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▶ **To cite this version:**

Charlotte Sy, Beatrice Gleize, Stéphanie Chamot, Olivier Dangles, Frédéric Carlin, et al.. Glycosyl carotenoids from marine spore-forming Bacillus sp. strains are readily bioaccessible and bioavailable . Food Research International, 2013, 51 (2), pp.914-923. 10.1016/j.foodres.2013.02.003 . hal-03202928

HAL Id: hal-03202928
https://hal.inrae.fr/hal-03202928

Submitted on 20 Apr 2021

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Glycosyl carotenoids from marine spore-forming *Bacillus* sp. strains are readily bioaccessible and bioavailable[‡]

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27 **RUNNING TITLE:** *Bacillus* sp. strains carotenoid bioavailability.

28 ǂ The present work has received funding from the European Community's Seventh

29 Framework Programme. The funding was attributed to the COLORSPORE project entitled

30 New Sources of Natural, Gastric Stable, Food Additives, Colorants and Novel Functional

31 Foods (small collaborative project N° 207948, topic (Bio-)Technologies for the production of

32 food additives, colorants, and flavours, KBBE-2007-2-3-03). The funding source had no

33 involvement in this publication. This publication reflects only the authors' view. The

34 Colorspore community is not liable for any use that may be made of the results. No author has

35 any conflict of interest. All authors have approved the final article.

36 **ABSTRACT**

37

38 The main human sources of carotenoids are fruits and vegetables. Some bacteria also
39 synthesize carotenoids that can have peculiar chemical structures that raise the question of
40 their bioavailability in humans. HU36 and GB1 *Bacillus* strains contain glycosyl carotenoids
41 that are partially acylated by linear fatty acids. The aim was to assess the bioaccessibility, the
42 uptake efficiency and the tissue distribution of these bacterial carotenoids. β-Carotene was
43 used as a model carotenoid for comparisons. Results of *in vitro* digestion experiments showed
44 that bioaccessibility of purified extracts of bacterial carotenoids was considerably higher
45 (about 4.5 times as high) than that of pure β-carotene. Bacterial carotenoids were also
46 bioaccessible when digested from their natural matrices, but about 2 times less than their
47 purified extracts. Bacterial carotenoids were absorbed by Caco-2 with similar efficiency than
48 β-carotene, i.e. about 10%. Bacterial carotenoids were recovered in significant amounts in
49 their native chemical forms in plasma, liver and in adipose tissue of rats, which were force-fed
50 for 3 days with either bacterial carotenoid extracts or lyophilised bacteria. Bacterial
51 carotenoids were found at higher concentrations in rat tissues than β-carotene, about 2-3 times
52 for GB1 carotenoids and 9 times for HU36 carotenoids. They were also more recovered in
53 adipose tissue than β-carotene. In conclusion, glycosyl carotenoids from the HU36 and GB1
54 *Bacillus* strains are readily bioavailable. This is due in part to their higher bioaccessibility but
55 perhaps also to their easier accumulation in tissues.

56

57 **KEYWORDS:** bacteria, β-carotene, micelles, absorption, bioavailability, probiotic.

58

59 ## 1. INTRODUCTION

60

61 Carotenoids are lipophilic dietary microconstituents that are not biosynthesized by
62 humans but may have interesting beneficial properties for human health. Epidemiological
63 studies have associated the consumption of carotenoid-rich foods (especially fruit and
64 vegetables) with reduced risks of several diseases: e.g. age-related macular degeneration,
65 some cancers, cardiovascular disease (Krinsky and Johnson, 2005; Stahl and Sies, 2005;
66 Tapiero, Townsend, & Tew, 2004). The beneficial effect of carotenoids on health was initially
67 assumed to be mediated by their antioxidant activity (Tyssandier et al., 2004). However recent
68 findings suggest that other mechanisms could be implicated such as effects on signalling
69 pathways or on gene expression (Gouranton et al., 2011).

70 The potential health-promoting properties of carotenoids have led to substantial
71 interest in carotenoids as nutritional supplements, especially from natural sources. β-Carotene
72 is one of the naturally occurring carotenoid that have been most intensively studied. It can be
73 used as a food colouring agent, as a dietary source of vitamin A (Krinsky and Johnson, 2005)
74 and for other bioactive properties (Britton, Liaaen-Jensen, & Pfander, 2008; Peto, Doll,
75 Buckley, & Sporn, 1981). Besides, bacterial producing carotenoids have gained interest in the
76 past years due to their ability to act as probiotics (Hempel et al., 2012; Ma, Choi, Choi,
77 Pothoulakis, Rhee, & Im, 2010) and their potential to provide a suitable biosource of
78 carotenoids with both C40 and C30 backbones (Perez-Fons et al., 2010) and with improved
79 solubility and stability for the food and feed industry (Duc, Fraser, Tam, & Cutting, 2006). A
80 diverse range of spore-forming *Bacillus* species has been isolated that contain various red,
81 pink or yellow-orange carotenoid pigments. Among *Bacillus* sp., the *B. indicus* HU36 and *B.*
82 *firmus* GB1 strains are of particular interest for their high production of carotenoids, the
83 resistance of their spores to UV radiations (Khaneja et al., 2010) and their amenable probiotic

84 properties (Hong, Huang, Khaneja, Hiep, Urdaci, & Cutting, 2008). The HU36 strain, which
85 has been isolated in human faeces, synthesizes yellow-orange pigments in variable
86 proportions depending on whether they are in the vegetative or spore-forming state. The
87 corresponding carotenoid extract displays maxima of visible absorption at 429, 454 and 485
88 nm (Khaneja et al., 2010). The most abundant pigments in the extract were determined as 1-
89 glycosyl-3-4-dehydro-8'-apolycopene and methyl-1-glycosyl-3-4-dehydro-8'-apolycopenoate
90 esters with saturated fatty acid secondary chains from C8 to C15 (Fig. 1 A) (Perez-Fons et al.,
91 2010). The GB1 strain, which has been isolated from human ileum, produces deep-pink
92 pigments with maxima of visible absorption at 463, 492 and 524 nm (Khaneja et al., 2010),
93 the main one being 4,4'-diglycosyl-4,4'-diapolycopenoic diester (Fig. 1 B).
94 Knowledge on the metabolism of carotenoids in humans has been considerably
95 increasing during the last ten years. It is now assumed that carotenoids have to be extracted
96 from their vegetable matrix to be transferred into mixed micelles and efficiently absorbed.
97 The efficiency of this transfer is called "bioaccessibility". Several factors have been identified
98 that affect carotenoid bioaccessibility (Borel, 2003; West and Castenmiller, 1998). Among
99 these factors we have recently shown (Sy, Gleize, Dangles, Landrier, Caris-Veyrat, & Borel,
100 2012) that the chemical structure of carotenoids is a key-factor that modulates their solubility
101 into mixed micelles. It has also been shown that the matrix in which carotenoids are
102 embedded can play a significant role on bioaccessibility (Gartner, Stahl, & Sies, 1997; Reboul
103 et al., 2005b; Stahl and Sies, 1992; Sy et al., 2012). After being incorporated into mixed
104 micelles, carotenoids are transported to the enterocyte, where they are absorbed via membrane
105 transporters (Reboul and Borel, 2011). It is not yet known how these transporters facilitate the
106 uptake of carotenoids and it has been hypothesized that carotenoid chemical structure might
107 play a role in the interaction between carotenoid and their transporters.

108 The peculiar chemical structure of HU36 and GB1 carotenoids and their localisation in
109 spores have raised question on their bioavailability, i.e. on the fraction of the dose which is
110 entering the systemic circulation to exert its function (or used for storage for later use). It was
111 indeed not known whether they efficiently solubilized into mixed micelles, whether they can
112 be absorbed by intestinal cells and whether they are transported within the body to reach the
113 main storage tissues of carotenoids, i.e. the liver and adipose tissue.

114 The aim of this study was therefore to compare the bioaccessibility, the uptake by
115 intestinal cell, and the tissue distribution of HU36 and GB1 carotenoids, with the most studied
116 carotenoid: β-carotene.

