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ORIGINAL ARTICLE

Bacterioruberin extracts from a genetically modified hyperpigmented *Haloferax volcanii* strain: antioxidant activity and bioactive properties on sperm cells

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[Correction added on 6 February 2019, after first online publication: An absorption coefficient ε^{490} = 2600 mol l^{-1} cm^{-1} was applied with wrong units for estimation of Bctr concentrations. A molar absorption coefficient for Bctr ε^{490} = 185,287.5 mol l^{-1} cm^{-1} was calculated. Using this factor, Bctr concentrations in the HVLON3 samples are 70 × lower than those reported in the article. This does not change the main conclusions reported in the article.]

Abstract

Aims: To examine the antioxidant activity of Bacterioruberin (Bctr)-rich extracts isolated from a hyperpigmented, genetically modified *Haloferax volcanii* strain (HVLON3) and to investigate the effect on cold-sensitive ram sperm cells.

Methods and Results: The strain HVLON3 produces higher Bctr amounts than most haloarchaea (220 ± 13 mg g^{-1} DW). HVLON3-Bctr extract has higher antioxidant activity than β-carotene (threefold) as evaluated using 2,2 diphenyl-1-picrylhydrazyl combined with Electron Paramagnetic Resonance analysis (EC_{50} $4{\cdot}5 \times 10^{-5}$ mol l^{-1} *vs* $13{\cdot}9 \times 10^{-5}$ mol l^{-1} respectively). Different concentrations of HVLON3-Bctr extracts were assayed on ram sperm after freezing/thawing and physiologically relevant parameters were examined. Extracts containing 7 and 20 μmol l^{-1} Bctr significantly improved cell viability ($P < 0{\cdot}0001$), total and progressive motility ($P < 0{\cdot}0001$) and sperm velocities ($P = 0{\cdot}0172$ for curvilinear velocity VCL, $P = 0{\cdot}0268$ for average path velocity VAP and $P = 0{\cdot}0181$ for straight line velocity VSL) and did not affect other parameters evaluated.

Conclusions: HVLON3 is an excellent source of natural microbial C_{50} carotenoids with applicability in Biotechnology, Biomedical and Veterinary fields. HVLON3 Bctr extract improves the quality of cryopreserved ram sperm cells and could be applied to increase insemination yields.

Significance and Impact of the Study: This study provides an insight on the bioactive properties of a bioproduct derived from haloarchaea (carotenoids) which are so far underexploited.

Introduction

Carotenoids are natural isoprenoid pigments synthesized by plants, algae and micro-organisms. Their conjugated polyene system determines their spectral behaviour (light absorbance ranging from 400 to 500 nm) as well as their chemical reactivity, showing antioxidant properties (Miller *et al.* 1996). They serve as accessory light-harvesting photosynthetic pigments, membrane stabilizers, precursors of plant hormones and protect the cells from oxidative damage (Gruszecki and Strzałka 2005; Vilchez *et al.* 2011; Sandmann 2014). Carotenoids such as provitamin A, lutein and zeaxanthin are essential for the vision in mammals and must be provided in the diet.

As carotenoids are effective scavengers of reactive oxygen and nitrogen species (ROS, NOS), they are beneficial to health and prevent some human pathologies including cancer, cardiovascular diseases and other oxidative stress-related disorders (Vilchez *et al.* 2011; Fiedor and Burda 2014; Galasso *et al.* 2017). Carotenoids such as β-carotene (βcar), lycopene, astaxanthin and fucoxanthin are known to stimulate immune defences, arrest the cell cycle and induce apoptosis in various cell types. An updated and comprehensive review on the biological effects of carotenoids on human health and their mechanism of action was recently published (Milani *et al.* 2017).

Due to their colour and health-promoting properties, carotenoids are applied in food, pharmaceutical, nutraceutical and cosmeceutical industries (Sandmann 2014; Galasso *et al.* 2017). Commercially relevant carotenoids are mainly produced by chemical synthesis (βcar, astaxanthin, lycopene) although there is increasing interest in the optimization of natural sources for the production of these compounds (bioproducts). To date, most of the carotenoids derived from plants and/or micro-organisms were not competitive due in part to low yields, thus, efforts have been made to improve their production by modifying the biosynthetic pathway (Sandmann 2014).

Haloarchaea thrive in hypersaline habitats containing NaCl concentration larger than 2 mol l^{-1}. To protect from the harmful effects of UV light most haloarchaea contain carotenoids within their cell membranes, producing a reddish appearance in the environments in which they predominate. Carotenoids derived from these micro-organisms have been identified by using chromatographic, spectroscopic and/or chemical methods, Bacterioruberin (Bctr) and their derivatives being the most abundant (Table S1). The biology and biotechnological potential of haloarchaeal carotenoids have been reviewed (Rodrigo-Baños *et al.* 2015). Bctr is a C_{50} isoprenoid with 13 conjugated double bonds which contains four OH groups. In contrast to C_{40} carotenoids derived from plants and algae, the bioactive properties of Bctr are scarcely known and its biotechnological potential is at present unexploited. A few examples that show the bioactivity of Bctr include *Halobacterium halobium* carotenoid extracts with antiproliferative activity against HepG2 human cancer cell lines (Abbes *et al.* 2013) and *in vitro* antihaemolytic and anticancer activities of carotenoids extracted from haloarchaea (Hou and Cui 2018).

