Carotenoid production in *Lactobacillus plantarum*

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Abstract

Eighteen strains of *Lactobacillus plantarum* from different origins were screened for carotenoid production, as many of them exhibited a deep yellow pigmentation when cultured as isolated colonies on MRS-agar plates. We found that most of them produced significative amounts of the yellow C_{30} carotenoid 4,4'-diaponeurosporene in the range 1.8 to 54 mg/kg of dry cell weight. Although some of the strains produced just trace amounts of this carotenoid, PCR studies showed that all of them harbored the genes *crtM* and *crtN* which, inferred from homology, had been predicted in the three *L. plantarum* complete genome sequences currently available. Our results suggest the full functionality of a C_{30} carotenoid biosynthesis pathway in this species, driven by the operon *crtNM*. DNA sequencing of the entire *crtNM* operon in the maximum carotenoid-producing strain found in this study, i.e. *L. plantarum* CECT7531, was accomplished. Genes *crtM* and *crtN* were annotated as dehydrosqualene synthase and dehydrosqualene desaturase, respectively, in this strain.

1. Introduction

Carotenoids are a group of colored terpenoids with antioxidant properties which are widespread in the plant and animal kingdoms, as well as in fungi and in photosynthetic and non-photosynthetic microorganisms (Weedon, 1971; Phadwal, 2005). In the later, although not essential for growing as heterotrophic organisms, they accomplish important biological functions. In Gram-positive bacteria, for instance, carotenoids play an important role in protecting from oxidative stress by scavenging free radicals with their conjugated double bonds (Clauditz et al., 2006). Also, it has been demonstrated a correlation between carotenoid production and decreased membrane fluidity, which provides resistance to oleic acid killing in *Staphylococcus aureus* (Chamberlain et al., 1991). In addition, carotenoids are used commercially as food colorants, animal feed supplements and, more recently, for nutraceuticals, cosmetical and pharmaceutical purposes (Lee and Schmidt-Dannert, 2002; Klein-Marcuschamer et al., 2007). A lot of effort has been made at selecting microorganisms that can provide a cost-effective source of carotenoids (Bhosale, 2004). At present, metabolic engineering of diverse microorganisms, including non-carotenogenic ones, is being exploited for the biotechnological production of large amounts of reasonably pure carotenoids as well as to synthesize novel carotenoid structures by using combinatorial and *in vitro* evolutionary strategies (Lee and Schmidt-Dannert, 2002; Umeno et al, 2005; Das et al. 2007; Klein-Marcuschamer et al., 2007; Wang et al., 2007).

Lactic acid bacteria (LAB) are Gram-positive, low-GC, microaerophilic, non-sporulating, rod or cocci that ferment sugars to produce primarily lactic acid and are associated by their common physiological characteristics (Makarova et al., 2006). These bacteria are historically linked with food and feed fermentations, and their industrial

66 importance is further evidenced by their Generally Regarded As Safe (GRAS) status
67 (Holzapfel et al., 1995). Among LAB, selected species of the *Lactobacillus* genus are
68 widely used as probiotics primarily in dairy products and dietary supplements (Reid,
69 1999; Ouwehand et al. 2002; Klaenhammer et al., 2005). One of these species,
70 *Lactobacillus plantarum,* is industrially important and is involved in many vegetable
71 fermentations (Buckenhüskes, 1997), as well as being a frequent inhabitant of the
72 human intestinal tract (Johansson et al., 1993) which is already being used as a probiotic
73 microorganism (Rodgers, 2008). In our laboratory, we have isolated a number of *L.*
74 *plantarum* strains from olive fermentations and used some of them as starter cultures
75 (Ruiz-Barba et al., 1994; Leal et al., 1998). Intrigued by the eye-catching deep yellow
76 pigmentation of some of these strains when growing as isolated colonies on MRS-agar
77 plates, we decided to search for the real nature of such coloring. Preliminary
78 characterization of some *L. plantarum* strains showed the involvement of carotenoid
79 compounds in the yellow pigmentation. Actually, Breithaupt *et al.* (2001) had described
80 one *L. plantarum* strain isolated from bakers yeast which was able to produce the
81 triterpenoid 4,4'-diaponeurosporene and its isomers, being the only report on the matter
82 up to date. In this report, we describe the widespread presence of carotenoid production
83 among *L. plantarum* strains from a wide variety of environments. This result was
84 reinforced by the finding of the genes *crtM* and *crtN*, first described in the annotated
85 complete genome sequence of *L. plantarum* WCFS1 as inferred by homology
86 (Kleerebezem et al., 2003), in all of the strains investigated. Our results suggest the
87 functionality of these genes in *L. plantarum*, although the nomenclature and function of
88 their predicted products should be updated.
89
90

