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

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ABSTRACT

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

1. Introduction

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

The potential health-promoting properties of carotenoids have led to substantial interest in carotenoids as nutritional supplements, especially from natural sources. β-Carotene is one of the naturally occurring carotenoids that have been most intensively studied. It can be used as a food colouring agent, as a dietary source of vitamin A (Krinsky & Johnson, 2005) and for other bioactive properties (Britton, Liaaen-Jensen, & Pfander, 2008; Peto, Doll, Buckley, & Sporn, 1981). Besides, bacterial producing carotenoids have gained interest in the past years due to their ability to act as probiotics (Hempel et al., 2012; Ma et al., 2010) and their potential to provide a suitable biosource of carotenoids with both C40 and C30 backbones (Perez-Fons et al., 2010) and with improved solubility and stability for the food and feed industry

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(Duc, Fraser, Tam, & Cutting, 2006). A diverse range of spore-forming *Bacillus* species has been isolated that contains various red, pink or yellow-orange carotenoid pigments. Amongst the *Bacillus* sp., the *B. indicus* HU36 and *B. firmus* GB1 strains are of particular interest for their high production of carotenoids, the resistance of their spores to UV radiations (Khaneja et al., 2010) and their amenable probiotic properties (Hong et al., 2008). The HU36 strain, which has been isolated from human faeces, synthesises yellow-orange pigments in variable proportions depending on whether they are in the vegetative or spore-forming state. The corresponding carotenoid extract displays maxima of visible absorption at 429, 454 and 485 nm (Khaneja et al., 2010). The most abundant pigments in the extract were determined as 1-glycosyl-3-4-dehydro-8′-apolycopene and methyl-1-glycosyl-3-4-dehydro-8′-apolycopenoate esters with saturated fatty acid secondary chains from C8 to C15 (Fig. 1A) (Perez-Fons et al., 2010). The GB1 strain, which has been isolated from human ileum, produces deep-pink pigments with maxima of visible absorption at 463, 492 and 524 nm (Khaneja et al., 2010), the main one being 4,4′-diglycosyl-4,4′-diapolycopenoic diester (Fig. 1B).

Knowledge on the metabolism of carotenoids in humans has been considerably increasing during the last ten years. It is now assumed that carotenoids have to be extracted from their vegetable matrix to be transferred into mixed micelles and efficiently absorbed. The efficiency of this transfer is called “bioaccessibility”. Several factors have been identified that affect carotenoid bioaccessibility (Borel, 2003; West & Castenmiller, 1998). Amongst these factors we have recently shown (Sy et al., 2012) that the chemical structure of carotenoids is a key-factor that modulates their solubility into mixed micelles. It has also been shown that the matrix in which carotenoids are embedded can play a significant role on bioaccessibility (Gartner, Stahl, & Sies, 1997; Reboul, Borel, et al., 2005; Stahl & Sies, 1992; Sy et al., 2012). After being incorporated into mixed micelles, carotenoids are transported to the enterocyte, where they are absorbed *via* membrane transporters (Reboul & Borel, 2011). It is not yet known how these transporters facilitate the uptake of carotenoids and it has been hypothesised that carotenoid chemical structure might play a role in the interaction between carotenoid and their transporters.

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

The aim of this study was therefore to compare the bioaccessibility, the uptake by intestinal cell, and the tissue distribution of HU36 and GB1 carotenoids, with β-carotene.

2. Material and methods

2.1. Supplies and chemicals

Canned steamed carrots (Daucy, Vannes, France) were purchased from a local supermarket in Marseille (France) and chosen without antioxidants, acidifiers or preservatives. Plain yogurt was from Danone (Paris, France) and was purchased from a local supermarket in Marseille (France). Mixtures of lyophilised spores and vegetative cells from *Bacillus* strains (*Bacillus* indicus HU36 and *Bacillus* firmus GB1) were provided by Paul Fraser and Reena Khaneja (Royal Holloway University of London) in the frame of the COLORSPORE project (European Small Collaborative Project No. 207948, FP7) (Duc et al., 2006). Pure all-*E* β-carotene (>95%) was kindly provided by DSM LTD (Basel, Switzerland). Salts ($NaHCO_3$, NaCl, KCl, $CaCl_2$, $2H_2O$ and K_2HPO_4), mucin, α-amylase, pepsin, porcine pancreatin, porcine bile extract, 2-Oleoyl-1-palmitoyl-*sn*-glycero-3-phosphocholine (phosphatidylcholine), 1-palmitoyl-*sn*-glycero-3-phosphocholine (lysophosphatidylcholine), monoolein, free cholesterol, oleic acid, sodium taurocholate, pyrogallol (used as antioxidant preservative) and apo-8′-carotenal (>95%) were purchased from Sigma-Aldrich (St Quentin-Fallavier, France). FBS (foetal bovine serum) was purchased from Biomedia (Issy-les-Moulineaux, France). DMEM (Dulbecco's Modified Eagle Medium) containing 4.5 g/L glucose and trypsin/EDTA (500 and 200 mg/L respectively), non-essential amino acids, penicillin/streptomycin, PBS (phosphate buffered saline solution) and PBS containing 0.1 mmol/L $CaCl_2$ and 1 mmol/L $MgCl_2$ (PBSCM) were from Invitrogen (Cergy-Pontoise, France). Hexane, methanol, dichloromethane and methyl-*t*-butylether (MTBE) were of HPLC grade and purchased from Carlo Erba Reactifs SDS (Val-de-Reuil, France). The small kit of the Uptima bicinchoninic acid (BCA) assay for protein quantification was from Interchim (Montluçon, France).