117

118 **2. MATERIAL AND METHODS**

119

120 **2.1 Supplies and chemicals.**

121 Canned steamed carrots (Daucy, Vannes, France) were purchased from a local supermarket
122 and chosen without antioxidants, acidifiers or preservatives. Plain yogurt was from Danone
123 (Paris, France). Mixtures of lyophilized spores and vegetative cells from *Bacillus* strains
124 (HU36 and GB1) were provided by Paul Fraser and Reena Khaneja (Royal Holloway
125 University of London) in the frame of the COLORSPORE project (European Small
126 Collaborative Project No. 207948, FP7) (Duc et al., 2006). Carotenoids in bacterial extracts
127 were purified by liquid chromatography before the study and a purity > 90% was obtained.
128 Pure all-*E* β-carotene (> 95%) was kindly provided by DSM LTD (Basel, Switzerland). Salts
129 ($NaHCO_3$, NaCl, KCl, $CaCl_2$, $2H_2O$ and K_2HPO_4), mucin, α-amylase, pepsin, porcine
130 pancreatin, porcine bile extract, 2-Oleoyl-1-palmitoyl-*sn*-glycero-3-phosphocholine
131 (phosphatidylcholine), 1-palmitoyl-*sn*-glycero-3-phosphocholine (lysophosphatidylcholine),
132 monoolein, free cholesterol, oleic acid, sodium taurocholate, pyrogallol (used as antioxidant
133 preservative) and apo-8'-carotenal (> 95%, used as internal standard to calculate recovery
134 yield during carotenoid extraction (Sy et al., 2012)) were purchased from Sigma-Aldrich (St
135 Quentin-Fallavier, France). FBS (foetal bovine serum) was purchased from Biomedia (Issy-
136 les-Moulineaux, France). DMEM (Dulbecco's Modified Eagle Medium) containing 4.5 g/L
137 glucose and trypsin/EDTA (500 and 200 mg/L respectively), non-essential amino acids,
138 penicillin/streptomycin, PBS (phosphate buffered saline solution) and PBS containing 0.1
139 mmol/L $CaCl_2$ and 1 mmol/L $MgCl_2$ (PBSCM) were from Invitrogen (Cergy-Pontoise,
140 France). Hexane, methanol, dichloromethane and methyl-*t*-butylether (MTBE) were of HPLC
141 grade and purchased from Carlo Erba Reactifs SDS (Val-de-Reuil, France). The small kit of

142 the Uptima bicinchoninic acid (BCA) assay for protein quantification was from Interchim

143 (Montluçon, France).

144

145 **2.2 Test meals used in the *in vitro* digestion experiments performed for assessing**

146 **carotenoid bioaccessibility.**

147 Experiments were performed twice for each carotenoid (or carotenoid mixture); first

148 using pure solid β-carotene and purified bacterial extracts, in order to measure the intrinsic

149 bioaccessibility of bacterial carotenoids (i.e. without the effect of the food matrix), then with

150 foods rich in the studied carotenoids, in order to measure the bioaccessibility of bacterial

151 carotenoids when they are digested in their usual matrix, i.e. bacteria. For experiments with

152 pure carotenoids, stock solutions of carotenoids in hexane were dispersed in commercial

153 groundnut oil, which did not contain quantifiable amounts of carotenoids as checked by

154 HPLC, and the solvent evaporated to obtain carotenoid concentrations of ca. 750 µmol/L in

155 oil. The first digestions were conducted on the oil alone, while for experiments with foods, the

156 food sources of carotenoids were codigested with a standard meal (Table 1). This was done in

157 order to be in nutritional conditions where carotenoid sources are usually ingested together

158 with other foods. Foods were chosen in order that i) they did not contained detectable amount

159 of carotenoids (checked by HPLC), and ii) their mix gave a meal composition in

160 macronutrients (lipids, carbohydrates and proteins) that was close to US-dietary

161 recommended intake in macronutrients (see table 1 legend). Potatoes were boiled in tap water,

162 peeled, and hand-pureed. Meat was fried in a frying-pan without added fat. Potato purée and

163 fried meat were divided into aliquots and frozen at -20°C. The food source of β-carotene was

164 commercial carrot purée. Lyophilized HU36 and GB1 cells + spores were homogenized in

165 plain yogurts (Table 2).

166

167 **2.3 *In vitro* digestion experiments for assessing carotenoid bioaccessibility.**

168 We used the *in vitro* digestion model developed by Garrett et al. (Garrett, Failla, &
169 Sarama, 1999). The experimental conditions were slightly modified (Reboul, Richelle, Perrot,
170 Desmoulins-Malezet, Pirisi, & Borel, 2006) to better take into account the physicochemical
171 conditions prevailing in the human gastrointestinal lumen (Tyssandier et al., 2003) and to
172 reduce as much as possible the amount of triglycerides left at the end of the digestion
173 experiments (Sy et al., 2012). Meal components, or only groundnut oil when the
174 bioaccessibility of carotenoids was studied without the effect of the food matrix (Table 1),
175 were mixed with 32 mL NaCl 0.9% and homogenized in a shaking water bath at 37°C for 10
176 min. The next steps of the *in vitro* digestion procedure are described in detail in a recent
177 publication of our team (Sy et al., 2012). Each experiment was run in triplicate.

178 Natural mixed micelles produced during *in vitro* digestion were separated as described
179 recently (Sy et al., 2012).

180

181 **2.4 Monitoring of the bacterial material during *in vitro* digestion**.

182 Aliquots (250 µL) were taken into the digestion medium at regular time intervals, i.e.
183 t=0 min (bacteria just added into the test meal), t = 10 min (after addition of artificial saliva), t
184 = 70 min (the end of gastric digestion), t = 130 min (mid-term of duodenal digestion), and t =
185 190 min (the end of duodenal digestion). Samples were serially diluted in sodium phosphate
186 buffer (0.2 M, pH 7.0) and 100 µL were manually spread on Luria-Bertani (LB) agar (Sigma-
187 Aldric, Steinheim, Germany) plates in duplicate. After 24 and 48 h incubation at 37°C,
188 concentrations of total bacterial population were expressed as colony forming units per
189 millilitre (CFU/mL). The limit of detection was taken as one colony on the lowest dilution
190 plate, *i.e.* 10 CFU/mL. To determine spore concentrations, the samples were additionally
191 heated at 75°C for 10 min previously to serial dilution in phosphate buffer and duplicate

192 spreading on LB agar plates. In a separate test, the tolerance of HU36 and GB1 spores to
193 porcine bile was estimated by following changes in bacterial counts in gastric media
194 containing concentrations of porcine bile ranging from 8.09 g/L to 80.90 mg/L (a control did
195 not contain bile salts). After 48 h incubation at 37°C, spore concentrations were expressed as
196 CFU/mL. The limit of detection was taken as one colony on the lowest dilution plate, i.e. 10
197 CFU/mL.
198

199 **2.5 Determination of the intrinsic ability of bacterial carotenoids to be**
200 **incorporated into synthetic mixed micelles.**

201 Synthetic mixed micelles either free of carotenoids or containing either pure β-
202 carotene or bacterial carotenoids were prepared as previously described (Reboul et al., 2005a;
203 Sy et al., 2012). Synthetic mixed micelle sizes were checked by photon correlation
204 spectroscopy (Zetasizer Nano Zs, Malvern Instruments, Malvern, UK) (Sy et al., 2012).
205 The maximal amount of either β-carotene or purified bacterial carotenoids that can be
206 incorporated into a fixed amount of synthetic mixed micelles was measured as previously
207 described (Sy et al., 2012).
208 To measure the transfer of either β-carotene, or purified bacterial carotenoids, from
209 their solid form into synthetic mixed micelles solubilised in water we used a protocol
210 described in detail in a recent publication (Sy et al., 2012).
211

212 **2.6 *In vitro* carotenoid uptake by Caco-2 cell monolayers.**

213 Caco-2 clone TC-7 cells, which were a gift from Dr M. Rousset (UMR S872, Paris, France),
214 were grown as recently described (Sy et al., 2012). Carotenoid uptake experiments were also
215 described in detail in this publication (Sy et al., 2012). In summary the apical side of the cell
216 monolayers received 1 mL carotenoid-rich natural mixed micelles, i.e. mixed micelles coming