2. Materials and methods

2.1. Bacterial strains, media and growth conditions

L. plantarum strains used in this study are described in Table 1. They were cultured in MRS agar (Oxoid, Basingstoke, UK) at 30 ºC. For preliminary optimization experiments, MRS broths from different sources were used, including the commercially available from Oxoid, Difco (Detroit, USA), Biokar (Beauvais, France), and Merck (Darmstadt, Germany). Also, for this purpose, two different defined media were used: DM1, containing, per litre, glucose (Panreac, 20 g), peptone (Difco, 10 g), beef extract (Oxoid, 8 g), yeast extract (Oxoid, 4 g), K_2HPO_4 (Fluka, 2 g), sodium acetate·$3H_2O$ (Fluka, 5 g), tri-ammonium citrate (Merck, 2 g), $MgSO_4 \cdot 7H_2O$ (Merck, 0.2 g), $MnSO_4 \cdot 4H_2O$ (Merck, 0.05 g), and Tween 80 (Sigma, 1 ml); and DM2, containing, per litre, glucose (Panreac, 22 g), yeast extract (Oxoid, 10 g), $(NH_4)_2HPO_4$ (Fluka, 2.5 g), $MgSO_4 \cdot 7H_2O$ (Merck, 0.05 g), $MnSO_4 \cdot H_2O$ (Merck, 0.005 g), and Tween 80 (Sigma, 0.2 ml). In all cases, pH was adjusted to 6.5 with 10N HCl and media were sterilised at 121 ºC, 1 atm, for 15 min.

2.2. Cell culture for carotenoid production and extraction

Five hundred ml of each of the MRS broths used were inoculated with a single colony of the *L. plantarum* strain to be tested and the culture was incubated for 24, 48, 72 or 96 h at 30 ºC without aeration. Cells were collected by centrifugation at 12,000 x *g*, at 4 ºC for 15 min, washed with sterile distilled water, and centrifuged again to obtain a pellet, which was submitted to lyophilization. Samples were stored at -20ºC until

116 carotenoid extraction and chromatographic analysis (within 24-48 h). Data obtained
117 were expressed in terms of dry cell weight (DCW).
118
119 *2.3. Carotenoid extraction and analysis by high performance liquid chromatography*
120 *(HPLC).*
121
122 One gram of each bacterial cell pellet was introduced in a 15-ml polypropylene
123 tube and extracted with 10 ml of N,N-dimethylformamide, at 65ºC for15 min. The cell
124 debris was separated by centrifugation at 5,000 rpm and the upper phase, containing the
125 carotenoid pigments, was transferred to a separator funnel. The operation was repeated
126 until the complete exhaustion of color (usually four extractions were enough). All
127 extracts were pooled and shaken with 100 ml of diethyl ether. A sufficient quantity of
128 10% NaCl was added at the end of the process to aid in the efficient separation of the
129 liquid phases. Subsequently, the organic phase was dried over anhydrous Na_2SO_4,
130 evaporated in a rotary evaporator, and taken up to 1 ml of acetone. Samples were
131 centrifuged at 12,000 rpm and stored at -30 °C until analyzed.
132 Monitoring and quantification of the bacterial carotenoid pigments were carried
133 out by reversed-phase HPLC (RP-HPLC) using a method previously developed in our
134 laboratory (Mínguez-Mosquera and Hornero-Méndez, 1993). The method involves a
135 C18 reverse-phase column (Waters Spherisorb ODS2 column; 250×4.6 mm I.D.,
136 particle size 5 μm; Waters Ltd., Hertsfordshire, UK) and a binary gradient elution
137 system of acetone-H_2O at a flow rate of 1.5 ml/min. Injection volume was 5 μl and
138 detection was carried out at 440 nm. Quantification was performed by using an external
139 standard calibration curve prepared with β-carotene (Sigma Chemical Co., St.Louis,
140 MO), a commercially available yellow carotenoid whose chromatic characteristics are

141 similar to the carotenoids found in the *L. plantarum* strains of this study. HPLC
142 analyses were performed with a Waters 600E quaternary pump equipped with a diode
143 array detector (PDA 996, Waters) and controlled with a Empower2 data acquisition
144 software (Waters Corporation, Milford, Massachusetts, USA). For the conditions used
145 in this study, the limit of detection was 0.01 mg/kg DCW of carotenoid in the sample,
146 while the limit of quantification was 1.5 mg/kg DCW. Carotenoids detected below the
147 limit of quantification are indicated as "traces" in Table 1.
148
149 *2.4 Pigment isolation and identification.*
150
151 Routine procedures for the isolation and identification of carotenoid pigments,
152 already described in detail in previous publications (Mínguez-Mosquera et al. 1990;
153 Mínguez-Mosquera and Hornero-Méndez, 1993) were used. Briefly, this consisted of:
154 separation and isolation of the pigments by thin layer chromatography on silicagel
155 60GF plates; acquisition of UV-visible spectra (Hewlett-Packard UV-vis diode array
156 spectrophotometer model 8452A) in different solvents and comparison with the values
157 reported in the literature (Foppen, 1971; Davies, 1976 and 1988; Britton, 1991 and
158 1995), as well as chemical tests for the examination of 5,6-epoxide groups investigated
159 by addition of 2% HCl in ethanol, acetylation with acetic anhydride-pyridine to test for
160 hydroxyl groups and reduction with $NaBH_4$ in ethanol to test for carbonyl groups
161 (Eugster, 1995).
162
163 *2.5. Liquid chromatography / mass spectrometry (LC/MS).*
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LC/MS was performed on a Waters 2695 XE separation module (Waters Corporation, Milford, USA) coupled with a Waters 2998 Photodiode Array detector and Micromass ZMD4000 (Manchester, UK) mass spectrometer equipped with an atmospheric pressure chemical ionization (APcI) interface. Chromatographic conditions and MS parameters were used according to Breithaupt and Schwack (2000). The MS system was operated in full scan mode (*m/z* 200-1200), and the UV-absorbance of carotenoids was recorded at 440 nm. A C30 YMC analytical column (YMC Europe GMBH, Germany) with 5µm particle size and 250 x 4.6 mm dimensions was used. The injection volume was 20 µl.

