 profiles, the molecules were identical. The pigments extracted
4 from the cheese rind represented all isomers of isorenieratene and hydroxyl derivatives
5 described in *Brevibacterium linens* by Kohl *et al.* (Kohl *et al.* 1983).

6 Pigments of the groups II and group III are not generally detected at this high level of
7 concentration in the extracts of pigments resulting from *Brevibacterium linens* cultivated in
8 synthetic media. It was thus the first time that the three groups of pigment with as much of
9 intensity and resolution are detected.

10 The presence of these molecules on the rind of cheeses raises some questions about their
11 production. Was the production of these molecules induced by factors (e.g biotic, physico-
12 chemical...) which are not present or effective on synthetic culture medium? Was an
13 interaction with other microorganisms (e.g yeasts, bacteria) necessary for the production of
14 substantial amounts of mono-hydroxy-isorenieratene and isorenieratene? In the literature the
15 study of the production of pigments from this bacterium was primarily and mainly conducted
16 with synthetic culture media. In the future, it would be judicious to use complex and/or dairy
17 media reflecting more the composition of a cheese, such as curd models or “cheese models”
18 used by Leclercq-Perlat *et al.* (Leclercq-Perlat *et al.* 2004).

19 Latest studies concerning the microflora of red-smear ripened soft cheeses showed a weak
20 presence of *Brevibacterium linens* group. Bockelmann (Bockelmann 2002) reported that
21 *Arthrobacter nicotianae*, pigmented in yellow, accounts for 5 to 10% of the bacteria isolated
22 from Tilsit whereas *Brevibacterium linens* accounts for 0.1 to 10% (Bockelmann *et al.* 2005).
23 In Comté, less than 1% of the total flora consists of *Brevibacterium linens* (Bockelmann *et al.*
24 2005). The 16S DNA sequences coding for *Brevibacterium linens* are almost not detected at
25 the end of the ripening of red-smear ripened soft cheeses, the bacteria present being especially

1 species of *Arthrobacter* (Feurer *et al.* 2004). In French PDO red smear cheeses, the
2 *Brevibacterium linens* pigment fingerprint was only slightly detected on the Maroilles cheese
3 (Galaup *et al.* 2007). With all these various works and results, the tendency was to minimize
4 the role of *Brevibacterium linens* to the profit of other microorganisms (Goerges *et al.* 2008;
5 Mounier *et al.* 2006).

6 Since 1997, Bockelmann and his team have studied which bacterium could lead to the
7 complete formation of cheese models close to Tilsit from a point of view of odor and color.
8 When the sole presence of *Brevibacterium linens* produced imperfect cheeses, the
9 combination of *Brevibacterium linens* with *Arthrobacter* sp. led to acceptable cheeses. More
10 complex combinations between *Debaryomyces hansenii*, *Arthrobacter* sp. (yellow strain) and
11 *Staphylococcus* sp. (pigmented and not pigmented strain) could give color and odor similar to
12 those of Tilsit (Bockelmann 2002; Bockelmann *et al.* 2005). The whole of this work led to an
13 effective cocktail of microorganisms for the ripening of Tilsit containing 5 species:
14 *Debaryomyces hansenii*, *Brevibacterium linens*, *Staphylococcus equorum* (*S. sciuri*),
15 *Corynebacterium ammoniagenes*, and *Arthrobacter nicotianae* (Bockelmann 2002;
16 Bockelmann *et al.* 2005).

17 Our study, in the case of this French industrial cheese Vieux-Pané, demonstrated that a strain
18 from *Brevibacterium linens* group was able to produce pigments and impart color to a cheese
19 with efficacy. The biodiversity of the bacterial flora on the surface of cheese is important
20 (Brennan *et al.* 2002; Bokulich and Mills 2013). In this case of industrial cheeses (Gori *et al.*
21 2013; Feligini *et al.* 2012), the composition of the various microorganisms seeded during the
22 process is not known and is confidential. It may occur that *Brevibacterium linens* did not have
23 to undergo competition with other microorganisms and that its development at the cheese
24 surface could happen in an optimal way.