217 from the *in vitro* digestion of test-meals containing the food sources of carotenoids (*i.e.* carrot
218 puree or yogurt enriched in bacterial lyophilised material). Cell monolayers were incubated at
219 37°C for 3 h. After the incubation period, media from each side of the membrane were
220 harvested. Cell monolayers were washed twice with 1 mL PBS, scraped, and collected in 500
221 µL PBS. All the samples were stored at -80°C under nitrogen until carotenoid extraction and
222 HPLC analysis.
223
224 **2.7 Measurement of carotenoid bioavailability in an animal model.**
225 The animal model to study carotenoid bioavailability was multiple-dose gavage
226 experiment lasting 3 days in young male albino Wistar rats (Sy et al., 2012). Multiple gavages
227 over 3 days were preferred to dietary supplementation for several weeks because: i) the
228 amounts of bacterial carotenoids available were limited, ii) gavage allows a better control of
229 the amounts of carotenoids ingested by the rats, and iii) gavage limits carotenoid oxidation
230 that can occur in foods given to rats and stored at room temperature. Experiments were
231 conducted according to animal ethics rules and were approved by the Aix-Marseille
232 University experimental animal ethic committee. Besides, the innocuousness of the bacterial
233 carotenoid extracts and of the bacterial strains producing the carotenoids were studied by
234 other partners of the project (Hong et al., 2008). Six groups of 8 rats were included in the
235 study: a control group was force-fed with 1 mL groundnut oil without carotenoids, three
236 groups were force-fed with the same amount of oil-containing purified carotenoids, i.e. pure
237 β-carotene, purified HU36 carotenoids, or purified GB1 carotenoids, two groups were force-
238 fed with yoghurts containing either HU36 or GB1 lyophilised bacterias. The gavage protocol
239 was described in detail recently (Sy et al., 2012). The doses of carotenoid sources
240 incorporated in oil and yoghurts and given to the rats were chosen so as to bring 0.15 mg
241 carotenoid/kg/day (Sy et al., 2012), which correspond to 10.5 mg/d for a man of 70 kg. This

242 dose of carotenoid remains nutritional as it is close to the daily total carotenoid intake, which
243 is estimated at 14 mg/d in Europe (O'Neill et al., 2001). The last gavage experiment was
244 carried out on fasting rats (Sy et al., 2012). Rats were killed exactly 4 h later, i.e. at the time
245 of maximal concentration of carotenoids in blood (Mathews-Roth, Welankiwar, Sehgal,
246 Lausen, Russett, & Krinsky, 1990). Blood and tissues samples (liver and adipose tissue) were
247 collected. The blood samples were collected in tubes with heparin and immediately
248 centrifuged in order to separate the plasma. All the plasma and tissue samples were
249 immediately plunged into liquid N_2 and kept at -80°C until carotenoid analysis.
250

251 **2.8 Extraction of carotenoids from the various *in vitro* and *in vivo* samples.**

252 The procedure was adapted to the diverse types of samples (digesta, micellar fractions,
253 Caco-2 experiments fractions, plasma, liver and adipose tissue samples) and described in
254 detail recently (Sy et al., 2012).
255

256 **2.9 HPLC analysis of the carotenoids.**

257 After evaporation to dryness, all dried extracts were dissolved in 200 µL methanol-
258 CH_2Cl_2 (65:35, v/v). Carotenoids were quantified as recently described (Gleize, Steib, Andre,
259 & Reboul, 2012; Sy et al., 2012) by reverse-phase HPLC on a Dionex system (equipped with
260 in line degasser, a P680 pump, a cooled automatic sample injector ASI-100 and a UV/visible
261 diode-array detector UVD340U, Dionex France, Voisins-le-Bretonneux, France). Carotenoids
262 and apo-8'-carotenal (used as internal standard to calculate recovery yield during carotenoid
263 extraction) were detected at their wavelength of maximal absorption (455 nm for β-carotene
264 and HU36 carotenoids and 495 nm for GB1 carotenoids). Bacterial carotenoids in micelles,
265 cells and tissue samples were identified and quantified by their HPLC chromatogram and UV-
266 visible spectrum (from 300 to 550 nm) in comparison with the HPLC chromatograms and

267 UV-visible spectrums of the purified molecules. Calibration curves were performed with the
268 purified bacterial carotenoids. Quantification was performed using Dionex Chromeleon
269 software (Dionex Chromatography Management system, version 6.80).
270

271 **2.10 Protein assay on the tissue samples from the *in vivo* study.**

272 This method was described in detail recently (Sy et al., 2012).
273

274 **2.11 Calculations and Statistics.**

275 The extinction coefficients of the purified carotenoid mixtures were calculated after
276 dilution of the mixtures in dichloromethane and OD measurements at 455 and 495 nm for the
277 HU36 and GB1 carotenoids, respectively. The values obtained were 165 000 and 225 300
278 $L.mol^{-1}.cm^{-1}$. The estimated mw of the bacterial carotenoid mixtures were 750 and 784 g/mol
279 for the HU36 and GB1 carotenoids, respectively. Bioaccessibility was defined as the
280 percentage of carotenoids recovered in the micellar fraction after *in* v*itro* digestion, in relation
281 to the amount of carotenoids measured in the digestive medium just before addition of
282 artificial saliva. The solubility of the carotenoids in synthetic mixed micelles was defined as
283 the percentage of carotenoids recovered in the micellar fraction, in relation to the total
284 carotenoids present in the medium. Uptake efficiency of the carotenoids was defined as the
285 percentage of carotenoids recovered in scraped Caco-2 cells, in relation to the amount of
286 carotenoids initially added on the apical sides of the cell monolayers. All the *in vitro*
287 experiments were run in triplicate. Results were expressed as means and standard deviations.
288 Differences between means were assessed using ANOVA followed by the post-hoc Tukey-
289 Kramer’s test for parametric data (*in vitro* results). In the case of non-parametric data (*in vivo*
290 results), they were assessed using the Kruskal-Wallis’ test followed by the Mann-Whitney *U*-
291 test when the Kruskal-Wallis test showed significant differences between groups. *P* values

292 under 0.05 were considered significant. Statistical comparisons were performed using

293 StatView software version 5.0 (SAS Institute Inc., Cary, NC).

294

295 **3. RESULTS**

296

297 **3.1 Fate of HU36 and GB1 bacteria during digestion.**

298 Most bacteria were present as vegetative cells in the lyophilised bacterial mixture, with
299 only around 1‰ bacteria as spores in both strain preparations. Initial concentrations of viable
300 cells were about 8 $x10^6$ cfu/mg lyophilised material for HU36 and $2x10^5$ cfu/mg lyophilised
301 material for GB1. Initial concentrations of spores were $8x10^3$ cfu/mg lyophilised material for
302 HU36 and $3x10^2$ cfu/mg lyophilised material for GB1. The number of viable cells and spores
303 decreased during the gastric digestion (Fig. 2B). During the duodenal digestion the number of
304 GB1 cells continued to diminish in the first hour than remained stable during the second hour.
305 The spore concentrations remained relatively unchanged (final spore populations were $8x10^4$
306 cfu for HU36 and $1x10^4$ cfu for GB1), showing in particular a rather low germination. Final
307 vegetative cell populations were $9x10^7$ cfu for HU36 and around $5x10^5$ cfu for GB1 (i.e. 66%
308 and 92% viability loss, respectively) (Fig. 2, A & B). In conclusion, the bioaccessibility of
309 carotenoids does not depend on cellular growth.

310

311 **3.2 Stability of bacterial carotenoids during digestion.**

312 HPLC chromatograms suggest that both yellow and orange HU36 glycosyl carotenoid
313 esters were transformed during the *in vitro* digestion experiment (Fig. 3, B vs A). The
314 digestion of GB1 carotenoids led to 2 new pigments (Fig. 4, B vs A), which were likely less
315 hydrophobic (more polar) than the native carotenoids, i.e. because they eluted earlier. New
316 products could result from autoxidation (oxidation by O_2 initiated by metal traces) and/or (in
317 the case of the bacterial carotenoids) hydrolysis of sugar and acyl moieties by the digestive
318 enzymes. GB1 carotenoids were the most stable with 81.0 ± 0.6% of area under the curves of

319 the initial carotenoids recovered at the end of digestion. The residual amounts of β-carotene
320 and native HU36 carotenoids were identical: 69.5 ± 1.2% and 69.5 ± 4.3% respectively.
321

322 **3.3 Bioaccessibility of bacterial carotenoids as measured by the *in vitro* digestion**
323 **model.**

324 Purified HU36 and GB1 carotenoids were equally bioaccessible with about 60% of the
325 initial carotenoid content recovered in the micellar fraction. Pure β-carotene was about 5
326 times less bioaccessible (Fig. 5). Comparison of HPLC chromatograms of bacterial
327 carotenoids recovered in the micellar fraction vs. those present in the whole medium at the
328 end of the duodenal digestion, showed that the different carotenoids that were present in the
329 bacterial carotenoid extract were incorporated into natural mixed micelles with similar
330 efficiencies (data not shown).