2.6. Genetic analyses

The presence of the operon *crtNM* in the different *L. plantarum* strains was detected by PCR using oligonuclotide primers designed from the published nucleotide sequence of these predicted genes in *L. plantarum* WCFS1 (GenBank accession number [acc.no.] AL935261; Kleerebezem et al., 2003). The primer pair crtN-for (*CGCG***GAATTC**ATGAAGCAAGTATCGATTATTGGC) and crtM-rev (*GATC***GAATTC**TTAAGCCTCCTTAAGGGCTAGTTC) was used to amplify a 2,379-bp DNA fragment including the coding sequences for both genes. Alternatively, in those cases when the first primer pair did not amplified any DNA fragment, the primer pair rbs-crtN-for (*CTAG***GGTACC**AAGGGGGAGATTTACTGATGAAGC) and crtM-rev was used to amplify a 2,396-bp DNA fragment which included part of the putative *crtN-crtM* promoter. *Eco*RI restriction sites were introduced at the 5' ends of primers crtN-for and crtM-rev, and *Kpn*I site at the 5' end of primer rbs-crtN-for to facilitate future cloning strategies (bold face in the primer sequences above). Also, hanging

sequences at the 5' ends of the primers were introduced to ensure proper restriction digestions (italics in the primer sequences above). Total DNA from isolated *L. plantarum* colonies was extracted with chloroform as previously described (Ruiz-Barba et al., 2005). Amplification of DNA fragments was performed in 25-µl reaction mixtures containing 2.5 mM MgCl, 1 x reaction buffer, 100 µM each of the deoxynucleoside triphosphates, 100 pmol of each primer, 5 U of *Taq* DNA polymerase (Promega), and 5 µl of total DNA solution prepared as described above as the template. A GeneAmp PCR system 2400 thermal cycler (Perkin-Elmer) was used with the following conditions: denaturation at 94 ºC for 2 min, followed by 30 cycles of denaturation at 94 ºC for 15 s, annealing at 60 ºC for 30 s, and polymerization at 72 ºC for 2 min, plus a final polymerization step at 72 ºC for 4 min. Alternatively, when no amplification was obtained with the later conditions, 58 ºC was used as the annealing temperature. Alternatively, when the amplicon was needed for sequencing purposes, the PCR Extender System (5Prime Gmbh, Hamburg, Germany) was used under the conditions recommended by the manufacturer for high fidelity performance. PCR-amplified DNA fragments were finally analyzed by agarose gel electrophoresis. Homology searches were carried out using the Blastn, Blastp and FASTA programmes, and sequence alignments were carried out using the EMBOSS alignment algorithm, all of them available at EMBL-EBI (www.ebi.ac.uk). DNA sequencing was carried out by the Servicio de Secuenciación Automática de DNA (SSAD), CIB-CSIC, Madrid, Spain, with an ABI PRISM 377 DNA sequencer (Applied Biosystems, Perkin-Elmer). Nucleotide sequences of *crtM* and *crtN* genes in *L. plantarum* CECT7531 have been assigned the GeneBank acc. no. GU474811.

3. Results

3.1. Most strains of L. plantarum analyzed produce the triterpenoid 4,4'-diaponeurosporene.

Chromatographic analysis of the carotenoids extracted from the *L. plantarum* cell pellets showed in virtually all of the cases a main chromatographic peak at a retention time of 14.8 min (peak 1, Fig. 1). The absorption spectrum showed maxima absorbance at 416, 438 and 468 nm (%III/II=95), which is consistent with a chromophore containing nine double conjugated bonds. In principle these properties suggested the presence of neurosporene in the analysed samples, however the chromatographic mobility for this peak was different, eluting at earlier retention times (about 1 min) than neurosporene (lower polarity), which was revealed after comparison with a Rose hips extract containing neurosporene (data not shown). None of the chemical tests carried out to investigate the presence of either epoxy 5,6, hydroxyl or carbonyl groups was positive, showing the absence of oxygen in the molecules under study and therefore its carotene, and not xanthophyll, character. The mass spectra showed a quasimolecular ion $[M+H]^+$ at *m/z* 403 which is in accord with the formula $C_{30}H_{42}$ of 4,4'-diaponeurosporene (Fig. 2). This is in agreement with data reported by Breithaupt et al. (2001) for *L. plantarum* LTH4936. Other two minoritary peaks were also found at retention times 15.3 and 15.6 min, and whose absorption maxima were 380,402, 426 (%III/II=93) and 333, 349, 368 (%III/II=81), respectively (peaks 2 and 3 in Fig. 1). Mass spectra of these peaks showed $[M+H]^+$ at *m/z* 405 and 407 respectively, which are in agreement with the molecular formulas $C_{30}H_{44}$ and $C_{30}H_{46}$, corresponding to 4,4'-diapo-ξ-carotene and 4,4'-diapophytofluene (also referred as 4,4'-diapo-7,8,11,12-tetrahydrolicopene) respectively, and corresponding to intermediates in the

biosynthetic pathway of 4,4'-diaponeurosporene in *S. aureus* (Wieland et al., 1994) (Fig. 2).