25

1 CONCLUSIONS

2 The weak occurrence of *Brevibacterium linens* and *Brevibacterium aurantiacum* sp. nov. at
3 the surface of red-smear ripened soft cheeses has been shown in previous studies and the role
4 of these bacteria on the development of cheese color has been minimized. In fact, cheese rind
5 coloration is a complex process and our results showed that this group of bacteria, under
6 certain conditions, was quite able to give color to cheese surface. So, the role of
7 *Brevibacterium linens* group is not negligible.

8 Future research would explain why this group of bacteria (always present at the earliest stage
9 of ripening) produced pigments on certain cheeses and not on others.

10

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12

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19 these fascinating chemical compounds.

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23

24

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1 **List of captions**

2

3 **Figure 1** Chromatographic profile of pigments from *Brevibacterium aurantiacum* ATCC
4 9175 methanolic extract (A) and chromatographic profile of pigments extracted from the rind
5 of Vieux-Pané red-smear soft cheese (B).

6

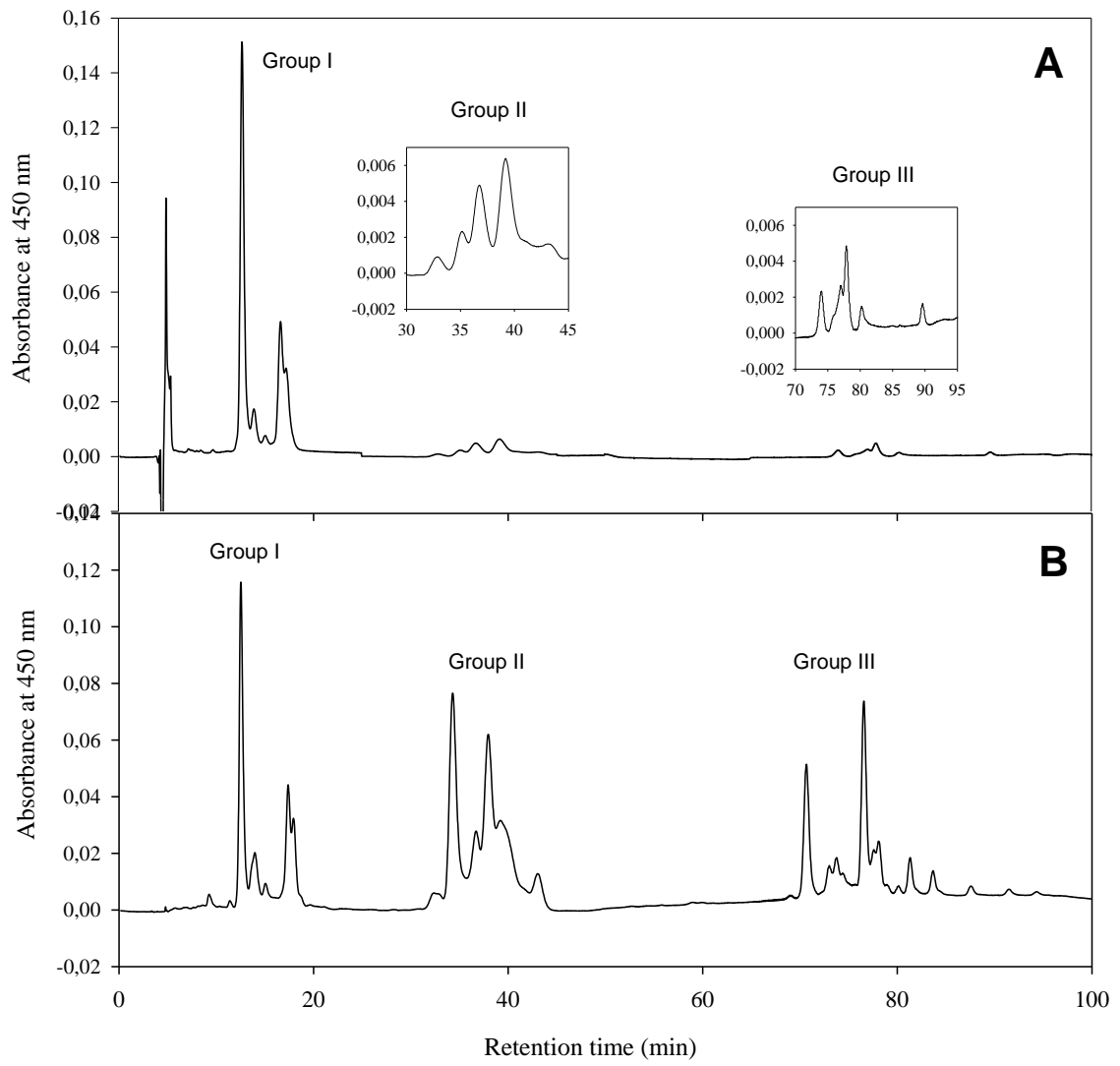
7

8 **Figure 2** Isomers of 3,3'-dihydroxy-isorenieratene, concerning only one isomerisation of the
9 ethylenic linkage (the most probable), i.e. the *all* trans isomer and the five *cis* isomers that
10 could occur. *Cis* isomers are named from A to E, starting from the middle of the molecule. *
11 indicates a steric hindrance. Similar isomers could be described for non hydroxylated
12 isorenieratene and 3-hydroxy-isorenieratene.

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Figure 1

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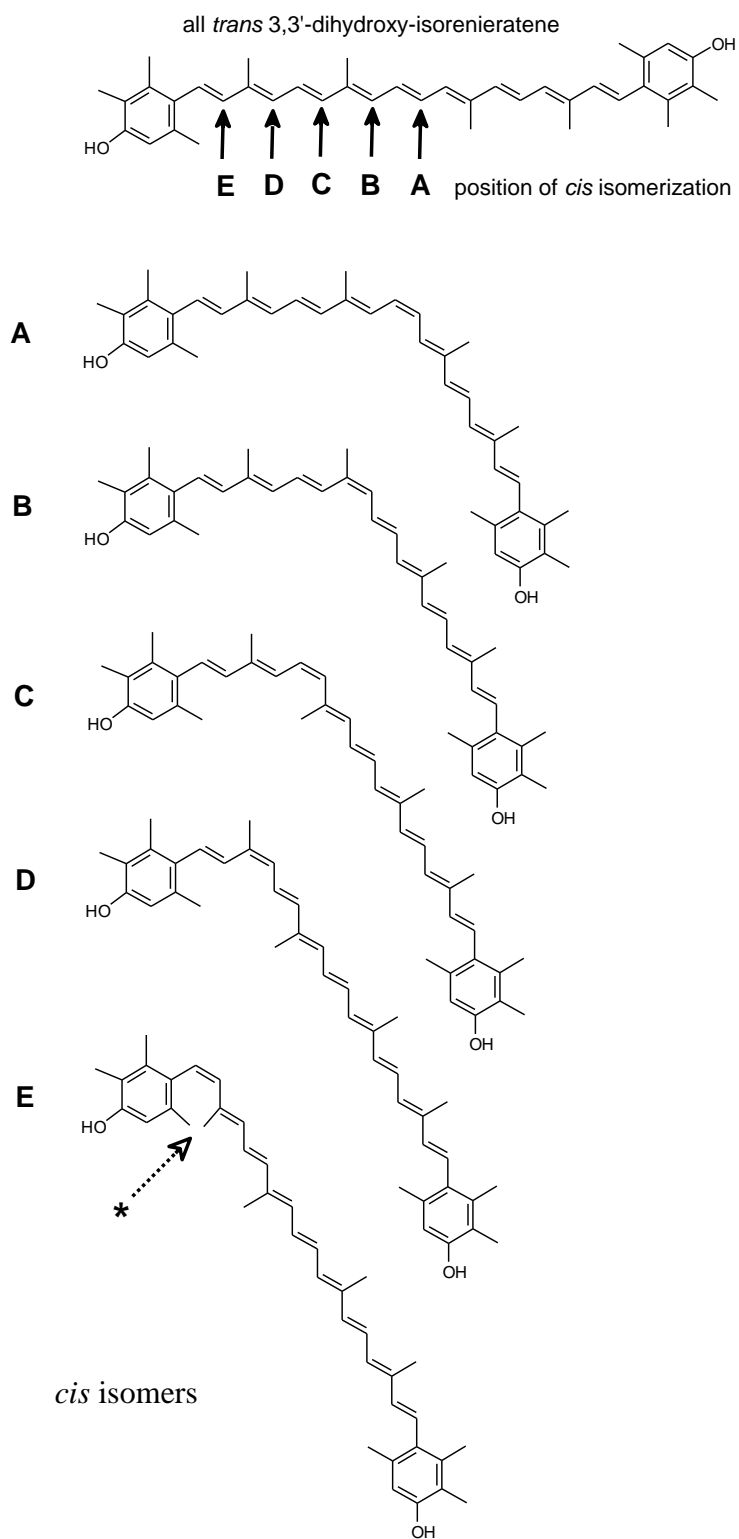