A target field for extracts enriched in molecules with antioxidant properties is the protection of sperm cells from cryodamage. Depending on the species, spermatozoa contain distinct concentrations of polyunsaturated fatty acids (Benson *et al.* 2012; Yimer *et al.* 2016). Sperm membrane composition impacts on recovery to freezing and thawing because it is responsible for differential stability, susceptibility to oxidative stress, mitochondrial functionality, signalling pathways and sperm survival (Bailey *et al.* 2003). Cryopreservation alters membrane permeability, induces the accumulation of ROS (Kim *et al.* 2011) and decreases the activity of some antioxidant enzymes (Marti *et al.* 2008) as well as chromatin integrity (Baumber *et al.* 2003).

Antioxidants reduce the impact of ROS-induced cold shock damage on sperm cells (Amidi *et al.* 2016). They have been used as dietary supplements or are added directly to the cooling, freezing and/or thawing media. Some carotenoids have been applied successfully on sperm cells. Semen quality was improved by lycopene in healthy men (Zareba *et al.* 2013) and by astaxanthin and βcar in goldfish (*Carassius auratus*; Tizkar *et al.* 2015) when added to the diet. Vitamin A and retinol promoted the production of motile sperm during *in vitro* maturation (Arkoun *et al.* 2015; Dumont *et al.* 2016). On the other hand, the addition of carotenoids (lycopene, canthaxanthin, lutein) to freezing extenders exhibited significant ROS-trapping and antioxidant properties preventing oxidative damage to frozen–thawed bovine (Tuncer *et al.* 2014; Tvrda *et al.* 2017), ram (Souza *et al.* 2017), boar (Varo-Ghiuru *et al.* 2015) and fish sperm (Liu *et al.* 2015) among others. Crocin applied to semen after thawing was an effective ROS scavenger and improved motility in bovine (Sapanidou *et al.* 2015) and red deer sperm (Domínguez-Rebolledo *et al.* 2010).

To advance in knowledge on the bioactive properties of Bctr as well as to implement their biotechnological and biomedical applications it is central to have a suitable biological resource with high capability of Bctr production. In our laboratory, we have previously constructed a genetically modified strain of the model haloarchaeon *Haloferax volcanii* (HVLON3) with a genetic modification in the carotenoid biosynthesis pathway which synthesizes higher levels of carotenoids (Bctr) compared to the wt (Cerletti *et al.* 2014).

In this context, we used the "hyperpigmented" strain HVLON3 as a source of Bctr and investigated the

antioxidant activity of HVLON3 Bctr-rich extracts as well as their effect on ram sperm cells after freezing/thawing.

Materials and methods

Micro-organism and growth conditions

Haloferax volcanii HVLON3 is a conditional mutant for a protease gene that synthesizes higher Bctr amounts compared to the parental H26 strain in the absence of Trp (Cerletti *et al.* 2014). *Haloferax volcanii* Δ *crtB* is a null mutant with a deletion in the *crtB* gene (HVO_2524) encoding phytoene synthase with "non-pigmented" phenotype due to the absence of Bctr (Cerletti *et al.* 2018). These strains and the wt H26 were grown in HV-Min medium (Dyall-Smith 2008) containing uracil 50 μg ml^{-1} at 42°C with agitation (200 rev min^{-1}) until the stationary growth phase (OD_{600} >1·5). The cells were harvested by centrifugation (12 000 ***g*** 20 min 4°C) and stored at −80°C protected from light until carotenoid extraction.

Carotenoid extraction

Cell pellets were suspended in an acetone : methanol mixture (1 : 1 v/v), incubated at room temperature for 1 h and then centrifuged (12 000 ***g*** 20 min 4°C) to obtain the carotenoid extract. This step was repeated until the cells were decolorated and the extracts were pooled. Alternatively, carotenoids were extracted with cold 100% acetone. Bctr concentration was calculated by measuring absorbance at 490 nm (A_{490}) and using $\varepsilon^{490} = 2600$ (mol l^{-1})$^{-1}$ cm^{-1} (acetone : methanol) or $\varepsilon^{490} = 2540$ (mol l^{-1})$^{-1}$ cm^{-1} (acetone) (Britton 1985). The Bctr extracts were stored at −20°C and protected from light until use (in solution or as dried powder).

High-performance liquid chromatography and GC-MS analysis of Bctr extracts

Carotenoid extracts were examined by high-performance liquid chromatography (HPLC) and gas chromatography coupled with mass spectrometry (GC-MS).

HPLC analysis was performed in a Shimadzu Prominence DAD SPD-M20A instrument equipped with an Agilent TC-C18 column (4·6 × 250 mm, 5 μm particle size). The extracts were filtered (0·2-μm, 13 mm diameter, nonsterile Phenex filter membranes) and injected into the column (20 μl). Carotenoids were eluted with an acetonitrile : methanol gradient (40 : 60 at $t = 0$; 20 : 80 at $t = 4{\cdot}5$ min; 20 : 80 at $t = 10$ min) with a flow rate of 1 ml min^{-1}. Chromatograms were examined at 370 and 490 nm.