331 As shown in Fig. 5, the carrot purée matrix had no significant effect on β-carotene
332 bioaccessibility. Conversely, the bacterial matrices significantly diminished bacterial
333 carotenoid bioaccessibility (by a factor ca. 2). Nevertheless note that the bioaccessibility of
334 bacterial carotenoids contained in mixtures of cells and spores, was significantly higher than
335 that of β-carotene, either digested pure or in carrot purée.
336

337 **3.4 Incorporation of purified bacterial carotenoids into synthetic mixed micelles.**

338 First of all we measured the solubility of purified bacterial carotenoids in water
339 because we suspected that they are partially soluble in water due to their glycosid groups. The
340 values measured were ca. 0.25 and 0.16 µmol/L for HU36 and GB1, respectively. These
341 values are of course higher than that of β-carotene (essentially insoluble in water) but
342 markedly lower than their solubility in synthetic micelles. Indeed, when homogenized with
343 phospholipids, cholesterol and glycerol esters (mixed micelle lipids) before the formation of

344 synthetic mixed micelles, incorporation efficiencies of purified HU36 carotenoids and GB1
345 carotenoids were over 90% (Table 3). This was significantly higher than that of pure β-
346 carotene (about 50%).

347 The percentage versus time of transfer of pure carotenoids from carotenoid powders to
348 pre-formed synthetic mixed micelles in water is shown in Fig. 6. In all cases, the maximal
349 solubilisation rates were nearly reached in the first 30 to 60 min. The maximal transfer of
350 carotenoids measured at the plateau revealed that bacterial carotenoids were significantly
351 more transferred from powder into synthetic mixed micelles (over 70%) than β-carotene
352 (around 8%). Moreover, maximal percentage values of carotenoids transferred into synthetic
353 mixed micelles were significantly lower than in the incorporation of carotenoids during the
354 preparation of synthetic mixed micelles (Table 3).

355

356 **3.5 Uptake of carotenoids by Caco-2 cells.**

357 The Caco-2 cells monolayers were incubated with carotenoid-rich natural mixed
358 micelles coming from the *in vitro* digestions of the test-meals containing the food sources of
359 carotenoids (carrot purée or yoghurts enriched with lyophilised mixtures of vegetative cells +
360 spores) and the uptake efficiencies of the carotenoids were compared. Note that no carotenoid
361 was detected in the basolateral media. This was not surprising because it is well known that
362 Caco-2 hardly secrete lipoproteins which are the carrier of carotenoids in the basolateral
363 medium. Note also that the following β-carotene metabolites: retinol, and retinyl palmitate,
364 were not detected in the cells, suggesting that the incubation conditions did not allow β,β-
365 carotene-15,15'-monooxygenase (Duszka, Grolier, Azim, Alexandre-Gouabau, Borel, &
366 Azais-Braesco, 1996; Grolier, Duszka, Borel, Alexandre-Gouabau, & Azais-Braesco, 1997;
367 Lobo, Amengual, Palczewski, Babino, & von Lintig, 2012) to significantly cleave β-carotene.
368 Both HPLC chromatograms showed the presence of native bacterial carotenoids and β-

369 carotene in the Caco-2 cell fractions. Uptake efficiency of β-carotene, HU36 carotenoids and
370 GB1 carotenoids were not significantly different and fell in the range 6-11% (Fig. 7).
371 To assess whether Caco-2 cell uptake was selective to some bacterial carotenoids, a
372 complementary experiment was conducted with synthetic mixed micelles. Indeed, synthetic
373 mixed micelles allowed incorporation of higher amounts of carotenoids, thus facilitating
374 identification. After 3 h incubation, all the bacterial carotenoids apparently exhibited similar
375 uptake efficiencies and did not undergo significant metabolism in Caco-2 cells. Indeed, HPLC
376 profiles were similar in synthetic mixed micelles and in Caco-2 cells (data not shown).
377

378 **3.6 Blood and tissue bacterial carotenoid responses, i.e. changes from initial**
379 **concentrations, to bacterial carotenoid gavages in rats.**

380 No carotenoids were detected either in rat plasma before the gavages or in plasma and
381 tissues of rats that were force-feed with carotenoid-free oil (data not shown). In addition,
382 retinyl palmitate concentrations, which can increase with provitamin A carotenoids, did not
383 significantly vary in either plasma or liver, either before or after gavage with the studied
384 carotenoid sources. Concentrations of carotenoids in plasma and tissues after 3-day gavage
385 with carotenoids are shown in table 4. Note that, for an unknown reason, the variabilities of
386 plasma and tissue concentrations of bacterial carotenoids were much higher than that β-
387 carotene. However it appears that all plasma, liver and adipose tissue concentrations of
388 bacterial carotenoids were higher than that of β-carotene.
389 Fig. 8 shows the total amounts and proportions of carotenoids recovered in plasma,
390 liver and adipose tissue. The total amount of bacterial carotenoids recovered in
391 plasma+liver+adipose tissue was significantly ($p<0.05$) higher after gavage with HU36
392 carotenoids (about 9 times as high) than with pure β-carotene. The total amount of GB1
393 carotenoids was about 2-3 times as high as β-carotene (not statistically significant likely

394 because of the high variability in bacterial carotenoid response). Bacterial carotenoids were

395 recovered in higher proportion in the adipose tissue (8-43%) than β-carotene (7%).

396

397 **4. DISCUSSION**

398

399 The objective of this study was to assess the bioavailability of glycosyl carotenoids
400 and glycosyl carotenoid esters from HU36 and GB1 *Bacillus* sp. strains. To that purpose,
401 complementary models were used to study the 3 key-steps that govern carotenoid
402 bioavailability: i) bioaccessibility, which was quantifed in a standard *in vitro* digestion model
403 (Fernández-García, Carvajal-Lérida, & Pérez-Gálvez, 2009; Garrett et al., 1999; Reboul et al.,
404 2006), ii) uptake efficiency by human intestinal cells, which was assessed by the widely used
405 Caco-2 cell model (Artursson and Karlsson, 1991; During, Albaugh, & Smith, 1998; Grès et
406 al., 1998), iii) distribution in tissues, which was assessed by a home-made force-feeding
407 model in rats with dietary doses of carotenoids (Sy et al., 2012). β-Carotene was selected as a
408 reference carotenoid.

409 The first limiting-step for carotenoid bioavailability is assumed to be their transfer into
410 mixed micelles in the duodenal lumen during digestion. Our results on the efficiency of
411 transfer of purified carotenoids from carotenoid powders to synthetic mixed micelles in water
412 (fig 6) clearly showed that HU36 and GB1 carotenoids were very efficiently incorporated into
413 synthetic mixed micelles, in fact much better than β-carotene. This is perhaps due to their
414 higher melting point brought about by their polar glycosyl moieties. Indeed, we have recently
415 shown that the incorporation efficiency of carotenoids in synthetic micelles was related to
416 carotenoid melting point (Sy et al., 2012). The very efficient transfer of bacterial carotenoids
417 to mixed micelles likely explains the higher bioaccessibility of these carotenoids, as compared
418 to β-carotene, in the *in vitro* digestion experiments (fig 5). Because bacterial carotenoids are
419 most likely to be developed as dietary supplements under the form of bacteria (probiotics)
420 (Hempel et al., 2012; Ma et al., 2010) it was interesting to compare the bioaccessibility of
421 these carotenoids either when they are embedded in their natural matrix, i.e. spores and

422 vegetative cells, or when they are extracted and purified. As expected, the bioaccessibility of
423 the bacterial carotenoids from lyophilized cells was only ca. twice as low as from pure
424 extracts. We hypothesize that this lower bioaccessibility was due to a less efficient release of
425 carotenoids from spores or vegetative cells, due to the double coat of *Bacillus* cells (plasmic
426 membrane + peptidoglycanes).