2.2. Test meals used in the in vitro bioaccessibility studies

Experiments were performed twice for each carotenoid (or carotenoid mixture); first using pure solid β-carotene and purified bacterial extracts (>90% pure), which were purified by liquid chromatography

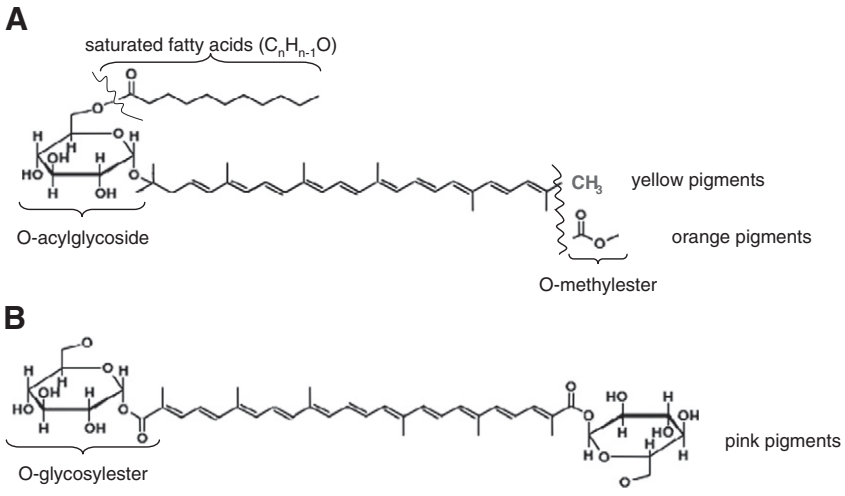

Fig. 1. Chemical structures of the main carotenoids synthesised by (A) HU36 and (B) GB1 spore-forming strains. Most HU36 carotenoids are oxygenated derivatives of apolycopene, which have undergone glycosylation and/or esterification. The most abundant apocarotenoids are glycosyl-apolycopene and glycosyl-4′-methyl-apolycopenoate esters (Perez-Fons et al., 2010). Concerning GB1 carotenoids, the main one is 4,4′-diglycosyl-4,4′-diapolycopenoic ester (Khaneja et al., 2010).

before the study (Perez-Fons et al., 2010; Steiger, Perez-Fons, Fraser, & Sandmann, 2012), in order to measure the intrinsic bioaccessibility of bacterial carotenoids, in other words without the effect of the food matrix. In a second time experiments were performed with foods rich in the studied carotenoids, in order to measure the bioaccessibility of bacterial carotenoids when they are digested in their bacteria matrix. For experiments with pure carotenoids, stock solutions of carotenoids in hexane were dispersed in commercial groundnut oil, which did not contain quantifiable amounts of carotenoids as checked by HPLC, and the solvent evaporated to obtain carotenoid concentrations of *ca.* 750 μmol/L in oil. The first digestions were conducted on the oil alone, whilst for experiments with foods, the food sources of carotenoids were codigested with a standard meal (Table 1). This was done in order to be in nutritional conditions where carotenoid sources are usually ingested together with other foods. Foods were chosen in order that i) they did not contained detectable amount of carotenoids (checked by HPLC), and ii) their mix gave a meal composition in macronutrients (lipids, carbohydrates and proteins) that was close to the US-dietary recommended intake in macronutrients (see Table 1 legend). Potatoes were boiled in tap water, peeled, and hand-pureed. Meat was fried in a frying-pan without added fat. Potato purée and fried meat were divided into aliquots and frozen at −20 °C. The food source of β-carotene was commercial carrot purée. Lyophilised HU36 and GB1 cells + spores were homogenised in plain yogurts (see Table 1 legend).

2.3. In vitro digestion experiments for assessing carotenoid bioaccessibility

Bioaccessibility was defined as the percentage of carotenoids recovered in the micellar fraction after *in vitro* digestion, in relation to the amount of carotenoids measured in the digestive medium just before addition of artificial saliva. To measure carotenoid bioaccessibility we used the *in vitro* digestion model developed by Garrett, Failla, and Sarama (1999). The experimental conditions were slightly modified (Reboul et al., 2006) to better take into account the physicochemical conditions prevailing in the human gastrointestinal lumen (Tyssandier et al., 2003) and to reduce as much as possible the amount of triglycerides left at the end of the digestion experiments (Sy et al., 2012). Meal components, or only groundnut oil when the bioaccessibility of carotenoids was studied without the effect of the food matrix (Table 1), was mixed with 32 mL NaCl 0.9% and homogenised in a shaking water bath at 37 °C for 10 min. The next steps of the *in vitro* digestion procedure are described in detail in a recent publication of our team (Sy et al., 2012). Each experiment was run in triplicate.

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

Table 1
Composition of the test meals used in the *in vitro* digestion experiments.

Test meal	Component(s)	Amount (g)[a]
With purified carotenoids	- groundnut oil[b]	0.1 ± 0.02
With carotenoids in foods or bacteria[c]	- potato purée	6.7 ± 0.02
	- beef meat	1.2 ± 0.02
	- olive oil	0.1 ± 0.02
	- foods or bacteria[d]	4.0 ± 0.02

[a] Mean ± SD of the amounts added in three experiments.
[b] Containing about, but precisely determined, 40 μg β-carotene (all-*E* β-carotene >95%, synthetic) or purified bacterial carotenoids.
[c] The test meal without the carotenoid-rich source contained 64.5% energy as carbohydrates, 14.5% as fat, and 21.0% as proteins. These proportions are close to the dietary recommended intake in macronutrients, *i.e.*, 45–65% carbohydrates, 20–35% fat, and 10–35% proteins. The macronutrient composition and % of energy were estimated by using dietary software (Nutrilog 1.3, Nutrilog SAS, Marans, France).
[d] As carotenoid sources. Carrot purée (cooked common commercial food) provided 44.2 ± 5.8 μg β-carotene. Natural yogurts provided bacteria carotenoids (1 g lyophilised bacteria were incorporated in 125 g yogurt). Test meals contained either 30.3 ± 0.9 μg HU36 carotenoids or 38.0 ± 1.0 μg GB1 carotenoids.

2.4. Monitoring of the bacterial material during in vitro digestion

Aliquots (250 μL) were taken into the digestion medium at regular time intervals: t = 0 min (bacteria just added into the test meal), t = 10 min (after addition of artificial saliva), t = 70 min (the end of gastric digestion), t = 130 min (mid-term of duodenal digestion), and t = 190 min (the end of duodenal digestion). Samples were serially diluted in sodium phosphate buffer (0.2 M, pH 7.0) and 100 μL was manually spread on Luria-Bertani (LB) agar (Sigma-Aldrich, Steinheim, Germany) plates in duplicate. After 24 and 48 h incubation at 37 °C, concentrations of total bacterial population were expressed as colony forming units per millilitre (CFU/mL). The limit of detection was taken as one colony on the lowest dilution plate (10 CFU/mL). To determine spore concentrations, the samples were additionally heated at 75 °C for 10 min previously to serial dilution in phosphate buffer and duplicate spreading on LB agar plates. In a separate test, the tolerance of HU36 and GB1 spores to porcine bile was estimated by following changes in bacterial counts in gastric media containing concentrations of porcine bile ranging from 8.09 g/L to 80.90 mg/L (a control did not contain bile salts). After 48 h incubation at 37 °C, spore concentrations were expressed as CFU/mL. The limit of detection was taken as one colony on the lowest dilution plate (10 CFU/mL).