3.2. Quantification of carotenoid production by L. plantarum strains.

Preliminary results in our laboratory showed wide differences in the final carotenoid production regarding the brand and composition of the media used. As an example, for *L. plantarum* CECT7531 we obtained up to 54.55 mg/kg DCW of carotenoids in DM1 medium, 40.79 mg/kg in Oxoid MRS, 39.13 mg/kg in Difco MRS, 29.57 mg/kg in Biokar MRS, 26.62 mg/Kg in Merck MRS and just 18.79 mg/kg in DM2 medium. In all cases, cell counts after 24 h of incubation at 30 ºC prior to carotenoid extraction was virtually identical (*ca*. 10^9 CFU/ml). As best results were obtained with DM1 medium, this was chosen as the standard medium for comparison among the strains tested. As it is shown in Table 1, most (72.2%) of the cell pellets of the screened *L. plantarum* strains looked fairly yellow. Broth cultures of selected strains incubated for 24, 48, 72 and 96 h showed that after the first 24 h of incubation the main carotenoid peak progressively disappeared. Thus, as an example, for *L. plantarum* CECT4185, we obtained 29.79 mg/kg DCW of carotenoids after 24 h, and only 19.29, 2.61 and 4.82 mg/kg after 48, 72 and 96 h, respectively. Therefore, broth cultures incubated at 30 ºC for 24 h were chosen as the standard for maximum carotenoid production. Among the strains tested, carotenoid production ranged from traces (distinguishable characteristic carotenoid chromatographic peaks, but not reliably quantifiable) up to 54.55 mg/kg DCW produced by *L. plantarum* CECT7531 (Table 1). Yellow color of the pellets to the naked eye was observed for those strains producing at least 4.95 mg of carotenoids per kg of DCW (Table 1).

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266 *3.3. All L. plantarum strains tested harbor the operon crtNM described in L. plantarum*
267 *WCFS1 genome sequence.*

268

269 Genetic analyses through PCR revealed that all of the *L. plantarum* strains tested
270 contained the genes *crtN* and *crtM* arranged as an operon, as described in the annotated
271 complete genome sequence of *L. plantarum* WCFS1 (acc. no. AL935261), a strain
272 isolated from human pharynx (Kleerebezem et al., 2003). A 2,379-bp amplicon was
273 obtained from all but one of the strains tested when primers crtN-for and crtM-rev were
274 used (Table 1), with either 58 or 60 ºC as the annealing temperature. Although no
275 amplification was obtained with *L. plantarum* CECT4185 using this primer pair, a
276 2,396-bp amplicon was obtained from this strain when primers rbs-crtN-for and crtM-
277 rev were used instead (Table 1). These results are in agreement with the expected sizes
278 of the corresponding predicted amplicons. Only in five strains, amplification could be
279 obtained with both primer pairs, suggesting a certain degree of genetic diversity in the
280 promoter region among the *L. plantarum* strains used (Table 1). DNA sequence of the
281 amplicon obtained when DNA from the maximum carotenoid-producing strain *L.*
282 *plantarum* CECT7531 was used as the template showed 99.0 % identity (id.) to the
283 sequences of *crtM* and *crtN* genes reported for *L. plantarum* WCFS1. When translated,
284 amino acid sequences of the putative proteins coded by *crtM* and *crtN* genes in the
285 strain CECT7531 showed 99.0 % and 99.6 % id., respectively, to those proteins coded
286 by homologous genes found in the strain WCFS1. The product of gene *crtM* in the
287 strain CECT7531 has been annotated as dehydrosqualene synthase (also diapophytoene
288 synthase; acc. no. GU474811) based on similarity (30.5% id.; 64% similarity [sim.]) to
289 a homologous protein from *S. aureus* ATCC25904 (acc. no. O07854), where carotenoid

biosynthesis has been extensively studied (Wieland et al., 1994; Pelz et al., 2005). In *S. aureus*, this enzyme catalyses the head-to-head condensation of two molecules of FPP into the colourless C_{30} carotenoid dehydrosqualene (diapophytoene) (Fig. 2). On the other hand, the product of gene *crtN* in the strain CECT7531 has been annotated as dehydrosqualene desaturase (also diapophytoene desaturase; acc. no. GU474811) based also on similarity (46% id.; 76% sim.) to a homologous protein from *S. aureus* ATCC25904 (acc. no. O07855). In *S. aureus*, this enzyme catalyses three successive dehydrogenation reactions that lead to the introduction of three double bonds into dehydrosqualene to render diaponeurosporene, with diapophytofluene and diapo-ξ-carotene as intermediates (Fig. 2).