Figure 2

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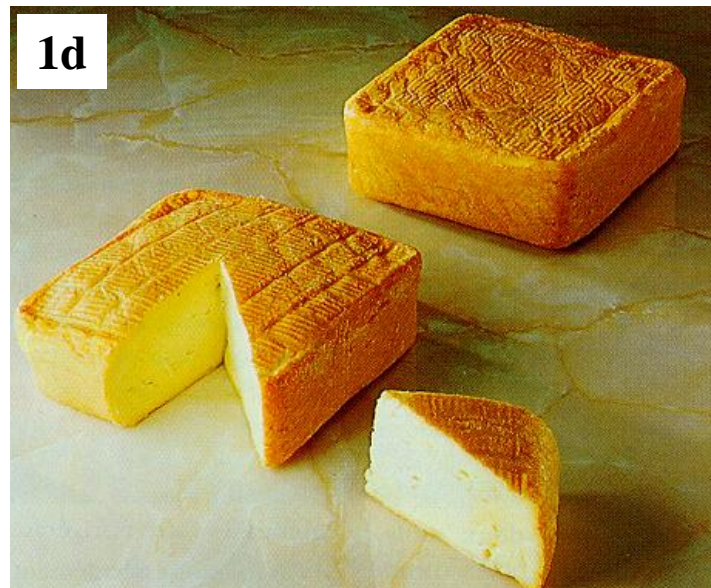
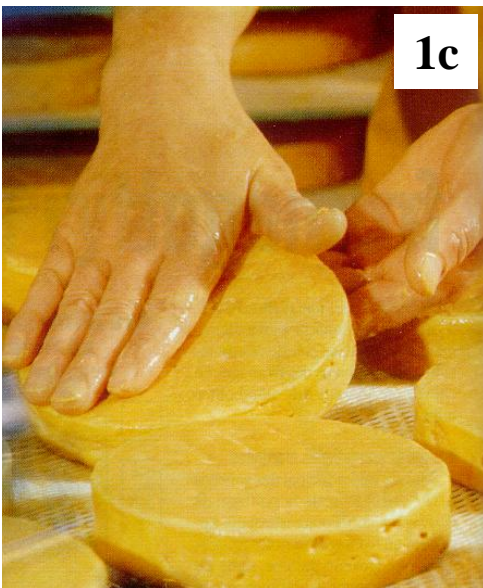
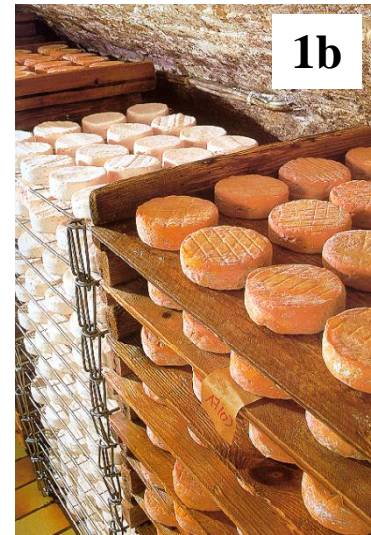
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1 **Supplementary material 1**

2
3 **Smear-cheeses with rinds colored by a bacterial microflora. 1a. Livarot, 1b. Rollot in the**
4 **ripening cellar, 1c. Hand-made washing of Munster, 1d. Maroilles.**



22 The appearance of foodstuffs, of cheeses is one of the only permitted ways to appreciate on-
23 sell food products (Aydemir and Dervisoglu 2010; Fresno and Alvarez 2012). For so-called
24 red-smear cheeses like Maroilles, Munster, Livarot, Epoisses, Limburger, Herve, Gubbeen,
25 Taleggio or Tilsit, a good aspect is mostly characterized by the occurrence of an
26 homogeneous orange-red-brown, sticky surface (above pictures).