427 The second limiting-step for carotenoid bioavailability is assumed to be their
428 absorption by the intestinal cell. The first significant result of the caco-2 cell experiments was
429 that native, i.e. esterified and glycosylated, bacterial carotenoids were found in Caco-2 cell
430 fractions, and thus assumed to be absorbed. This was surprising because most glycosides and
431 esters of nutrients and micronutrients are hydrolyzed in the gastrointestinal tract lumen, and it
432 is assumed that only the free forms of the nutrients and micronutrients are absorbed by the
433 intestinal cell. Nevertheless this result is supported by the fact that native bacterial carotenoids
434 were also recovered in blood and tissue of rats. It is not known whether the mechanism of
435 uptake of bacterial carotenoids involves scavenger receptors, as reported for common
436 carotenoids (Moussa et al., 2011; Moussa et al., 2008; Reboul et al., 2005a; Reboul and Borel,
437 2011), but the similar absorption efficiency observed herein between bacterial carotenoids and
438 β-carotene suggests that similar mechanisms are involved. In fact this is in agreement with
439 recent results showing that structural differences in carotenoids do not have a significant
440 impact on the uptake efficiency of carotenoids in intestinal cells (Sy et al., 2012).

441 After absorption, carotenoids are mainly transported by the chylomicrons to the liver,
442 then to other tissues by lipoproteins (Tyssandier, Choubert, Grolier, & Borel, 2002). In the rat
443 experiments, much higher amounts of bacterial carotenoids than β-carotene were recovered in
444 the liver and in the adipose tissue. Because there was no significant conversion of carotenoids
445 in vitamin A in this experiment (as suggested by the lack of significant increase in liver
446 retinyl palmitate concentrations or plasma retinol concentrations following the β-carotene or

447 bacterial carotenoid gavages), we conclude that HU36 carotenoids were about nine times as
448 bioavailable as β-carotene and that GB1 carotenoids were about two to three times as
449 bioavailable as β-carotene. Moreover, by comparing these results with results on the
450 bioavailability of other carotenoids, results obtained with the same protocol by our team (Sy
451 et al., 2012), we concluded that HU36 carotenoids are also more bioavailable than
452 astaxanthin, lutein and lycopene. The very high concentration of HU36 carotenoids in tissues,
453 as compared to β-carotene, can be explained by their higher bioaccessibility, but also by a
454 very inefficient degradation of these carotenoids in the body. Although we were not able to
455 verify this hypothesis with the model we used, this raises the question of the potential
456 accumulation of these carotenoids in the body and its physiological consequences. Additional
457 studies are therefore necessary to study the metabolism of these bacterial carotenoids in
458 tissues and the potential biological effects of their metabolites.
459
460 On the whole, this study suggests that bacterial carotenoids from the *Bacillus* HU36
461 and GB1 strains are more bioavailable than common carotenoids. This higher bioavailability
462 apparently reflects their very high bioaccessibility, which is likely due to the presence of
463 glycosyl groups that increase their micellar solubility. Finally, significant amount of bacterial
464 carotenoids were recovered under their native forms in liver and adipose tissue of rats. Thus,
465 their long-term tissue accumulation, metabolism and potential bioactivity and toxicity in
466 humans deserve additional investigations.
467

468 **5. AUTHORS' CONTRIBUTION TO THE ARTICLE**

469

470 PB and CS designed the protocol and have primary responsibility for the final content. CS
471 performed all the experiments with the help of BG, for the rat experiments, and of SC, for the
472 microbiology experiments. CS and PB drafted the manuscript. All authors contributed to the
473 final version of the manuscript.

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FIGURES

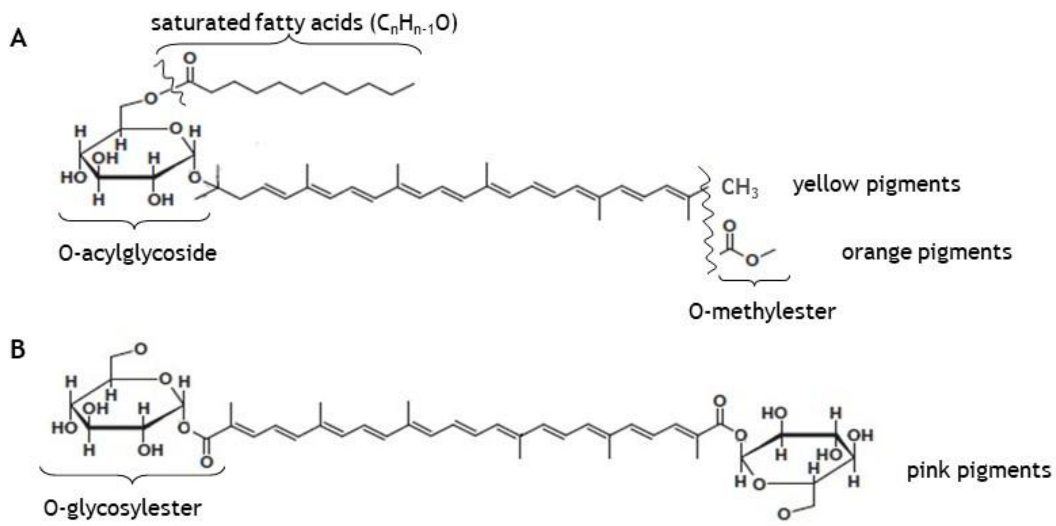

Figure 1: Chemical structures of the main carotenoids synthesised by **(A)** HU36 and **(B)** GB1 spore-forming strains.