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

This experimentation was carried out in order to measure the solubility of bacterial carotenoids in micelles formed during human digestion. Synthetic mixed micelles, which mimic the lipid composition of those found in the human gastrointestinal tract, and which were either free from carotenoids or which contained either pure β-carotene or bacterial carotenoids, were prepared as previously described (Reboul, Abou, et al., 2005; Sy et al., 2012). Synthetic mixed micelle sizes were checked by photon correlation spectroscopy (Zetasizer Nano Zs, Malvern Instruments, Malvern, UK) (Sy et al., 2012).

The maximal amount of either β-carotene or purified bacterial carotenoids that can be incorporated into a fixed amount of synthetic mixed micelles was measured as previously described (Sy et al., 2012).

To measure the transfer of either β-carotene, or purified bacterial carotenoids, from their solid form into synthetic mixed micelles solubilised in water we used a protocol described in detail in a recent publication (Sy et al., 2012).

The solubility of the carotenoids in synthetic mixed micelles was defined as the percentage of carotenoids recovered in the micellar fraction, in relation to the total carotenoids present in the medium.

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

Caco-2 clone TC-7 cells, which were a gift from Dr M. Rousset (UMR S872, Paris, France), were grown as recently described (Sy et al., 2012). Carotenoid uptake experiments were also described in detail in this publication (Sy et al., 2012). In summary the apical side of the cell monolayers received 1 mL carotenoid-rich natural mixed micelles, *i.e.* mixed micelles coming from the *in vitro* digestion of test meals containing the food sources of carotenoids (carrot puree or yogurt enriched in bacterial lyophilised material). Cell monolayers were incubated at 37 °C for 3 h. After the incubation period, media from each side of the membrane were harvested. Cell monolayers were washed twice with 1 mL PBS, scraped, and collected in 500 μL PBS. All the samples were stored at −80 °C under nitrogen until carotenoid extraction and HPLC analysis. Uptake efficiency of the carotenoids was defined as the percentage of carotenoids recovered in scraped Caco-2 cells, in relation to the amount of carotenoids initially added on the apical sides of the cell monolayers.

2.7. Measurement of carotenoid bioavailability in an animal model

The animal model to study carotenoid bioavailability was a multiple-dose gavage experiment lasting 3 days in young male albino Wistar rats (Sy et al., 2012). Multiple gavages over 3 days were preferred to dietary supplementation for several weeks because: i) the amounts of bacterial carotenoids available were limited, ii) gavage allows a better control of the amounts of carotenoids ingested by the rats, and iii) gavage limits carotenoid oxidation that can occur in foods given to rats and stored at room temperature. Experiments were conducted according to animal ethics rules and were approved by the Aix-Marseille University experimental animal ethic committee. Besides, the innocuousness of the bacterial carotenoid extracts and of the bacterial strains producing the carotenoids were studied by other partners of the project (Hong et al., 2008). Six groups of 8 rats were included in the study: a control group was force-fed with 1 mL groundnut oil without carotenoids, three groups were force-fed with the same amount of oil-containing purified carotenoids (pure β-carotene, purified HU36 carotenoids, or purified GB1 carotenoids), and two groups were force-fed with yoghurts containing either HU36 or GB1 lyophilised bacteria. The gavage protocol was described in detail recently (Sy et al., 2012). The doses of carotenoid sources incorporated in oil and yoghurts and given to the rats were chosen so as to bring 0.15 mg carotenoid/kg/day (Sy et al., 2012), which correspond to 10.5 mg/day for a man of 70 kg. This dose of carotenoid remains nutritional as it is close to the daily total carotenoid intake, which is estimated at 14 mg/day in Europe (O'Neill et al., 2001). The last gavage experiment was carried out on fasting rats (Sy et al., 2012). Rats were killed exactly 4 h later, which has been shown to be the time of maximal concentration of carotenoids in blood (Mathews-Roth et al., 1990). Blood and tissue samples (liver and adipose tissue) were collected. The blood samples were collected in tubes with heparin and immediately centrifuged in order to separate the plasma. All the plasma and tissue samples were immediately plunged into liquid N_2 and kept at −80 °C until carotenoid analysis.

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

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

2.9. HPLC analysis of the carotenoids

After evaporation to dryness, all dried extracts were dissolved in 200 μL methanol-CH_2Cl_2 (65:35, v/v). Carotenoids were quantified as recently described (Gleize, Steib, Andre, & Reboul, 2012; Sy et al., 2012) by reverse-phase HPLC on a Dionex system (equipped with in line degasser, a P680 pump, a cooled automatic sample injector ASI-100 and a UV/visible diode-array detector UVD340U, Dionex France, Voisins-le-Bretonneux, France). Carotenoids and apo-8′-carotenal (used as internal standard to calculate recovery yield during carotenoid extraction) were detected at their wavelength of maximal absorption (455 nm for β-carotene and HU36 carotenoids and 495 nm for GB1 carotenoids). Bacterial carotenoids in micelles, cells and tissue samples were identified and quantified by their HPLC chromatogram and UV–visible spectrum (from 300 to 550 nm) in comparison with the HPLC chromatograms and UV–visible spectra of the purified molecules. Analytical curves were performed with the purified bacterial carotenoids. Quantification was performed using Dionex Chromeleon software (Dionex Chromatography Management system, version 6.80). The linear range, regression coefficient >0.95, ranged between 1 ng and 200 ng. Based on the extraction method and the UV limit of quantification (signal-to-noise ratio >5), it was possible to quantify vitamin A (retinol and retinyl palmitate) down to 0.003 μg per injection, β-carotene down to 0.001 μg, and bacterial carotenoids down to 0.015 μg. The limits of detection of these molecules were around 2 ng per injection. The extinction coefficients of the purified carotenoid mixtures were calculated after dilution of the mixtures in dichloromethane and OD measurements at 455 and 495 nm for the HU36 and GB1 carotenoids, respectively. The values obtained were 165 000 and 225 300 $L \cdot mol^{-1} \cdot cm^{-1}$. The estimated mw of the bacterial carotenoid mixtures were 750 and 784 g/mol for the HU36 and GB1 carotenoids, respectively.