Carotenoid extracts were also analysed using a Shimadzu GCMS-QP2100ULTRA-AOC20i with a Zebron ZB-5MS column (30 m × 0·25 mm × 0·10 μm) in splitless mode and the injection volume was 1 μl. Helium chromatographic grade (99·9999%) was used as the carrier gas with a constant linear velocity of 52·1 cm seg^{-1}. The injector temperature, interface and the ionization source were kept at 320°C, 300°C and 230°C respectively. Electron impact ionization was used at 70 eV in the full scan mode from 35 to 800 amu. The oven temperature program was as follows: 80°C, held for 1 min, increased at 18°C min^{-1} to 320°C, held for 5 min. The peaks were tentatively identified based on library search using NIST and Wiley Registry 8 edition.

Antioxidant activity

Antioxidant activities of HVLON3 carotenoid extracts and the reference carotenoid βcar (Sigma-Aldrich, St. Louis, MO, USA C9750) were evaluated by measuring reactivity against 2,2 diphenyl-1-picrylhydrazyl (DPPH) radical (Sigma-Aldrich, D9132) through Electron Paramagnetic Resonance (EPR) spectroscopy. As a control, antioxidant activity was also determined in the nonpigmented Δ*crtB* strain. Measurements were performed in an ELEXSYS E500T (X band) spectrometer under the following parameters: magnetic field 3480 ± 50 G; modulation frequency 100 kHz; microwave frequency 9·75 Hz; modulation amplitude 10 G; scan time 10 s; sample g-factor 2. Each spectrum was obtained from the average of three scans. Since the signal corresponds to the first derivative of the EPR absorption spectrum, its intensity was determined as the area under the curve obtained from integration of the original trace along the field values. The carotenoid stock solution (Bctr in 100% acetone or βcar in acetone : methanol 1 : 1 v/v) was added to 50 μl of DPPH (in methanol) and completed to a final volume of 200 μl with acetone : methanol. Final carotenoid concentration was varied from 2·21 × 10^{-5} mol l^{-1} (22·1 μmol l^{-1}) to 1·71 × 10^{-4} mol l^{-1} (171 μmol l^{-1}) while that of DPPH was kept at 1·83 × 10^{-4} mol l^{-1} (183 μmol l^{-1}). The Bctr content in the stock solution was estimated spectrophotometrically. Similarly, DPPH concentration was determined by measuring A_{515}, $\varepsilon^{515} = 11\,563$ (mol l^{-1})$^{-1}$ cm^{-1} (Villano *et al.* 2007). Reaction mixtures were incubated in Eppendorf tubes for 90 min protected from light and then a constant aliquot (50 μl) was transferred to an EPR quartz tube for measurement (Molyneux 2004). The effective carotenoid concentration that reduced the DPPH signal to 50% (EC_{50}) was determined for Bctr and βcar.

As a control assay, the percentage of the DPPH signal decrease was evaluated as a function of time in the presence of extracts from Δ*crtB* or from HVLON3, prepared by processing equivalent amounts of biomass. In each case, a 100-μl aliquot of the cell extract was added to 100 μl of the DPPH acetone : methanol solution. The final concentration of Bctr in the HVLON3 mixture was estimated as *c.* 7×10^{-4} mol l^{-1}. The area under the integrated EPR signal (see above) was registered at different time intervals after tuning the instrument once an aliquot of each sample transferred in the quartz tube was located in the EPR cavity.

Biological assays on sperm cells

For the biological assays, Bctr was extracted by using acetone : methanol (1 : 1 v/v) and the solvents were evaporated in a vacuum rotary evaporator (Savant) at room temperature and suspended in ethanol. βcar was suspended in DMSO.

Sample collection and cryopreservation process

Sperm samples were obtained from fertile mature ram animals (Manchega sheep, $n = 5$ or Texel breed, $n = 3$, 5 years old with a healthy condition and mean body condition score) according to Marco-Jimenez *et al.* (2008). Animal handling and maintenance was performed in accordance with local regulation (Spanish Animal Protection Regulation, RD 1201/2005, which conforms to European Union Regulation 2010/63 or Ethics Committee of the National University of Mar del Plata RD 150/15).

Semen with good wave motion (4; range 0–5) and at least 60% of total motile sperm, were cryopreserved using Tris–Egg Yolk–Glycerol-based extender (to a final concentration of 50×10^6 spermatozoa) according to García-Álvarez *et al.* (2009).

Experimental design

Thawing was carried out by dropping the semen straws in a water bath at 37°C for 30 s. A motile sperm population was selected by a glass wool filtration and diluted to 10×10^6 cell per ml in phosphate-buffered saline, pH 7·4 (PBS) to avoid extender and seminal plasma effect. Sperm suspension (in PBS medium) was incubated with different Bctr extract or βcar concentrations (2·5, 7, 15 and 20 μmol l^{-1}) for at least 120 min (Fernandez-Santos *et al.* 2007; Sapanidou *et al.* 2015; Souza *et al.* 2017). Controls were performed with the corresponding amount of ethanol (0·125% (v/v), 0·35% (v/v), 0·75% (v/v) or 1% (v/v) in PBS respectively) or the maximum concentration of DMSO (1% (v/v) in PBS).