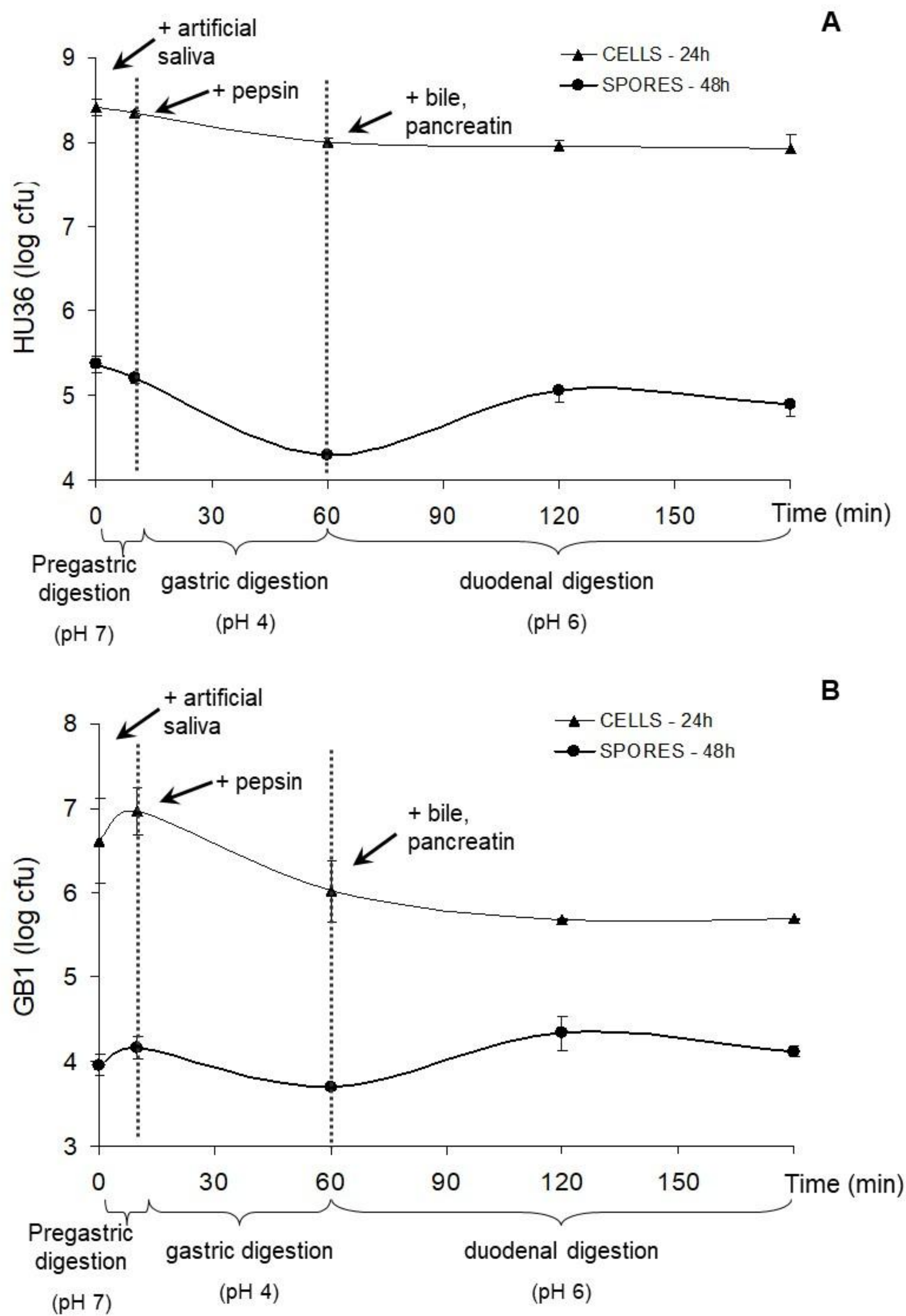

Figure 2: Evolution of the number of vegetative bacterial cells and of spores during *in vitro* digestion conducted with 32 mg lyophilized bacterial material of **(A)** HU36 and **(B)** GB1 strains. Means ± SD of three independent experiments. For more details see the material and methods section.

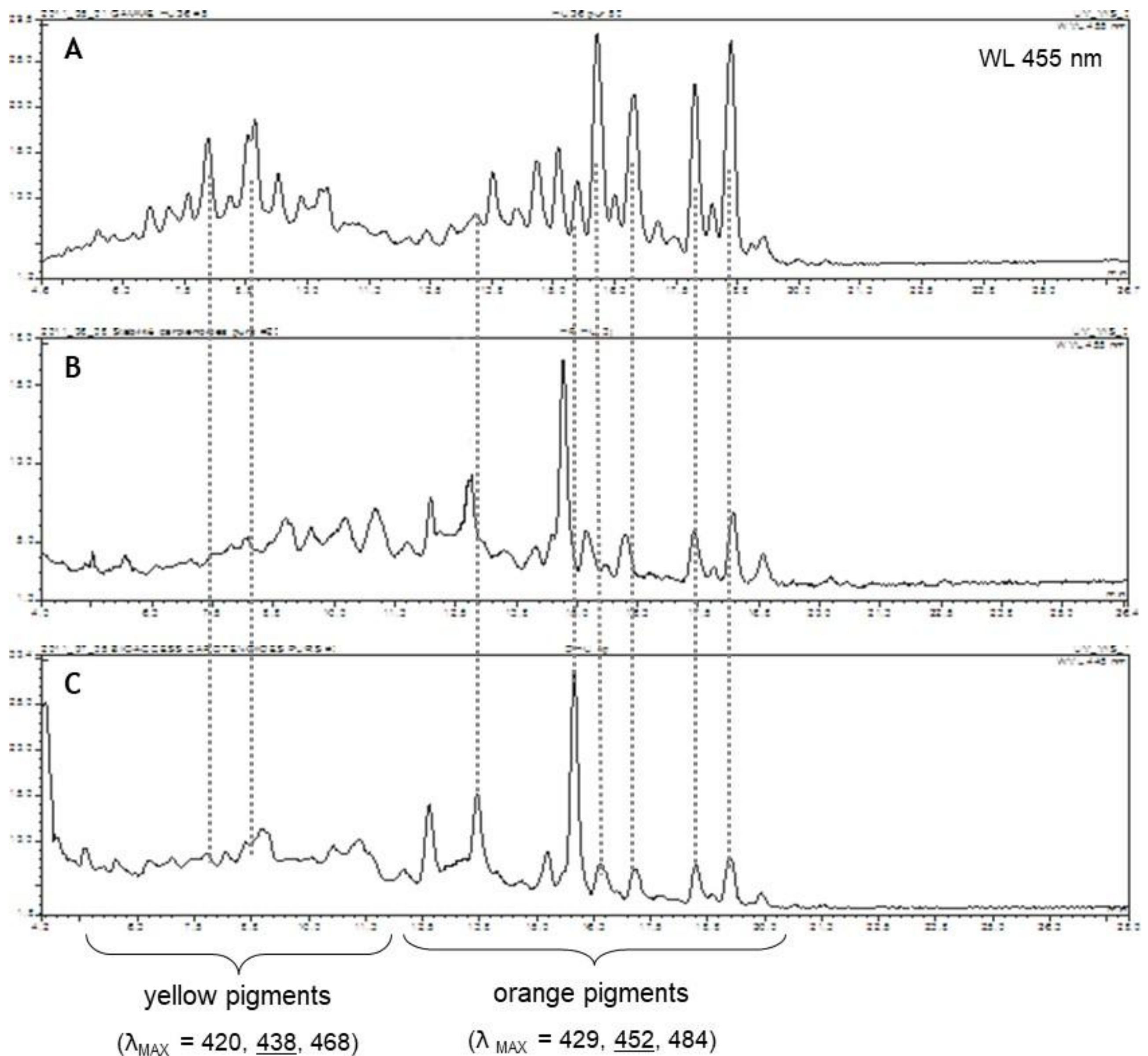

Figure 3: HPLC chromatograms with detection at 455 nm showing HU36 carotenoids in samples of **(A)** food mixture before *in vitro* digestion, **(B)** digestive medium at the end of the *in vitro* digestion, and **(C)** mixed micelle fraction at the end of the *in vitro* digestion. Dotted lines indicate similar compounds as suggested by their similar UV-visible spectra.