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

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

2.11. Calculations and statistics

All the *in vitro* experiments were run in triplicate. Results were expressed as means and standard deviations. Differences between means were assessed using ANOVA followed by the *post-hoc* Tukey–Kramer's test for parametric data (*in vitro* results). In the case of non-parametric data (*in vivo* results), they were assessed using the Kruskal–Wallis' test followed by the Mann–Whitney *U*-test when the Kruskal-Wallis test showed significant differences between groups. *P* values under 0.05 were considered significant. Statistical comparisons were performed using StatView software version 5.0 (SAS Institute Inc., Cary, NC).

3. Results

3.1. Fate of HU36 and GB1 bacteria during digestion

Most bacteria were present as vegetative cells in the lyophilised bacterial mixture, with only around 1‰ bacteria as spores in both strain preparations. Initial concentrations of viable cells were about 8.2×10^6 CFU/mg ($= 2.6 \times 10^8$/32 mg) lyophilised material for HU36 and 1.8×10^5 CFU/mg lyophilised material for GB1. Initial concentrations of spores were 7.5×10^3 CFU/mg lyophilised material for HU36 and 2.9×10^2 CFU/mg lyophilised material for GB1. The number of viable cells and spores decreased during the gastric digestion (Fig. 2B). During the duodenal digestion the number of GB1 cells continued to diminish in the first hour than remained stable during the second hour. The spore concentrations remained relatively unchanged (final spore populations were 8×10^4 CFU for HU36 and 1×10^4 CFU for GB1), showing in particular a rather low germination. Final vegetative cell populations were 9×10^7 CFU for HU36 and around 5×10^5 CFU for GB1 (66% and 92% viability loss, respectively) (Fig. 2, A & B).

3.2. Stability of bacterial carotenoids during digestion

HPLC chromatograms suggest that both yellow and orange HU36 glycosyl carotenoid esters were transformed during the *in vitro* digestion experiment (Fig. 3, B *vs* A). The digestion of GB1 carotenoids led to 2 new pigments (Fig. 4, B *vs* A), which were likely less hydrophobic (more polar) than the native carotenoids because they eluted earlier. New products could result from autoxidation (oxidation by O_2 initiated by metal traces) and/or (in the case of the bacterial carotenoids) hydrolysis of sugar and acyl moieties by the digestive enzymes. GB1 carotenoids were the most stable with 81.0 ± 0.6% of the initial carotenoids recovered at the end of digestion. The residual amounts of β-carotene and native HU36 carotenoids were identical: 69.5 ± 1.2% and 69.5 ± 4.3% respectively.

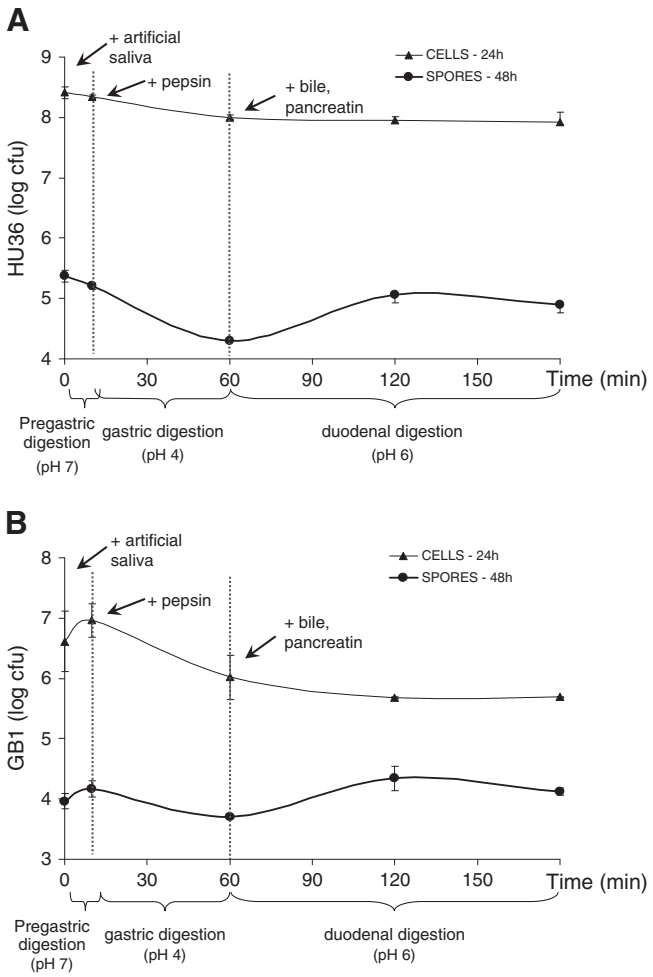

Fig. 2. Evolution of the number of vegetative bacterial cells and of spores during *in vitro* digestion conducted with 32 mg lyophilised bacterial material of (A) HU36 and (B) GB1 strains. Means ± SD of three independent experiments. For more details see the Material and methods section.

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

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

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

3.4. Incorporation of purified bacterial carotenoids into synthetic mixed micelles

First of all we measured the solubility of purified bacterial carotenoids in water because we suspected that they are partially soluble in water due to their glycosid groups. The values measured were *ca.* 0.25 and 0.16 μmol/L for HU36 and GB1, respectively. These values are of course higher than that of β-carotene (essentially insoluble in water) but markedly lower than their solubility in synthetic micelles. Indeed, when homogenised with phospholipids, cholesterol and glycerol esters (mixed micelle lipids) before the formation of synthetic mixed micelles, incorporation efficiencies of purified HU36 carotenoids and GB1 carotenoids were over 90% (Table 2). This was significantly higher than that of pure β-carotene (about 50%).

The percentage *versus* time of transfer of pure carotenoids from carotenoid powders to pre-formed synthetic mixed micelles in water is shown in Fig. 6. In all cases, the maximal solubilisation rates were nearly reached in the first 30 to 60 min. The maximal transfer of carotenoids measured at the plateau revealed that bacterial carotenoids were significantly more transferred from powder into synthetic mixed micelles (over 70%) than β-carotene (around 8%). Moreover, maximal percentage values of carotenoids transferred into synthetic mixed micelles were significantly lower than in the incorporation of carotenoids during the preparation of synthetic mixed micelles (Table 2).