Computer-assisted semen analysis

Objective motility was assessed with a Makler® counting chamber (10 μm depth) and analysed by using the Sperm Class Analyzer software (SCA® 2002; Microptic, Barcelona, Spain) equipped with a Basler A302fs digital camera (Basler Vision Technologies, Ahrensburg, Germany). Acquisition 25 fps obtained 5–10 videos per replicate and between 300 and 700 cells per independent replicate ($n = 5$) were analysed. The motility parameters assessed over each spermatozoon were defined by its current head velocity descriptors (Buzón 2013): curvilinear velocity (VCL), average path velocity (VAP) and straight line velocity (VSL) as defined in Fig. S1. Complementary, total motility (%) and progressive motility (%) were calculated.

Flow cytometer analysis

An aliquot of sperm suspension from each treatment was diluted to $1{\cdot}5 \times 10^6$ cells per ml in Sp-TALP medium (2 mmol l^{-1} $CaCl_2$, 3·1 mmol l^{-1} KCl, 0·4 mmol l^{-1} $MgCl_2$, 0·3 mmol l^{-1} NaH_2PO_4, 21·6 mmol l^{-1} sodium lactate, 87 mmol l^{-1} NaCl, 1 mmol l^{-1} sodium pyruvate, 10 mmol l^{-1} $NaHCO_3$, 40 mmol l^{-1} HEPES, 0·1% (w/v) Phenol Red y 0·1% (w/v) Polyvinyl alcohol, pH 7·5, 282–298 mOsm). Flow cytometric analyses were carried out on a Cyflow Space (Partec, Görlitz, Germany) and data acquisition was performed by Flomax® software. A 488-nm argon-ion laser was used for exciting all probes. An acquisition template was set up in order to discriminate spermatozoa from debris within the events acquired (FSC *vs* SSC dot-plots). A total of 10 000 spermatozoa were acquired per sample. The analysis of the flow cytometry data was carried out using Weasel ver. 3.2 (WEHI, Melbourne, Australia).

A combination of YO-PRO-1 (50 nmol l^{-1}, 491/509, Invitrogen, Carlsbad, CA, USA Y3603) and propidium iodide (PI) (12 μmol l^{-1}, 535/617 nm, Sigma-Aldrich, P4170) was employed for plasma membrane permeability, JC-1 (485/520 and 590 nm, 2·8 μmol l^{-1}, Invitrogen T3168) was used to asses mitochondrial membrane potential. Lipid peroxidation and ROS content were evaluated with BODIPY® (2 μmol l^{-1}, 581/591 nm C11, Invitrogen, D3861) and CM-H_2DCFDA (500 μmol l^{-1}, 492/517–527 nm, Invitrogen) combined with PI, respectively. For DNA damage assay, Tunnel (terminal deoxynucleotidyl transferase dUTP nick-end labelling 11684795910 ROCHE, Roche Diagnostics GmbH, Mannheim, Germany) was used according to the

manufacturer's instructions. Negative control (without enzyme) and positive control (incubation with 3 U μl^{-1} de DNAsa) were performed.

Statistical analysis

Data from the biological assays were analysed by GLMM (generalized linear mixed effect model) to determine statistical significance between treatments and control. Data associated with cell percentages were analysed through models with binomial distribution, whereas fluorescence intensities and motility parameters were analysed by models with Gaussian error distribution (Zuur *et al.* 32009). A *post hoc* analysis was conducted with the "lsmeans" package. All analyses were performed using R software ver. 3.3.3 (R Core Team 2017), with the "lme4" package for binomial models and the "nlme" package for Gaussian models. For all analyses, statistically significant differences were determined at $P < 0{\cdot}05$.

Results

HVLON3 as a resource of microbial carotenoids

We have previously shown that HVLON3 produces higher Bctr amounts (10–15 fold) than the parental strain (H26) as estimated in relative units (Cerletti *et al.* 2014). In this work, we determined the Bctr content per biomass units (mg g^{-1} cell dry weight, DW) which on average was $13{\cdot}8 \pm 2{\cdot}3$ mg g^{-1} cell DW for H26 and $219{\cdot}8 \pm 13$ mg g^{-1} cell DW for HVLON3, in agreement with our initial observation. We also measured the total amount of Bctr produced in stationary phase cultures of HVLON3 which was about $183{\cdot}1$ mg $l^{-1} \pm 15{\cdot}1$ ($OD_{600} = 1{\cdot}7$). To the best of our knowledge, the cellular production of Bctr in HVLON3 is the highest among those reported for most environmental isolates of haloarchaea, taking into account that the values are very variable and are expressed in different units. A summary on the production, chemical composition and bioactive properties of haloarchaeal carotenoids is shown in Table S1.

HVLON3 carotenoid extracts are enriched in Bctr

Carotenoids were extracted from HVLON3 using either an acetone : methanol mixture or 100% acetone with similar yields. The composition of the HVLON3 acetone : methanol extracts was analysed by HPLC and GC-MS chromatographies.