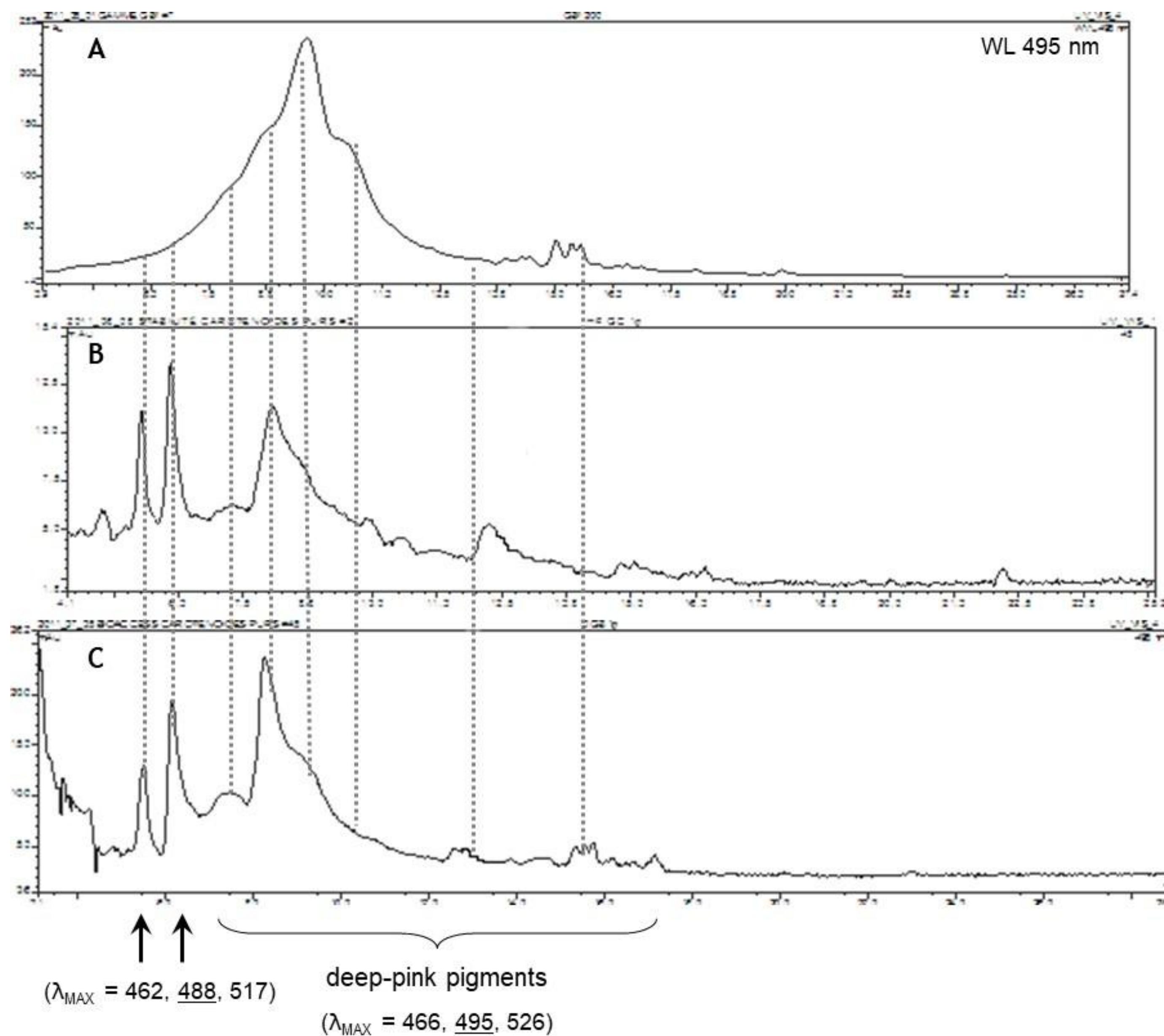

Figure 4: HPLC chromatograms with detection at 495 nm showing GB1 carotenoids in samples of **(A)** food mixture before *in vitro* digestion, **(B)** digestive medium at the end of the *in vitro* digestion, and **(C)** mixed micelle fraction at the end of the *in vitro* digestion. Dotted lines indicate similar compounds as suggested by their similar UV-visible spectra. Arrows show pigments that appeared at the end of the *in vitro* digestion.

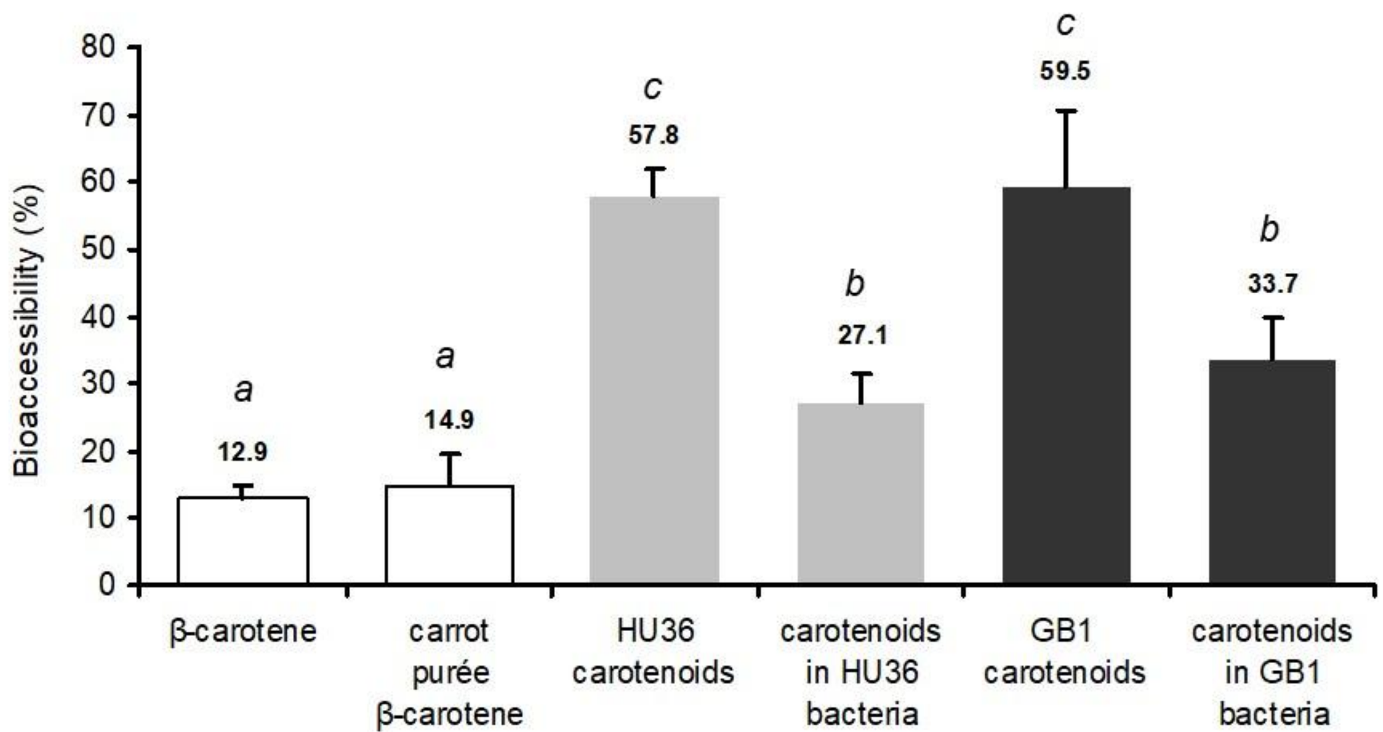

Figure 5: Bioaccessibility of β-carotene and bacterial carotenoids, either incorporated as pure molecules in oil or provided in their natural food matrix (carrot purée and bacteria), measured using the *in vitro* digestion model. Means ± SD of three independent experiments. Different letters indicate significant ($P < 0.05$) differences between means (ANOVA and Tukey-Kramer test).

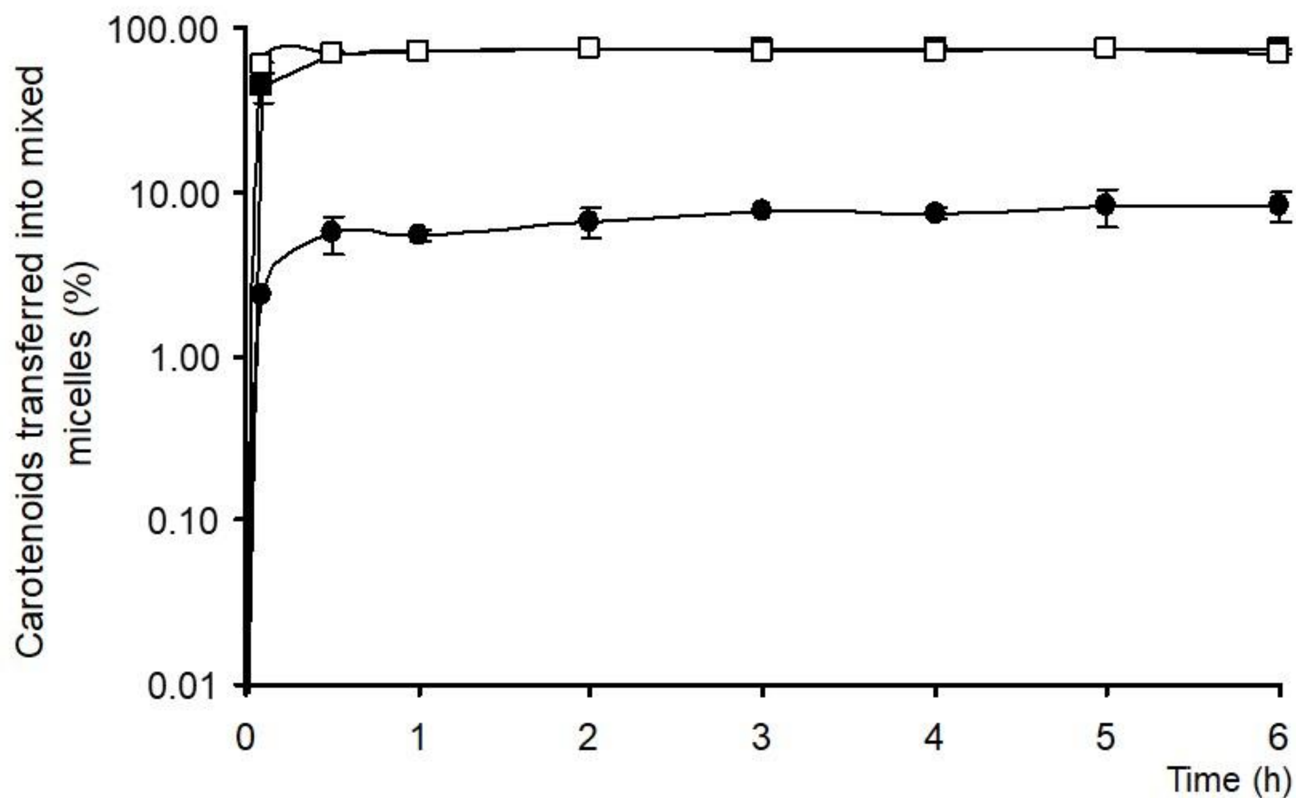

Figure 6: Amount of pure carotenoids transferred from carotenoid powders to synthetic mixed micelles solubilized in water as a function of time. **(●)** Pure β-carotene, **(■)** purified HU36 carotenoids and **(□)** purified GB1 carotenoids. Experiments performed protected from light at 37°C. Means ± SD of three independent experiments.