4. Discussion

Although most carotenoids found in bacteria are tetraterpenoids (C_{40}) (Phadwal, 2005), triterpenoids (C_{30}) have been reported in three species of non-photosynthetic bacteria, namely *S. aureus* (Marshall and Wilmoth, 1981), *Enterococcus faecium* (Taylor and Davies, 1974) and *Methylobacterium rhodinum* (Raisig and Sandmann, 1999), as well as in all tested species of the photosynthetic heliobacteria (Takaichi et al., 1997). Here we demonstrate that C_{30} carotenoids are also produced by most strains of *L. plantarum*. C_{30} and C_{40} carotenoid biosynthetic pathways are shown in Fig. 2. In the C_{30} pathway, condensation of two farnesyl pyrophosphate (FPP) molecules by diapophytoene synthase (coded by *crtM* gene) renders diapophytoene (also named dehydrosqualene; Fig. 2), the first colorless C_{30} carotenoid. Subsequently, successive desaturation reactions increase the number of conjugated double bonds in diapophytoene to produce colored carotenoids such as diaponeurosporene and

315 diapolycopene (Lee and Schmidt-Dannert, 2002) (Fig. 2). Although desaturases are
316 specific of the C_{30} or C_{40} routes, some interchangeability, either natural or induced
317 through molecular engineering, have been reported (Raisig and Sandmann, 2001;
318 Umeno et al., 2002 and 2005). In the few microorganisms known to produce C_{30}
319 carotenoids, the end product is either diaponeurosporene or compounds with further
320 modifications, involving for instance oxidation and glycosilation steps, as it is in the
321 case of staphyloxanthin in *S. aureus* (Pelz et al., 2005). The triterpenoid nature of these
322 compounds avoids cyclation of end groups rendering ionone rings which can be further
323 modified to diversify the number of carotenoid compounds with different colors and
324 properties (Lee and Schmidt-Dannert, 2002; Umeno et al., 2005). Therefore, genetic
325 manipulation of *L. plantarum* to increase the range of carotenoids being produced
326 should start with the introduction of heterologous genes coding for geranylgeranyl
327 pyrophosphate (GGPP) synthesis and appropriated desaturase and cyclase enzymes, so
328 that C40 carotenoids could be obtained (see Fig. 2).
329 No strong correlation between carotenoid production and the origin of the
330 different strains tested was observed, although all of the strains isolated from olive
331 fermentations produced high amounts, including the maximum producer CECT7531
332 (Table 1). Reported carotenoid production by microorganisms is very diverse regarding
333 net amounts produced. The different units and quantification methods used by different
334 authors makes it very difficult to compare between species. On top of this, different
335 authors have reported a number of environmental and cultural enhancers of carotenoid
336 production by microorganisms (Bhosale, 2004). Thus, two-fold up to one thousand-fold
337 increases have been obtained changing light irradiation or culture temperature, as well
338 as addition of chemical compounds, intermediates of the tricarboxylic acid cycle, metal
339 ions or salts to the culture medium (Bhosale, 2004). In our case, carotenoid production

was found to be dependent on the actual culture medium composition, i.e. brand and actual chemical components. This result illustrates the importance of the actual composition and source of the different nutrients of the culture medium used in carotenoid production even when the basic formulation of the media used, i.e. MRS (de Man et al., 1960), is nominally identical. Optimization experiments with high carotenoid-producer strains to obtain higher carotenoid yields will be carried out considering different growth conditions and medium composition, using appropriated statistical factorial designs.

The ability to produce diaponeurosporene together with the presence of the genes *crtN* and *crtM* encoding the putative enzymes necessary for its synthesis, suggest the full functionality of the operon *crtNM* in *L. plantarum,* whose previously assigned function in the strain WCFS1 had been predicted based on similarity data. The ubiquitous presence of this operon in all of the strains tested and its functionality in most of them, suggests that this function plays a role in this species survival. In *S. aureus*, a species where the functions of the enzymes coded by the genes *crtM* and *crtN* have been well established, carotenoid biosynthesis has been associated to resistance to different stress conditions, especially the oxidative stress (Clauditz et al., 2006). Heat resistance (Cebrián et al., 2007), desiccation susceptibility (Wieland et al., 1994), oleic and linolenic acid resistance (Chamberlain et al., 1991; Wieland et al., 1994), and impairing of neutrophil killing (Liu et al., 2005) have also been demonstrated to be linked to the carotenoid content of *S. aureus* strains. In all cases, these effects have been related to either the antioxidant properties of carotenoids or their ability to stabilize bacterial cell membranes. Optimization experiments with trace-amount-producing strains are necessary to find out whether low production is due to defective *crtN-crtM* genes, in particular at the promoter regions, or other strain-specific metabolic