3.5. Uptake of carotenoids by Caco-2 cells

The Caco-2 cells monolayers were incubated with carotenoid-rich natural mixed micelles coming from the *in vitro* digestions of the test meals containing the food sources of carotenoids (carrot purée or yoghurts enriched with lyophilised mixtures of vegetative cells + spores) and the uptake efficiencies of the carotenoids were compared. Note that no carotenoid was detected in the basolateral media. This was not surprising because it is well known that Caco-2 hardly secrete lipoproteins which are the carrier of carotenoids in the basolateral medium. Note also that the following β-carotene metabolites: retinol, and retinyl palmitate, were not detected in the cells, suggesting that the incubation conditions did not allow β,β-carotene-15,15′-monooxygenase (Duszka et al., 1996; Grolier, Duszka, Borel, Alexandre-Gouabau, & Azais-Braesco, 1997; Lobo, Amengual, Palczewski, Babino, & von Lintig, 2012) to significantly cleave β-carotene. Both HPLC chromatograms showed the presence of native bacterial carotenoids and β-carotene in the Caco-2 cell fractions. Uptake efficiency of β-carotene, HU36 carotenoids and GB1 carotenoids was not significantly different and fell in the range 6–11% (Fig. 7).

To assess whether Caco-2 cell uptake was selective to some bacterial carotenoids, a complementary experiment was conducted with synthetic mixed micelles. Indeed, synthetic mixed micelles allowed incorporation of higher amounts of carotenoids, thus facilitating identification. After 3 h incubation, all the bacterial carotenoids apparently exhibited similar uptake efficiencies and did not undergo significant metabolism in Caco-2 cells. Indeed, HPLC profiles were similar in synthetic mixed micelles and in Caco-2 cells (data not shown).

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

No carotenoids were detected either in rat plasma before the gavages or in plasma and tissues of rats that were force-feed with carotenoid-free oil (data not shown). In addition, retinyl palmitate concentrations, which can increase with provitamin A carotenoids, did not significantly vary in either plasma or liver, either before or after gavage with the studied carotenoid sources. Concentrations of carotenoids in plasma and tissues after 3-day gavage with carotenoids are shown in Table 3. Note that, for an unknown reason, the variabilities of plasma and tissue concentrations of bacterial carotenoids were much higher than that β-carotene. However it appears that all plasma, liver and adipose tissue concentrations of bacterial carotenoids were higher than that of β-carotene.

Fig. 8 shows the total amounts and proportions of carotenoids recovered in plasma, liver and adipose tissue. The total amount of bacterial carotenoids recovered in plasma + liver + adipose tissue was significantly ($p < 0.05$) higher after gavage with HU36 carotenoids (about 9

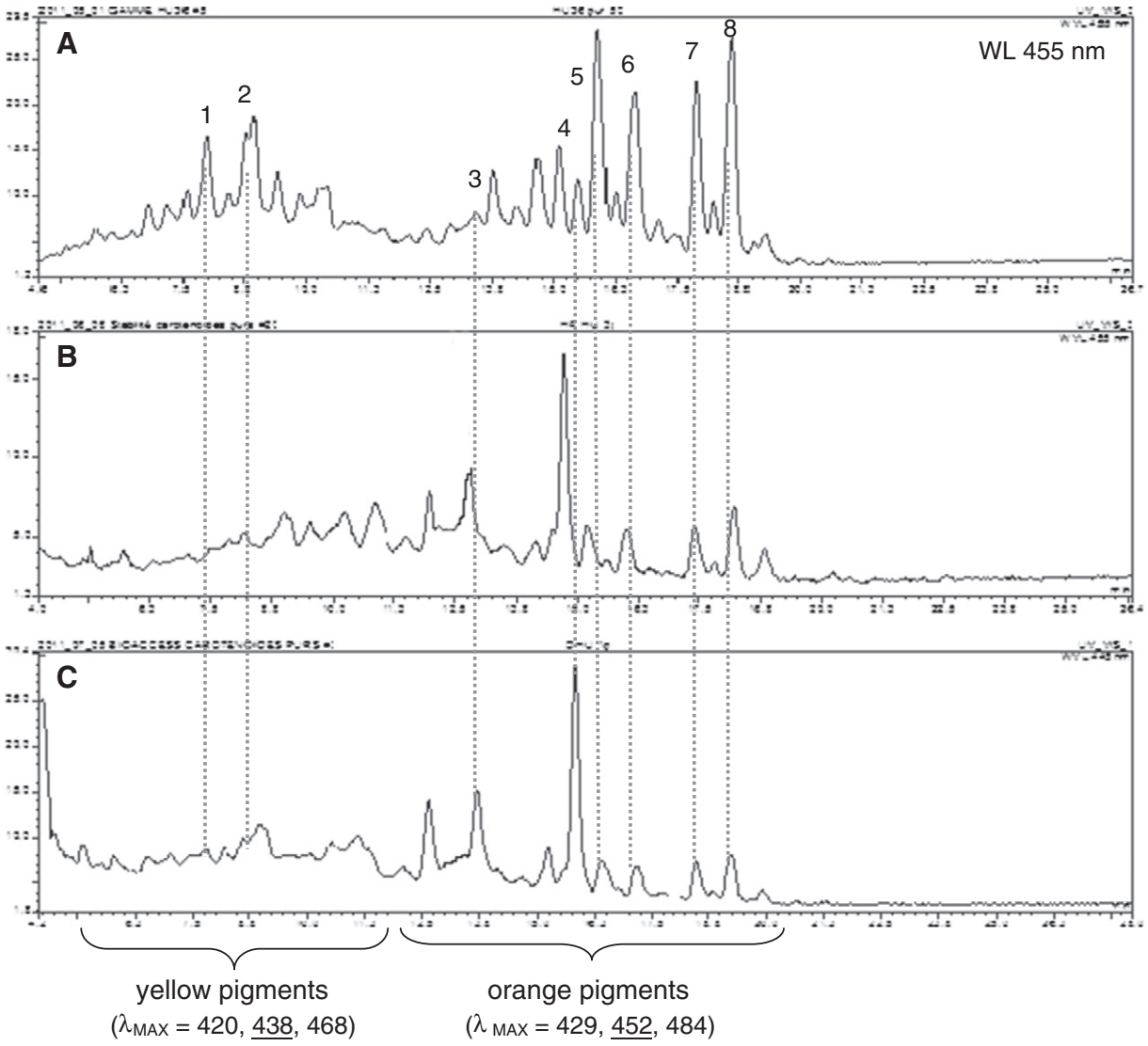

Fig. 3. HPLC chromatograms with detection at 455 nm showing HU36 carotenoids in samples of (A) food mixture before *in vitro* digestion, (B) digestive medium at the end of the *in vitro* digestion, and (C) mixed micelle fraction at the end of the *in vitro* digestion. Dotted lines indicate similar compounds as suggested by their similar UV–visible spectra characteristics. λ max peak n°1 (410, 436, 464); n°2 ((cis) 264, 420, 439, 465); n°3 ((cis) 343, 449, 476), n°4 ((cis) 345, 426, 448, 477), n°5 (420, 450, 474), n°6 ((cis) 268, 435, 466, 491), n°7 ((cis) 280, 429, 452, 482), n°8 ((cis) 280, 431, 453, 483).