1 Except for artificially colored cheeses, the color is due to pigments produced by the cheese
2 microflora during ripening. For a long time the coloration of red-smear ripened soft cheeses
3 was only imputed to *Brevibacterium linens*. The pigments produced by *Brevibacterium linens*
4 were first identified as aromatic carotenoids (Supplementary material 2) (Kohl *et al.* 1983),
5 and later characterized within the L*a*b* color system (Guyomarc'h *et al.* 2000; Dufossé *et*
6 *al.* 2001). In fact, cheese rind coloration is a complex process involving physical and
7 chemical parameters, such as temperature, dissolved oxygen, pH, culture medium, lightness,
8 and/or biotic interactions, all important for bacterial development and pigment production
9 (Irlinger and Mounier 2009). Studies have shown that the ripening of red-smear ripened soft
10 cheeses progresses through the succession of microbial communities on the cheese surface
11 (Mounier *et al.* 2009). This surface microflora, named the smear, is mainly composed of yeasts
12 and surface bacteria. First, from the earliest hours of cheese-making and during the first six
13 days of ripening, the yeasts make up the main species and *Debaryomyces hansenii* grow by
14 consuming lactose and lactate at the same time but with different specific consumption rates
15 (Leclercq-Perlat *et al.* 2000). Moreover, they produce a lot of compounds such as aroma
16 compounds and growth factors essential for bacterial development as supported by Corsetti *et*
17 *al.* (Corsetti *et al.* 2001). Through this ripening period (day 0 – day 6), yeasts deacidify the
18 surface and when pH increases to a value higher than 6, surface bacteria begin to grow and
19 eventually cover the entire cheese surface, forming the main population at the end of ripening.
20 However, some bacterial strains such as *Arthrobacter* sp. can grow at a lower pH (5.4). The
21 surface bacterial composition depends on the smear cheeses under study. These bacteria
22 belong to *Arthrobacter* sp., *Brevibacterium* sp., *Corynebacterium* sp., *Microbacterium* sp.,
23 *Micrococcus* sp., and *Rhodococcus* sp. (Bockelmann 2002). Even if *Brevibacterium linens* is
24 considered as the main pigmentation and flavouring bacterium for this type of cheeses, it does
25 not form the main bacterial species of the smear throughout the ripening. Bockelmann *et al.*

1 (Bockelmann *et al.* 2005) have also shown that the pigmentation of the smear is due to some
2 interactions between surface bacteria and the matrix. Interactions between the yeast
3 *Debaryomyces hansenii* and coryneform bacteria were shown to influence cheese surface
4 pigmentation (Masoud and Jakobsen 2003; Leclercq-Perlat *et al.* 2004). Among these
5 bacteria, the well-known bacterium species on the smear are belonging to the *Brevibacterium*
6 *linens* group (Gavrish *et al.* 2004), and the species essentially isolated from the smear cheeses
7 are *Brevibacterium linens* and *Brevibacterium aurantiacum*.

8 Considering the small content of *Brevibacterium linens* group in the smear, Bockelmann
9 (Bockelmann 2002) expressed reserves that *Brevibacterium linens* alone would contribute to
10 the rind coloration. Using cheese model system, they first reported about the importance of
11 *Brevibacterium linens* interactions with other bacteria, such as *Arthrobacter* sp. in the
12 development of cheese coloration (Bockelmann *et al.* 2005). More recently, it was shown by
13 molecular biology methods that in some types of red-smear ripened soft cheeses,
14 *Brevibacterium linens* was not even found at the end of the ripening process (Feurer *et al.*
15 2004).