365 characteristics are involved. Random and directed mutagenesis will be also very useful
366 to increase the amount of carotenoids produced by a specific strain.
367 DNA sequence of the genes *crtM* and *crtN* from *L. plantarum* CECT7531, the
368 maximum carotenoid producer strain in this study, showed that these genes are virtually
369 identical to homologous genes reported in the three *L. plantarum* strains whose genome
370 has been sequenced up to date: WCFS1, ATCC14917^T (isolated from pickled cabbage;
371 acc. no. ACGZ00000000.1), and JDM1 (Zhang et al., 2009; used as a probiotic strain in
372 China; acc. no. CP001617). *L. plantarum* ATCC14917^T is the type strain for the species
373 *L. plantarum*, and it is equivalent to *L. plantarum* CECT748^T, used in this study (Table
374 1). Our results suggest that the operon *crtNM* is well conserved in *L. plantarum*,
375 independently of the actual origin of the strain considered. In contrast to the C_{30}
376 carotenoid biosynthetic pathway found in *L. plantarum*, in *S. aureus* diaponeurosporene
377 is further converted to staphyloxanthin, the orange carotenoid present in most
378 staphylococci strains (Pelz et al., 2005) (Fig. 2). For this, *S. aureus* harbour up to three
379 extra enzymes coded by genes *crtO*, *crtP* and *crtQ*, which are located in the same
380 operon as *crtM* and *crtN* (Pelz et al., 2005). Neither staphyloxanthin nor any of the
381 intermediates in its biosynthesis (Fig. 2) was found in any of the *L. plantarum* strains
382 tested in this study. Moreover, no gene sharing homology with *crtO*, *crtP* or *crtQ* could
383 be found when analysed the *L. plantarum* WCFS1, ATCC14917^T or JDM1 complete
384 genome sequences. Finally, under specific conditions, i.e. high heterologous expression
385 levels and the effective concentration of substrates, the desaturase coded by *crtN* in *S.*
386 *aureus* is able to perform a fourth desaturation step, rendering 4,4'-diapolycopene in
387 amounts up to 50% of the total carotenoids produced (Umeno et al., 2002) (Fig. 2). This
388 carotenoid was not found in any of the *L. plantarum* strains tested under the standard
389 conditions used by us.

In conclusion, this study has shown the presence of C_{30} carotenoid biosynthesis in most of the *L. plantarum* strains studied, regardless their origin. The ubiquitous presence of the genes *crtM* and *crtN*, involved in the biosynthesis of the yellow C_{30} carotenoid 4,4'-diaponeurosporene, in all of the strains tested as well as the actual carotenoid production by most of them suggests that the role of carotenoids in *L. plantarum* environmental fitness must be important. On the other hand, considering that *L. plantarum* is a bacterial species which is extensively used to ferment food and feed products while having a GRAS status, the use of selected high-carotenoid-producing strains could contribute to increase the total amount of antioxidants supplied in the human and animal diet. In addition, as *L. plantarum* is a recognized inhabitant of the gastrointestinal tract, the use of selected strains of *L. plantarum* as probiotics could provide with a regular supply of antioxidant molecules, such as carotenoids, in a place where their protective action is quite welcome. Therefore, carotenoid production should be considered as an important feature for the selection of novel probiotic *L. plantarum* strains.

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596 **Legends of the Figures**

597

598 **Figure 1.**Typical HPLC chromatographic analysis and UV-visible absorption spectra of
599 the carotenoids extracted from *Lactobacillus plantarum* cell pellets. Peaks: 4,4'-
600 diaponeurosporene (1), 4,4'-diapo-ξ-carotene (2) and 4,4'-diapophytofluene (3).
601 Absorption maxima, expressed in nm, are indicated.

602

603 **Figure 2.** Biosynthetic pathways of the triterpenoid (C_{30}) and tetraterpenoid (C_{40})
604 carotenoids in microorganisms. Abbreviations: IPP, isopentenyl pyrophosphate; GPP,
605 geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranyl geranyl
606 pyrophosphate. Genes: (C_{30} and C_{40} pathways) *ispA*, geranyltranstransferase; (C_{30}
607 pathway) *crtM,* dehydrosqualene synthase (also, diapophytoene synthase); *crtN,*
608 dehydrosqualene desaturase (also, diapophytoene desaturase); *crtP*, diaponeurosporene
609 oxidase; *crtQ*, glycosyl transferase; *crtO*, acyl transferase; (C_{40} pathway) *crtE*, GGPP
610 synthase; *crtB,* phytoene synthase; *crtI,* phytoene desaturase. *Alternative common
611 name.** Under specific conditions (see text), the desaturase coded by *crtN* in *S. aureus*
612 is able to perform a fourth desaturation step, rendering 4,4'-diapolycopene.