times as high) than with pure β-carotene. The total amount of GB1 carotenoids was about 2–3 times as high as β-carotene (not statistically significant likely because of the high variability in bacterial carotenoid response). Bacterial carotenoids were recovered in higher proportion in the adipose tissue (8–43%) than β-carotene (7%).

4. Discussion

The objective of this study was to assess the bioavailability of glycosyl carotenoids and glycosyl carotenoid esters from HU36 and GB1 *Bacillus* sp. strains. To that purpose, complementary models were used to study the 3 key-steps that govern carotenoid bioavailability: i) bioaccessibility, which was quantified in a standard *in vitro* digestion model (Fernández-García, Carvajal-Lérida, & Pérez-Gálvez, 2009; Garrett et al., 1999; Reboul et al., 2006), ii) uptake efficiency by human intestinal cells, which was assessed by the widely used Caco-2 cell model (Artursson & Karlsson, 1991; During, Albaugh, & Smith, 1998; Grès et al., 1998), and iii) distribution in tissues, which was assessed by a home-made force-feeding model in rats with dietary doses of carotenoids (Sy et al., 2012). β-carotene was selected as a reference carotenoid. Groundnut oil was chosen as a source of dietary fat in all the experiments because it is rich in long-chain fatty acids, which are the main fatty acids present in usual Western diet, and because it does not contain detectable amount of carotenoids that can interfere with absorption of the studied carotenoids (Reboul, Abou, et al., 2005).

The first observation of this study was that there was a significant metabolism of the bacterial carotenoids during the *in vitro* digestion. Indeed, although some peaks of the native bacterial carotenoids remained present in the digestive medium and in the micelles at the end of digestion, some peaks diminished, some increased, and new peaks appeared. Nevertheless we had no mass spectrometry facility to identify the different compounds.

The first limiting-step for carotenoid bioavailability is assumed to be their transfer into mixed micelles in the duodenal lumen during digestion, which is called their bioaccessibility. The observation that HU36 and GB1 carotenoids were very efficiently incorporated into synthetic mixed micelles, in fact much better than β-carotene, is