16
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2 **Supplementary material 2**

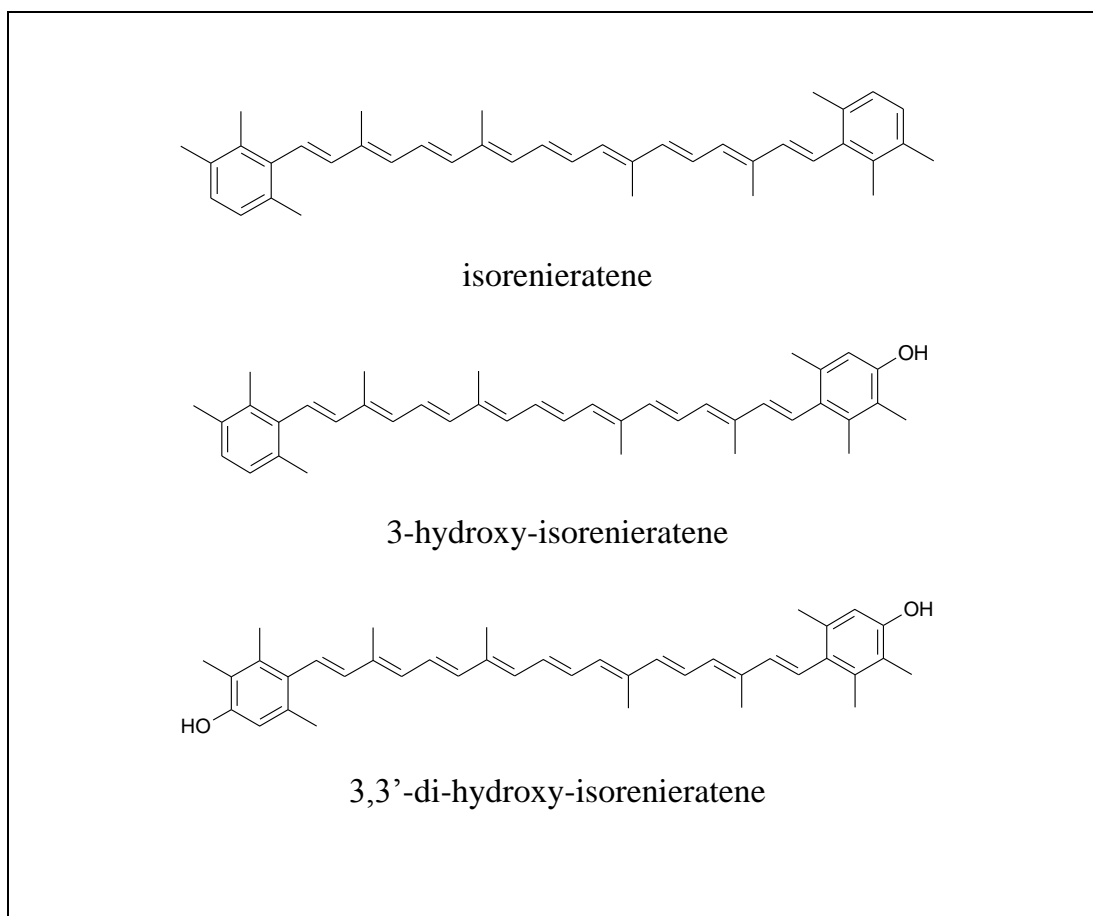
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5 **Aromatic carotenoids described in *Brevibacterium linens* according to Kohl *et al.* (1983).**

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11 aromatic carotenoids. *Phytochemistry* **22** 207-210.

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2 **Supplementary material 3**

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4 **Material and Methods**

5 ***Brevibacterium linens* analysis**

6 *Bacterial strains and culture medium*

7 A collection of 30 strains of *Brevibacterium linens* group was used, consisting of references
8 strains, industrial strains and strains isolated from the French red-smear cheeses' rinds.
9 Strains ATCC 9172 (*B. linens* reference strain), ATCC 9175 (*B. aurantiacum* reference
10 strain) and ATCC 19391 were obtained from the American Type Culture Collection
11 (Manassas, VA, USA) and NRRL B-4210 was obtained from the Northern Regional Research
12 Laboratory (Peoria, IL, USA).

13 Cultures were grown in 250 mL Erlenmeyer flasks containing 50 mL of a medium composed
14 of 20 g/L D-glucose (Carlo Erba), 5 g/L casamino acids (Difco), 1 g/L yeast extract (Biokar),
15 5 g/L NaCl and 1 g/L KH₂PO₄. The pH was adjusted to 6.9 and the medium was heat-
16 sterilized at 121°C, 15 min. Flasks were inoculated with 1% of 72 hour-old preculture (v/v)
17 (25°C; 150 rpm), and incubated at 25°C for 4 days with stirring (150 rpm) to oxygenate the
18 medium.