Figure

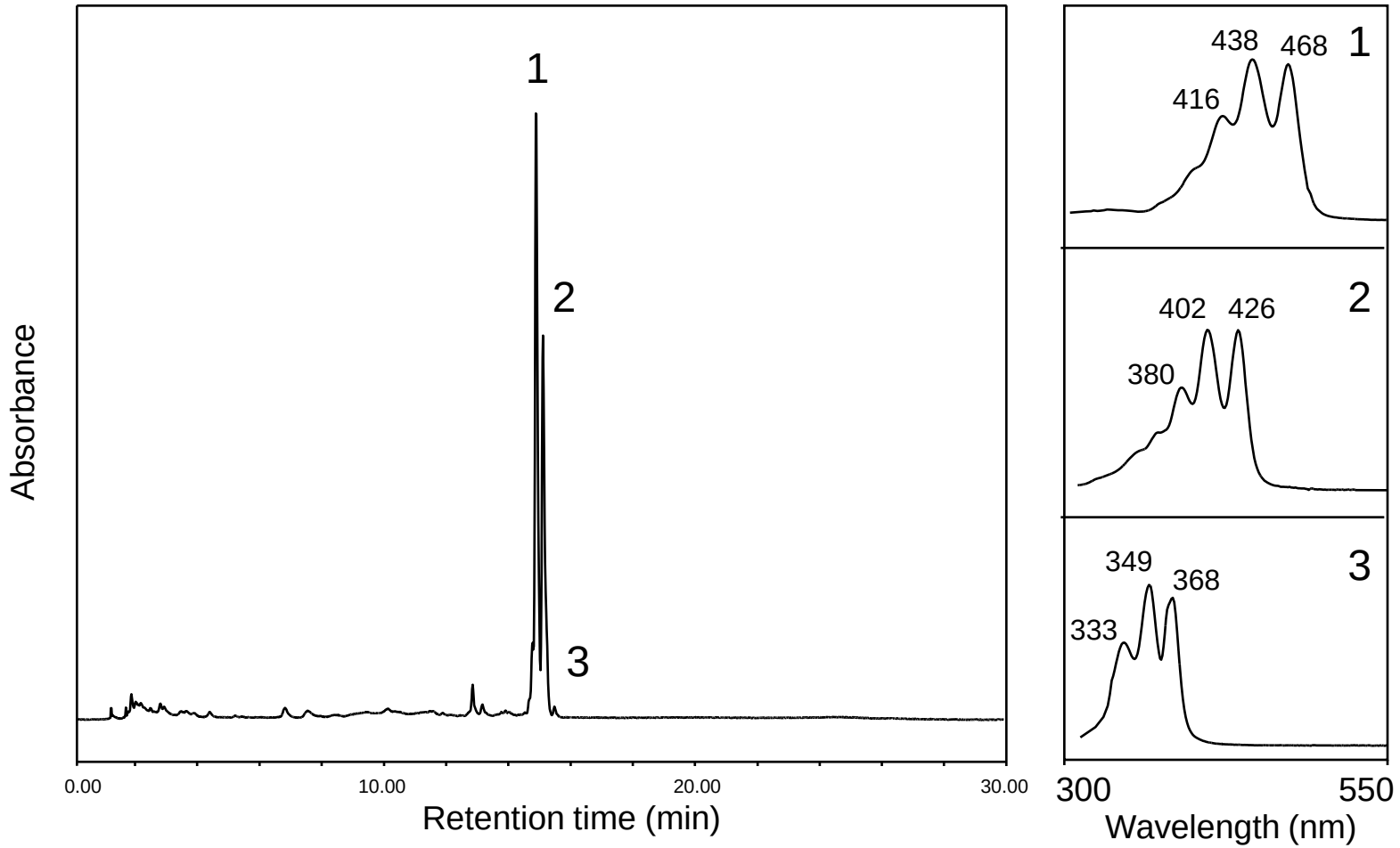

Figure 1, Garrido-Fernández, Maldonado-Barragán, Caballero-Guerrero, Hornero-Méndez, and Ruiz-Barba