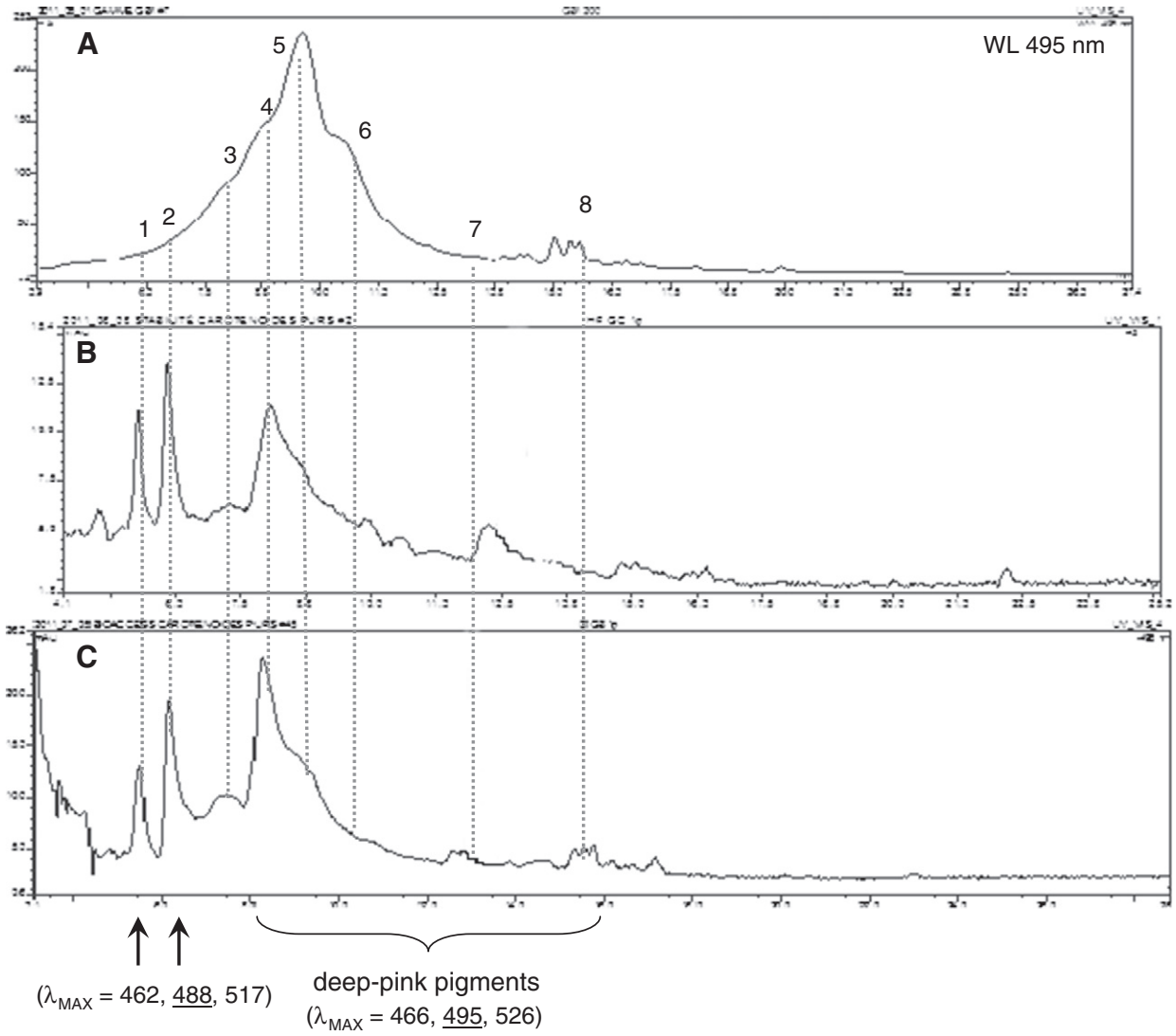

Fig. 4. HPLC chromatograms with detection at 495 nm showing GB1 carotenoids in samples of (A) food mixture before *in vitro* digestion, (B) digestive medium at the end of the *in vitro* digestion, and (C) mixed micelle fraction at the end of the *in vitro* digestion. Dotted lines indicate similar compounds as suggested by their similar UV–visible spectra characteristics. λ max peak n°1 ((cis) 310, 462, 488, 517); n°2 ((cis) 383, 462, 488, 517); n°3 ((cis) 260, 380, 460), n°4 ((cis) 320, 460, 495, 525), n°5 ((cis) 320, 460, 495, 527), n°6 (460, 495, 523), n°7 (472, 498, 537), n°8 (430, 465, 510). Arrows show pigments that appeared at the end of the *in vitro* digestion.

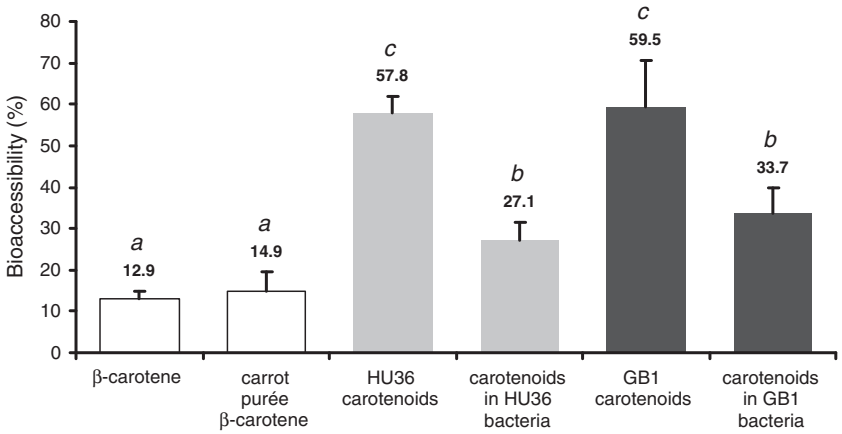

Fig. 5. Bioaccessibility of β-carotene and bacterial carotenoids, either incorporated as pure molecules in oil or provided in their natural food matrix (carrot purée and bacteria), measured using the *in vitro* digestion model. Means ± SD of three independent experiments. Different letters indicate significant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

Table 2
Incorporation efficiency and transfer efficiency of different carotenoids.

	β-carotene	HU36 carotenoids	GB1 carotenoids
Incorporation efficiency (%)[a]	48.6 ± 3.21*	93.1 ± 1.3	92.2 ± 0.6
Transfer efficiency (%)[b]	8.3 ± 2.2*	74.5 ± 0.8	72.7 ± 0.9

[a] Maximal proportion of carotenoids that were incorporated into synthetic mixed micelles during their preparation (mixture of lipids and carotenoids followed by sonication).
[b] Maximal proportion of carotenoids that were transferred from their solid form into synthetic mixed micelles solubilised in water during concentration *versus* time experiments (see Fig. 2). Values are means ± SD of 3 experiments. For each row, a mean bearing an asterisk indicates a significant ($P<0.05$) difference between this mean and the two other means (as assessed by ANOVA and Tukey–Kramer test).

perhaps due to their higher melting point brought about by their polar glycosyl moieties. Indeed, we have recently shown that the incorporation efficiency of carotenoids in synthetic micelles was related to carotenoid melting point (Sy et al., 2012). The very efficient transfer of bacterial carotenoids to mixed micelles likely explains the higher bioaccessibility of these carotenoids, as compared to β-carotene, in the *in vitro* digestion experiments (Fig. 5).

Because bacterial carotenoids are most likely to be developed as dietary supplements under the form of bacteria (probiotics) (Hempel et al., 2012; Ma et al., 2010) it was interesting to compare the bioaccessibility of these carotenoids either when they are embedded in their natural matrix (spores and vegetative cells) or when they are extracted and purified. Furthermore we co-digested the carotenoids incorporated in their natural matrix with a standard meal in order to mimic what will likely happen in the real life. Indeed carotenoid sources are seldom ingested without any food. As expected, the bioaccessibility of the bacterial carotenoids from lyophilised cells was only ca. twice as low as from pure extracts. We hypothesise that this lower bioaccessibility was due to a less efficient release of carotenoids from spores or vegetative cells, due to the double coat of *Bacillus* cells (plasmic membrane + peptidoglycanes).

The second limiting-step for carotenoid bioavailability is assumed to be their absorption by the intestinal cell. The first significant result of the caco-2 cell experiments was that native, *i.e.* esterified and glycosylated, bacterial carotenoids were found in Caco-2 cell fractions, and thus assumed to be absorbed. This was surprising because most glycosides and esters of nutrients and micronutrients are hydrolyzed in the gastrointestinal tract lumen, and it is assumed that only the free forms of the nutrients and micronutrients are absorbed by the intestinal cell. Nevertheless this result is supported by the fact that native bacterial carotenoids were also recovered in the blood and tissue of rats. It is

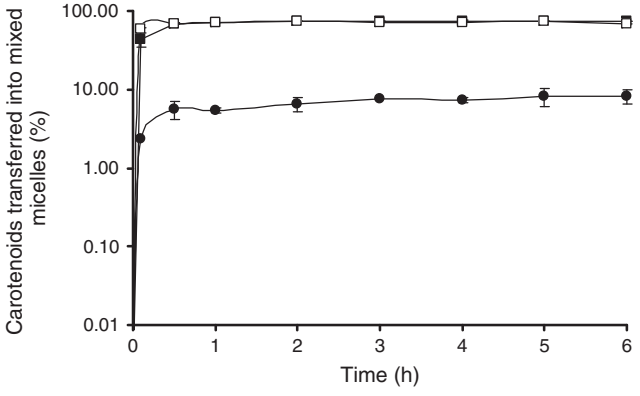

Fig. 6. Amount of pure carotenoids transferred from carotenoid powders to synthetic mixed micelles solubilised in water as a function of time. (●) Pure β-carotene, (■) purified HU36 carotenoids and (□) purified GB1 carotenoids. Experiments were performed protected from light at 37 °C. Means ± SD of three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