Initially, the HPLC elution profiles of acetone : methanol extracts of the wt strain H26 and the mutant HVLON3 were compared at A_{370} (Fig. 1). Both strains showed a similar chromatographic pattern with main peaks at retention times (Rt) around 3·2 and 3·9 min. In addition, HVLON3 evidenced minor peaks at Rt 5–6 min. Based on the spectral examination, the species eluting at 3·9 min and beyond showed typical peaks at 370, 386, 470, 489 and 522 nm compatible with Bctr and Bctr isomers (Ronnekleiv 1995). This figure also shows the higher concentration of Bctr in the HVLON3 strain compared to the wt H26 (see Absorbance scales).

To confirm the presence of Bctr in H26 and HVLON3, in parallel, acetone : methanol extracts were also prepared from a nonpigmented *H. volcanii* strain (Δ*crtB*) which contains a null mutation in the gene encoding phytoene synthase (PSY), the key enzyme in the carotenoid biosynthesis pathway. This mutant was previously constructed in our laboratory (Cerletti *et al.* 2018). The HPLC spectral profile at 3·9 min showed that the typical Bctr peaks were absent in the nonpigmented sample. Altogether, this analysis indicated that HVLON3 extract contains Bctr as the major carotenoid. HPLC chromatograms at A_{370} evidenced minor peaks with Rt 2·8–3·4 min in all the samples with maximal absorbance at 270 nm at Rt 3·2 min (Fig. 1 and Fig. S3).

A portion of each extract, HVLON3 and Δ*crtB*, was subjected to GC coupled with MS analysis. Both extracts contained the compounds identified as methyl octadecanoate (Rt 9·83 min) and dihydrofarnesol (Rt 12·63 min), the latter being more concentrated in the HVLON3 extracts (Fig. 2). Under the conditions of the assay, Bctr was not detected in the HVLON3 extract.

HVLON3 Bctr-rich extracts have potent antioxidant activity

The antioxidant activity of the HVLON3 Bctr-rich extracts was evaluated by quantifying the reactivity against the DPPH radical by EPR analysis and comparing it to that of βcar as a reference carotenoid (Fig. 3a–c). Various dilutions of the carotenoids were assayed. EC_{50} yielded $4{\cdot}5 \times 10^{-5}$ and $13{\cdot}9 \times 10^{-5}$ mol l^{-1} for HVLON3 Bctr-containing extracts and βcar, respectively, indicating threefold higher antioxidant activity compared to βcar. To address that the antioxidant activity was due to the presence of Bctr, we compared the reactivity of the HVLON3 and the nonpigmented strain Δ*crtB* extracts against DDPH by EPR (Fig. 3d). The results of this experiment showed that the activity of the Δ*crtB* extract was almost negligible, thus supporting the assignation of the antioxidant activity to Bctr.

The HVLON3 extracts maintained antioxidant activity upon storage at 20°C in the dark for at least 3 months, which represents an advantage for the potential application of this molecule.