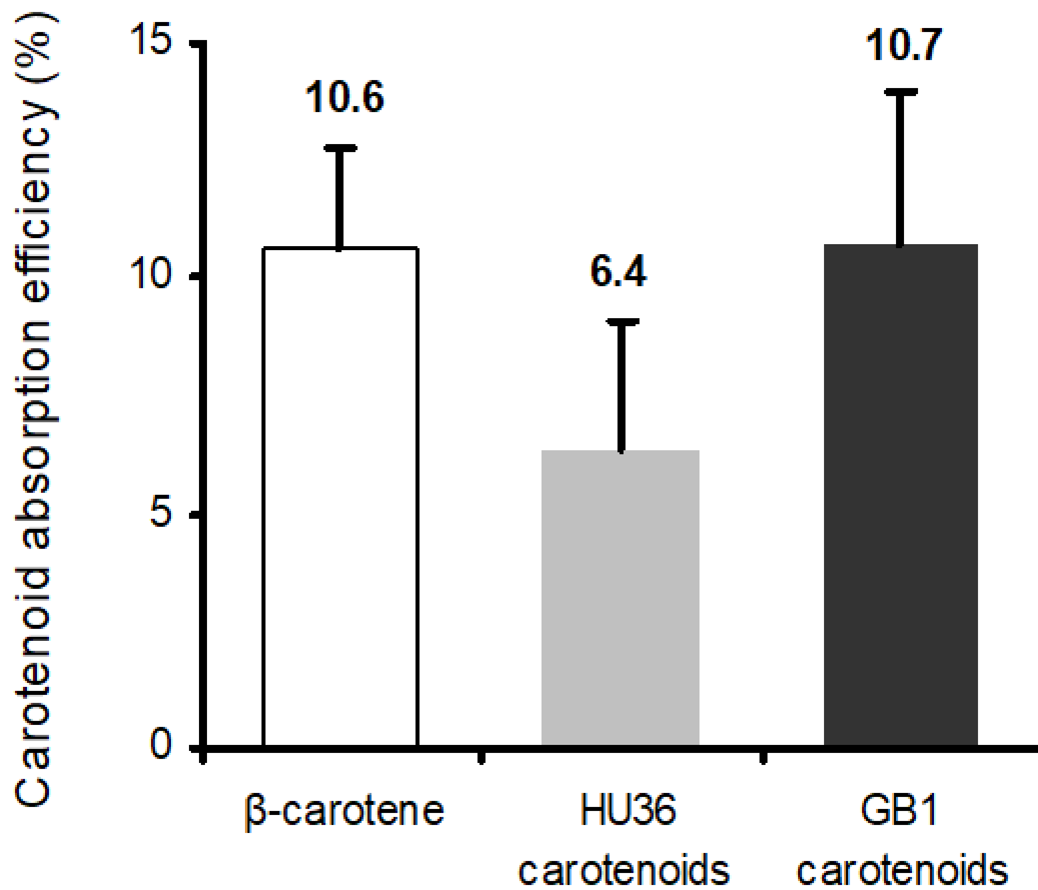

Figure 7: Uptake, by Caco-2 cell monolayers, of carotenoids incorporated in mixed micelles that came from *in vitro* digestion experiments, expressed as uptake efficiency (% of the carotenoid amount added onto cell monolayers). Means ± SD of three independent experiments. There was no significant ($P < 0.05$) differences between means (ANOVA and Tukey-Kramer test).

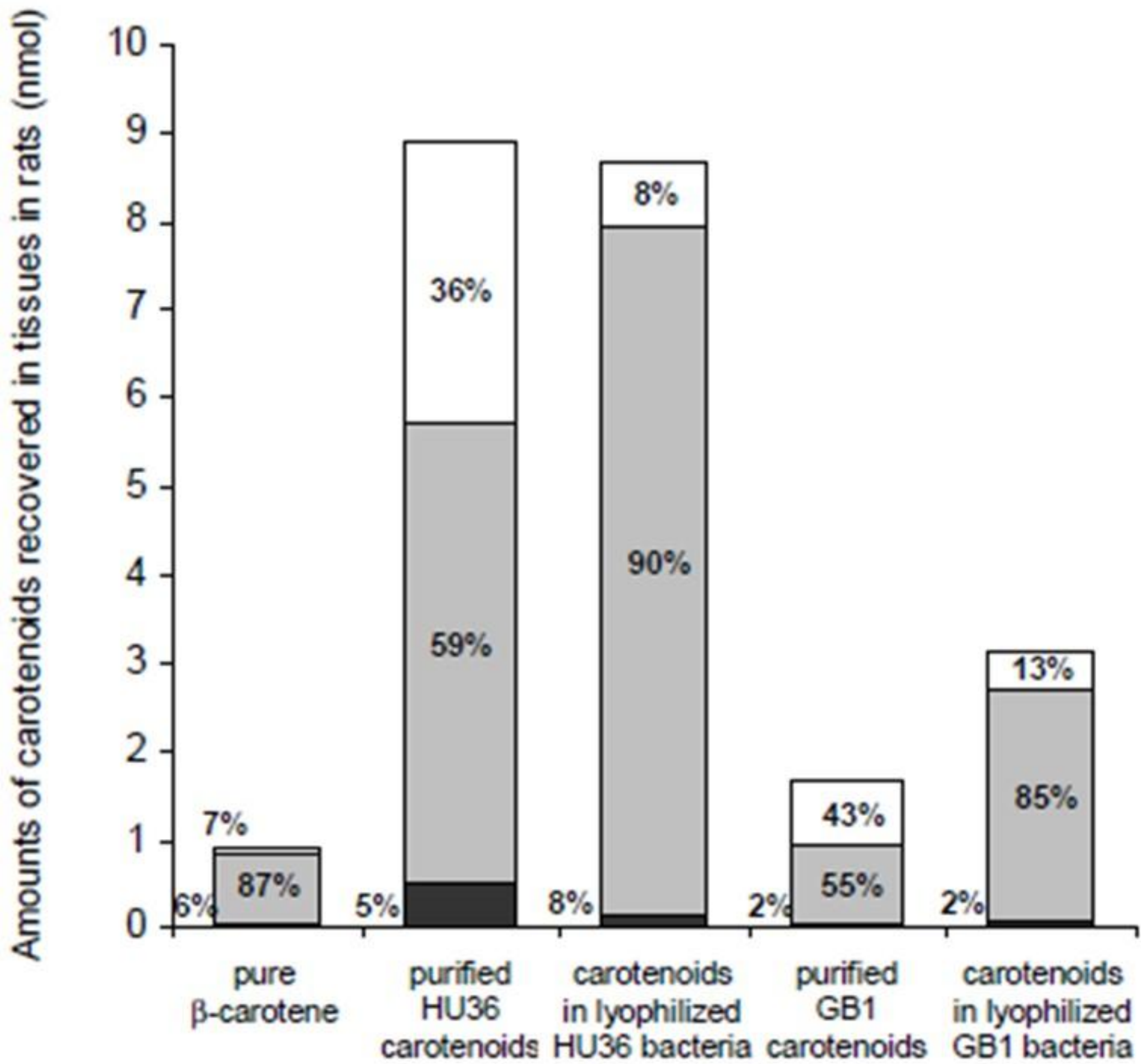

Figure 8: Amounts of carotenoids recovered in rat tissues after Wistar rats were force-fed for 3 days with either purified carotenoids or carotenoids in lyophilized bacteria. Numbers show the relative distributions (%) of these carotenoids between plasma (black section), liver (grey section) and adipose tissue (white section).