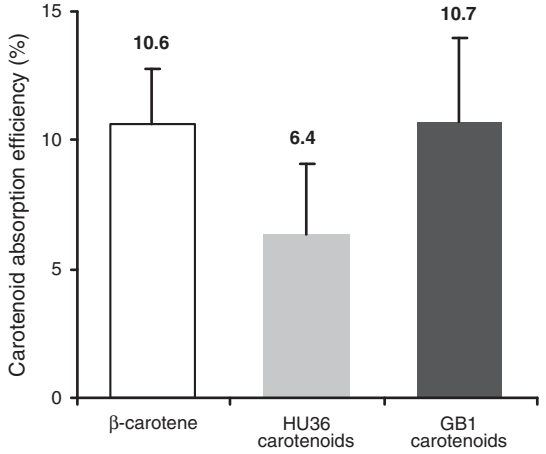

Fig. 7. Uptake, by Caco-2 cell monolayers, of carotenoids incorporated in mixed micelles that came from *in vitro* digestion experiments, expressed as uptake efficiency (% of the carotenoid amount added onto cell monolayers). Means ± SD of three independent experiments. There were no significant ($P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

not known whether the mechanism of uptake of bacterial carotenoids involves scavenger receptors, as reported for common carotenoids (Moussa et al., 2011, 2008; Reboul, Abou, et al., 2005; Reboul & Borel, 2011), but the similar absorption efficiency observed herein between bacterial carotenoids and β-carotene suggests that similar mechanisms are involved. In fact this is in agreement with recent results showing that structural differences in carotenoids do not have a significant impact on the uptake efficiency of carotenoids in intestinal cells (Sy et al., 2012).

After absorption, carotenoids are mainly transported by the chylomicrons to the liver, then to other tissues by lipoproteins (Tyssandier, Choubert, Grolier, & Borel, 2002). In the rat experiments, much higher amounts of bacterial carotenoids than β-carotene were recovered in the liver and in the adipose tissue. We were aware of the fact that β-carotene bioavailability could be underestimated because of a conversion of a fraction of β-carotene into vitamin A. Thus we checked whether liver retinyl palmitate and blood retinol concentrations, which are marker of vitamin A status, varied after ingestion of β-carotene. This was not the case thus we assumed that we can compare bioavailability of β-carotene and bacterial carotenoids by measuring their blood and tissue concentrations. This comparison allowed us to conclude that HU36 carotenoids were about nine times as bioavailable as β-carotene and that GB1 carotenoids were about two to three times as bioavailable as β-carotene. Moreover, by comparing these results with the results on the bioavailability of other carotenoids, the results obtained with the same protocol by our team (Sy et al., 2012), we concluded that HU36

Table 3
Concentrations of β-carotene and bacterial carotenoids in plasma, liver and adipose tissues of rats.[a]

	Plasma (nmol/L)[b]	Liver (nmol/g)[c]	Adipose tissue (nmol/g)[c]
Pure β-carotene	6.7 ± 0.9	0.6 ± 0.1	0.2 ± 0.3
Purified HU36 carotenoids	57.3 ± 38.6	3.9 ± 3.2	11.5 ± 4.1
Purified GB1 carotenoids	17.5 ± 9.8	0.7 ± 1.3	2.6 ± 3.1
Carotenoids in HU36[d]	18.8 ± 26.9	5.9 ± 3.1	2.6 ± 2.9
Carotenoids in GB1[d]	7.5 ± 10.3	2.0 ± 1.8	1.5 ± 1.8

[a] Eight rats per group were force-fed each day for 3 days with 55 nmol carotenoids/gavage experiment. Values are means ± SD.
[b] Carotenoid concentration measured in plasma and rat tissues 4 h after the last gavage.
[c] per g proteins.
[d] Lyophilised bacteria.

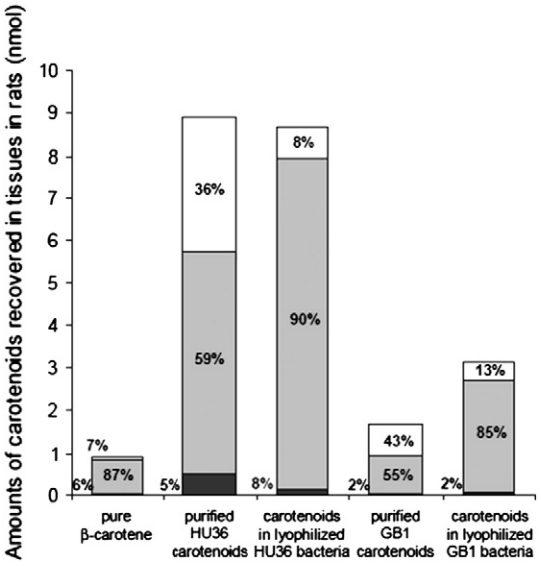

Fig. 8. Amounts of carotenoids recovered in rat tissues after Wistar rats were force-fed for 3 days with either purified carotenoids or carotenoids in lyophilised bacteria. Numbers show the relative distributions (%) of these carotenoids between plasma (black section), liver (grey section) and adipose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

carotenoids are also more bioavailable than astaxanthin, lutein and lycopene. The very high concentration of HU36 carotenoids in tissues, as compared to β-carotene, can be explained by their higher bioaccessibility, but also by a very inefficient degradation of these carotenoids in the body. Although we were not able to verify this hypothesis with the model we used, this raises the question of the potential accumulation of these carotenoids in the body and its physiological consequences. Additional studies are therefore necessary to study the metabolism of these bacterial carotenoids in tissues and the potential biological effects of their metabolites.

On the whole, this study suggests that bacterial carotenoids from the *Bacillus* HU36 and GB1 strains are more bioavailable than common carotenoids. This higher bioavailability apparently reflects their very high bioaccessibility, which is likely due to the presence of glycosyl groups that increase their micellar solubility. Finally, significant amounts of bacterial carotenoids were recovered under their native forms in the liver and adipose tissue of rats. Thus, their long-term tissue accumulation, metabolism and potential bioactivity and toxicity in humans deserve additional investigations.

Contributors

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

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