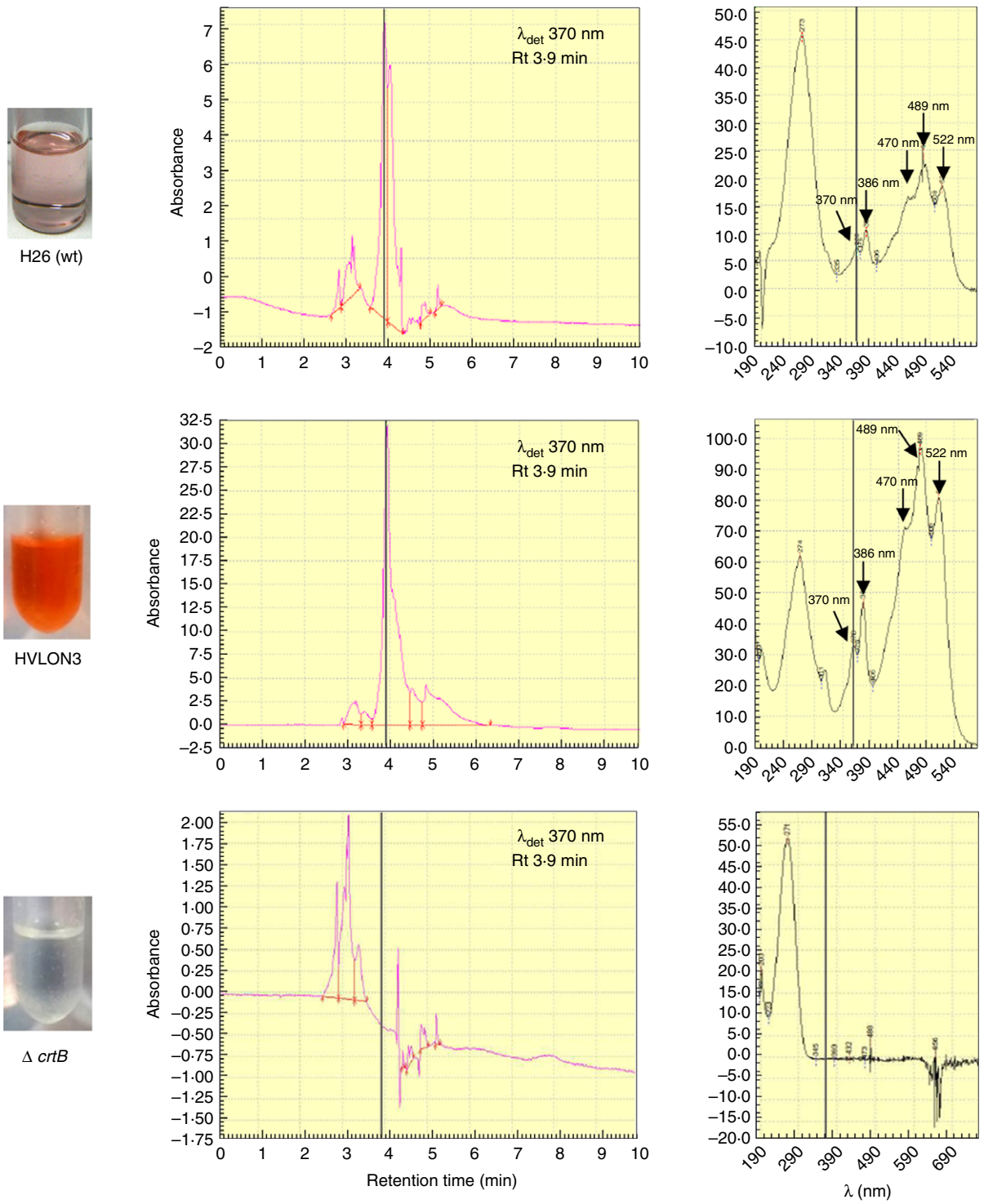

Figure 1 HPLC analysis of carotenoid extracts from H26, HVLON3 and the nonpigmented *Haloferax volcanii* Δ*crtB strains*. Carotenoid extracts were prepared from equal amounts of biomass (stationary phase cells) using acetone: methanol (50 : 50, v/v). Carotenoids were eluted with an acetonitrile : methanol gradient (40 : 60 at t = 0; 20 : 80 at t = 4·5 min; 20 : 80 at t = 10 min) with a flow rate of 1 ml min^{-1}. Chromatograms were examined at 370 nm (left panels). The vertical line points to the Rt = 3·9 min (Bctr). Absorption spectra (right panels) of species eluted at 3·9 min denote the presence of Bctr-related carotenoids (typical peaks at 370, 386, 470, 489 and 522 nm) in the wt and HVLON3 extracts and its absence in the nonpigmented strain. [Colour figure can be viewed at wileyonlinelibrary.com]

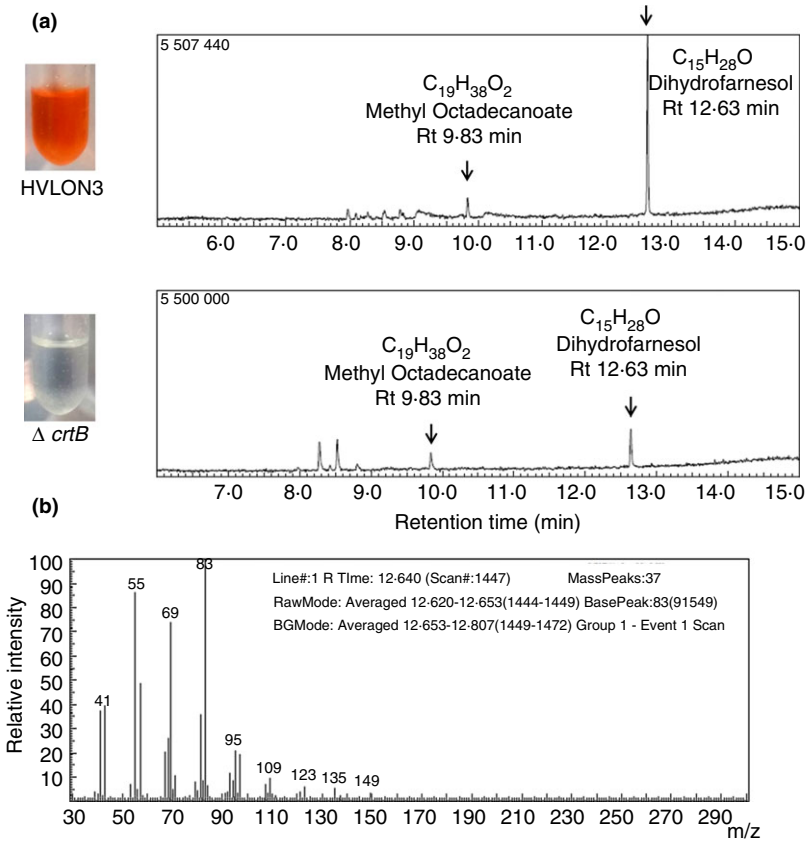

Figure 2 GC-MS analysis of carotenoid extracts from HVLON3 and nonpigmented *Haloferax volcanii* Δ *crtB* strains. Carotenoids extracts were prepared from equal amounts of biomass (stationary phase cells) using acetone: methanol (50 : 50, v/v) and were subjected to GC coupled to MS analysis as described in the Materials and methods section. The peaks were tentatively identified (arrows) based on library search using NIST and Wiley Registry 8 Edition. (a) Chromatograms, (b) Mass spectrum of Dihydrofarnesol. [Colour figure can be viewed at wileyonlinelibrary.com]

HVLON3 Bctr extracts improve viability and motility on cryopreserved ram sperm cells