Figure

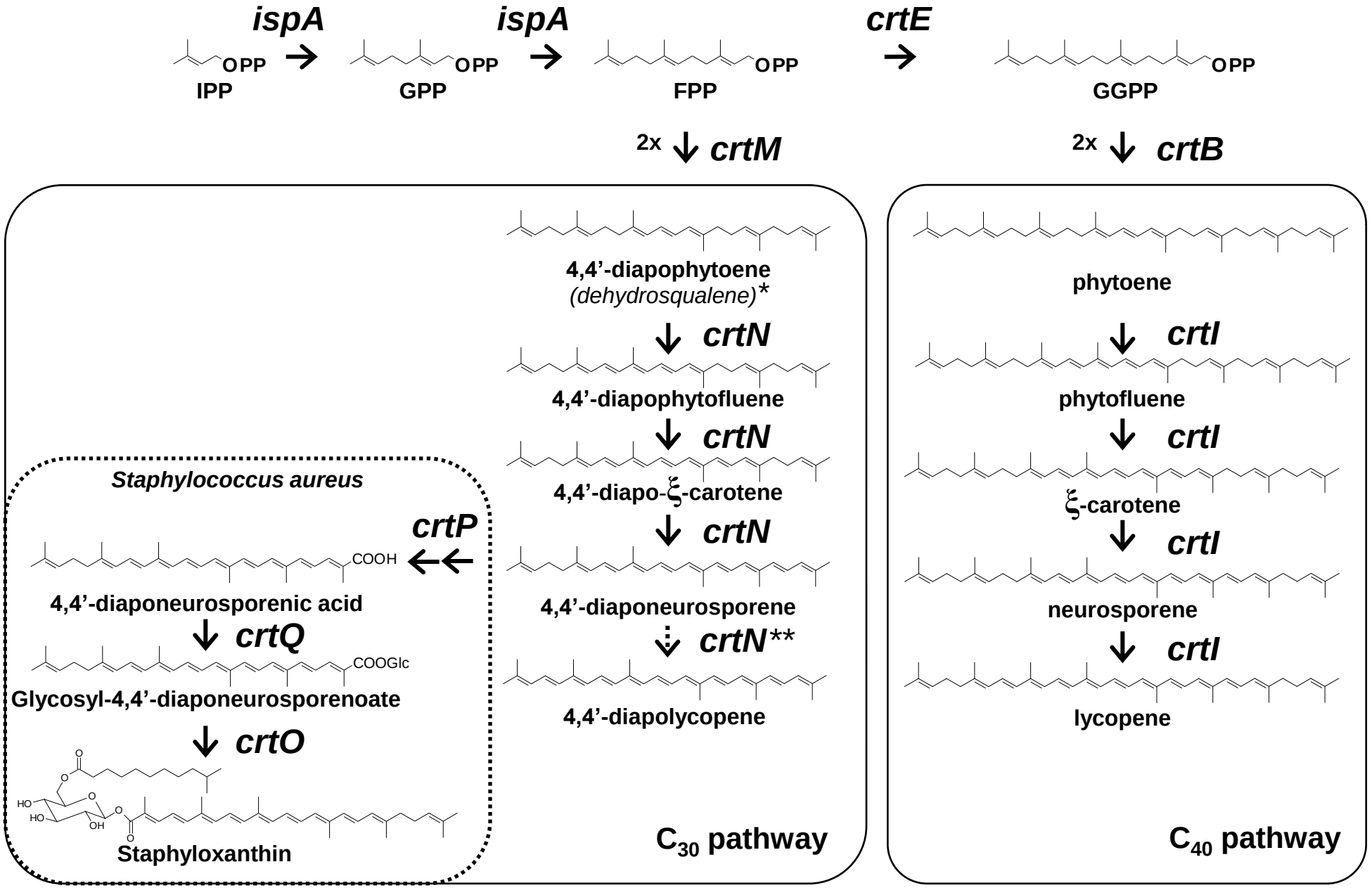

Figure 2, Garrido-Fernández, Maldonado-Barragán, Caballero-Guerrero, Hornero-Méndez, and Ruiz-Barba

Table 1. *Lactobacillus plantarum* strains and carotenoid production.

Strain	Origin	Colour[1]	Carotenoid production[2] (mg/kg dry cell weight±SD)[5]	PCR[3]	Source[4]
CECT7531	olive fermentation	Y	54.55 ± 0.65	1,2	IG-CSIC
CECT4185	silage	Y	29.79 ± 1.10	2	CECT
LPT70/3	olive fermentation	Y	29.74 ± 1.20	1	IG-CSIC
LB6	wine	Y	29.13 ± 0.75	1,2	UV
LPT57/1	olive fermentation	Y	27.41 ± 0.83	1	IG-CSIC
WCFS1	human pharynx	Y	22.53 ± 1.03	1,2	WCFS
LPT49/6	olive fermentation	Y	19.18 ± 0.90	1,2	IG-CSIC
LPT44/1	olive fermentation	Y	18.97 ± 0.70	1	IG-CSIC
LPJ10	olive fermentation	Y	14.78 ± 0.25	1	IG-CSIC
LPT57/2	olive fermentation	Y	9.19 ± 0.44	1	IG-CSIC
NC8	grass silage	Y	8.83 ± 0.67	1,2	Matforsk
ATCC10241	pickled cabbage	Y	6.33 ± 0.56	1	ATCC
RP1	commercial inoculum	Y	4.95 ± 0.88	1	Rhône-Poulenc
ATCC14431	grass silage	W	2.30 ± 0.78	1	ATCC
CECT 748^{T}	pickled cabbage	W	1.78 ± 0.20	1	CECT
ATCC8014	corn silage	W	traces[6]	1	ATCC
CECT 220	corn silage	W	traces	1	CECT
LL441	cheese	W	traces	1	IPLA-CSIC

[1] Colour to the naked eye of cell pellets obtained after centrifugation: Y = yellow; W = white.

[2] The majoritary carotenoid produced is 4,4'-diaponeurosporene.

[3] Primer pair that amplified the crtN-crtM gene cluster in that particular strain: 1 = crtN-for/crtM-rev; 2= rbs-crtN-for/crtM-rev.

[4]Sources: IG-CSIC: Instituto de la Grasa-CSIC, Sevilla, Spain; CECT: Colección Española de Cultivos Tipo (Spanish Type-Culture Collection), Burjassot, Spain; UV: Sergi Ferrer and Isabel Pardo, University of Valencia, Valencia, Spain; WCFS: Michiel Kleerebezem, Wageningen Centre for Food Sciences, Wageningen, The Netherlands; Matforsk, Lars Axelsson, Norwegian Food Research Institute, Osloveien, Norway; ATCC: American Type Culture Collection, Manassas, Virginia, USA; Rhône-Poulenc: Rhône-Poulenc Industries SA, Courbevoie, France; IPLA-CSIC: Baltasar Mayo, Instituto de Productos Lácteos de Asturias-CSIC, Asturias, Spain.

[5]Cells were collected from 500-ml 24-h cultures in DM1 medium (see text). Figures are mean values of three independent assays ± standard deviations.

[6]Carotenoids detected below the limit of quantification (1.5 mg/kg)

[T]Type strain, it is equivalent to *L. plantarum* ATCC14917^{T}.