19

20 *Extraction of pigments from bacteria*

21 Extracts were obtained from 20 mL of culture. Cells were first centrifuged at 6,000 g for 15
22 min. The supernatant was discarded and the cell aggregate was rinsed with 5 mL deionized
23 water, vortexed and centrifuged at 6,000 g for 15 min. The cell aggregate was then mixed
24 with 8 mL methanol, blended to prevent clotting, and extracted with constant agitation (50
25 rpm), protected from direct light with aluminium foil, until cells were bleached (within 2
26 hours). The sample was then centrifuged (6,000 g, 15 min), the cell aggregate was discarded

1 and the supernatant further centrifuged (10,000 g for 15 min). The resulting supernatant, i.e.
2 methanol extract, was then injected in the HPLC system.

3

4 *Biomass reaction with strong alkali*

5 The pigment of *Brevibacterium linens*, located in the cell membrane, reacted with strong
6 alkali, leading to a deep pink-red color. This is explained by the ionization of phenolic rings at
7 high pH, giving a subsequent bathochromic shift and seems to be specific to the diol
8 molecule. Extracted biomass was laid out on an inert white surface (i.e. Whatman paper), and
9 a few drops of 5 M NaOH were added. The test was positive if a characteristic carmine-red
10 color appeared.

11

12 *HPLC analysis of the pigments extracted from the microbial biomass*

13 Methanol extracts were evaporated to dry under reduced pressure at 75°C in a Büchi
14 rotavapor, within 5 minutes. Dry pigment extracts were dissolved in 1 mL methanol, filtered
15 through Millex-GV 0.2-µm hydrophilic membrane (Millipore), and injected (20 µL) onto a
16 LichroCART 250-4 RP-18 (250 x 4 mm, 5-µm particle size) column (Merck). The HPLC
17 apparatus consisted of Waters 600 constant flow pump and controller, and Waters 996
18 photodiode array detector (detection between 300 and 600 nm). Separation was achieved
19 using reverse phase HPLC at a flow rate of 0.5 mL/min. Solvents and conditions used for
20 separation were as follows: 0 to 45 min, 100% methanol; 45 to 80 min, 100% methanol to
21 80% methanol/20% chloroform; 80 to 130 min, 80% methanol/20% chloroform.

22

23 **Cheese analyses**

24 *Cheeses*

25 Vieux-Pané cheeses were purchased in retail outlets (at least 3 cheeses from the same batch).

26 At reception, cheeses were placed into polyethylene bags and stored at -20°C.

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Alkaline hydrolysis and extraction of pigments from the cheese rind

The carotenoid extraction of milk and its derivatives is very difficult because this matrix has a high lipidic content (Oliver and Palou 2000). Alkaline hydrolysis of the dairy material and pigment extraction were adapted from Ollilainen *et al.* (Ollilainen *et al.* 1989). Prior to pigment extraction, cheeses were thawed in the dark at 4°C. The smear was collected by scraping the surface of the cheese, weighed, and hydrolysed in 40 mL of concentrated KOH solution (100 g KOH dissolved in 100 mL water), 40 mL water supplemented with 1 g ascorbic acid, and 100 mL ethanol for 16 hours in the dark at 4°C. No adverse effect of alkaline hydrolysis on pigment contents and quality was observed. The hydrolysate was transferred into a 500 mL separatory funnel. The flask used for hydrolysis was rinsed with 100 mL of 10% NaCl. One hundred mL of hexane:diethyl ether (70:30) were added and the funnel was hand shaken for 5 min. The aqueous phase was re-extracted two times with 100 mL hexane-diethyl ether. The emulsion was carefully broken with few drops of ethanol. The pooled organic phases were evaporated to dry with a Büchi rotavapor on a water bath, set at 40°C. The residue was reconstituted in 1.5 mL methanol:chloroform (80:20) for pigment quantification and HPLC analysis.

HPLC analysis of pigments extracted from the cheese rind

The pigment extracts resulting from cheeses are analyzed under the same conditions as the pigment extracts resulting from the bacterial cultures.