Taking into account the potent antioxidant activity of HVLON3-Bctr extracts (Fig. 2) we established an assay to test whether these extracts could reverse the damage produced on ram sperm cells as a consequence of freezing and thawing. Cell viability as well as different motility parameters, ROS content, lipid peroxidation, mitochondrial activity and DNA damage were analysed after incubation of thawed sperm samples with different Bctr extract or βcar concentrations (2·5, 7, 15 and 20 μmol l^{-1}) for 120 min. These concentrations were below the EC_{50} for Bctr and βcar (0·046 and 0·14 mmol l^{-1} respectively) to avoid toxic effects of the diluents. Carotenoid concentrations in the μmol l^{-1} range (2–70) have been used by others (Wojcik *et al.* 2008; Andrisani *et al.* 2015). Based on previous reports (Blumenthal 2005; Abbes *et al.* 2013; Jamalzadeh *et al.* 2016; Tsakmakidis *et al.* 2011), Bctr extracts and βcar were added to the cells reaching a maximal diluent concentration of 1% (v/v).

Despite the antioxidant activity of the HVLON3-Bctr extracts detected with DPPH, none of the concentrations tested (2·5–20 μmol l^{-1}) reduced ROS content in the cryopreserved sperm samples under the experimental conditions assayed. However, the viable cell population was significantly higher than the controls when they were incubated with 7 and 20 μmol l^{-1} of Bctr extracts for 120 min ($P < 0{\cdot}0001$ and $P < 0{\cdot}0001$, respectively, Fig. 3a). Similar results were observed when sperms were treated with the same concentrations of βcar ($P < 0{\cdot}0001$ and $P < 0{\cdot}0001$, respectively, Fig. 3b). Consistently, 7 and 20 μmol l^{-1} Bctr and βcar improved viability at the expense of the decrease in both necrotic and/or apoptotic cell populations (Fig. 3a,b). Both diluents (ethanol and DMSO) did not significantly affect cell viability up to 1% (v/v) compared to the control (absence of solvent) (51·88 ± 14·11%; 45·91 ± 13·08% and 36·29 ± 6·25% respectively). Figure S4 shows that ethanol was not cytotoxic at any of the concentrations employed. Besides, as known (Ordas *et al.* 2012), 1% DMSO was despicably toxic to ram cryopreserved sperm cells at the times assayed (Fig. S4).

Sperm progressive motility is required after artificial insemination allowing the fertile sperm population to reach the egg in the isthmus where fertilization takes place. Progressive sperm swim forward in an essentially straight line and several kinetic parameters of sperm motility can be analysed by video microscopy. Taking into account the improvement on sperm viability with 7 or 20 μmol l^{-1}