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2 **Supplementary material 4**

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5 **UV-visible spectra of 3,3'-dihydroxy-isorenieratene (a: all-*trans* isomer, a': *cis* isomer),**

6 **3-hydroxyisorenieratene (b: all-*trans* isomer, b': *cis* isomer), and isorenieratene (c: all-**

7 ***trans* isomer, c': *cis* isomer). Arrows indicate the peak in the 320-380 nm range, specific**

8 **for *cis* rearrangement.**

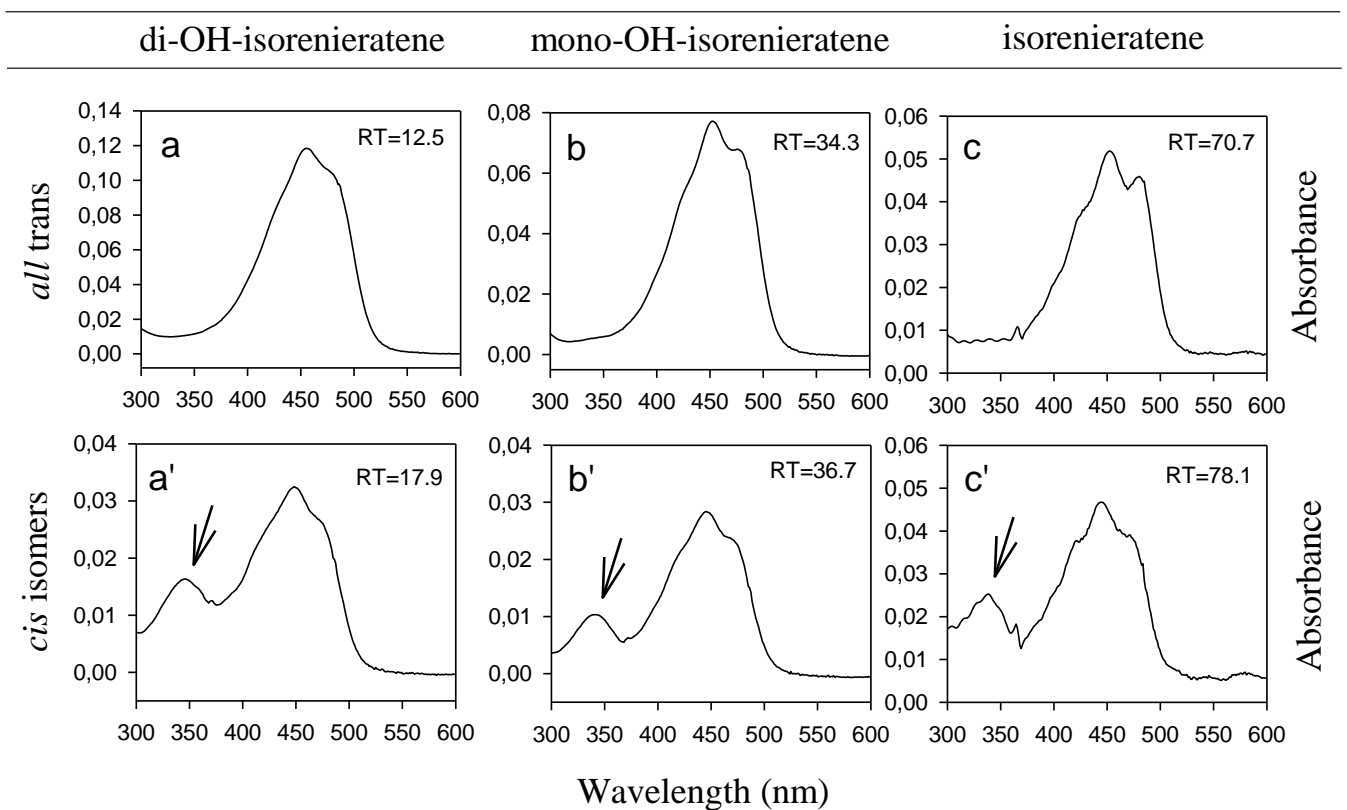
9 In neutral conditions, the phenolic hydroxyl groups in dihydroxy-isorenieratene have little

10 effect on the spectrum (isorenieratene λ_{\max} 426; 448; 475 – 3,3'-dihydroxy-isorenieratene

11 λ_{\max} 427; 449; 476). In basic conditions, ionization causes a substantial bathochromic shift,

12 from orange to carmine-red.

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