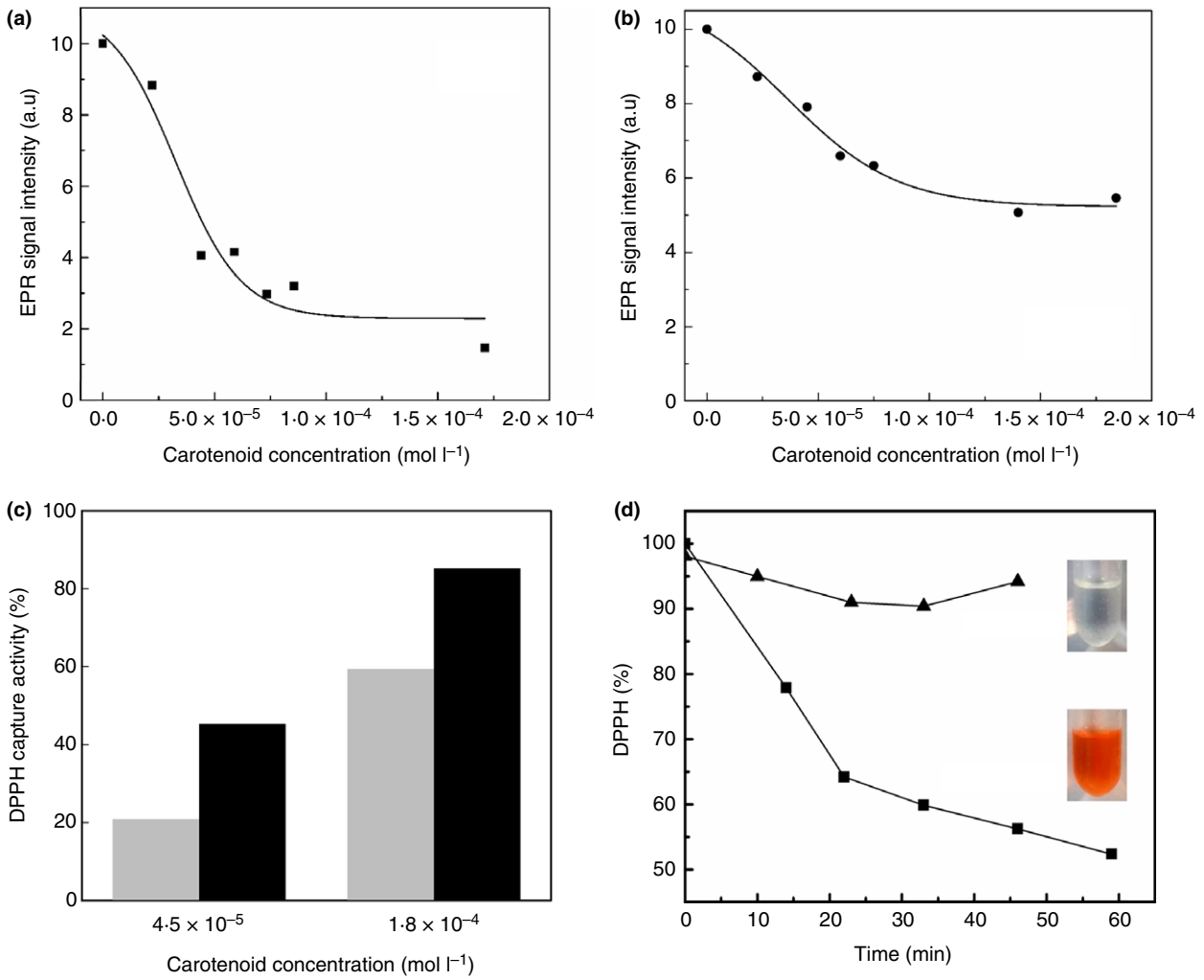

Figure 3 Antioxidant activity of HVLON3 Bctr-rich extracts. (a) Relative EPR signal intensity for $1{\cdot}83 \times 10^{-4}$ mol l^{-1} DPPH as a function of Bctr concentration, EC_{50} yields $4{\cdot}46 \times 10^{-5}$ mol l^{-1}; (b) Relative EPR signal intensity for $1{\cdot}83 \times 10^{-4}$ mol l^{-1} DPPH as a function of βcar concentration, EC_{50} yields $1{\cdot}39 \times 10^{-4}$ mol l^{-1}. (c) Comparison of the antioxidant activities determined as the percentage of DPPH capture for two different concentrations of carotenoids, Bctr (black bars) and βcar (grey bars). (d) Time course of the EPR signal intensity for DPPH (expressed as percentage of the initial value) in the presence of HVLON3 extract ($7 \times 10 \times 4$ mol l^{-1} in carotenoids, square symbols) and nonpigmented extract (triangle symbols). [Colour figure can be viewed at wileyonlinelibrary.com]

Bctr extracts, sperm motility was only analysed at these concentrations. It should be noted that the progressive motility of ram sperm cells decreased after freezing and thawing overtime (Fig. S2, $\chi^2 = 144{\cdot}96$, df = 1, $P < 0{\cdot}0001$). Interestingly, both the proportion of motile sperm and sperm with progressive motility were significantly improved by treatment with 7 μmol l^{-1} Bctr extract after at least 120 min of incubation ($P < 0{\cdot}0001$ in both cases, Fig. 4a). The kinetic values of this movement (VAP, VCL and VSL) were also enhanced with this concentration of the Bctr extract compared to the control without Bctr ($P = 0{\cdot}0172$ for VCL, $P = 0{\cdot}0268$ for VAP and $P = 0{\cdot}0181$ for VSL, Fig. 4b). Conversely, at 20 μmol l^{-1} the Bctr extract was only competent for ameliorating the proportion of sperm with total ($P = 0{\cdot}0110$) or progressive motility ($P = 0{\cdot}0069$) (Fig. 5a). However, while velocity values were not increased by the carotenoid treatment ($P = 0{\cdot}1131$ for VCL, $P = 0{\cdot}1459$ for VAP and $P = 0{\cdot}1404$ for VSL) the Bctr-treated sperm population was more homogeneous reaching the highest velocities observed for the control (Fig. 5b).

None of the other sperm parameters evaluated by flow cytometry (mitochondrial membrane potential, membrane lipid peroxidation or DNA damage) were improved by the treatments with Bctr extracts or βcar at any of the concentrations assessed (data not shown).

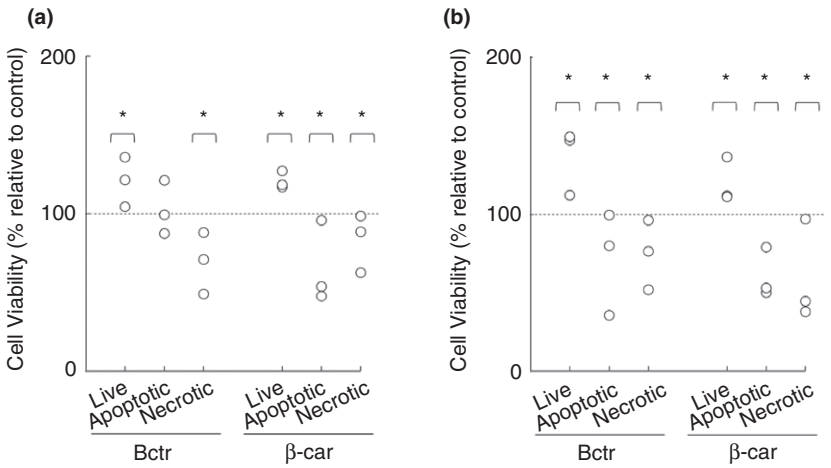

Figure 4 Effect of Bctr on the viability of cryopreserved sperm cells**.** Sperm was incubated with or without 7 μmol l^{-1} (a) or 20 μmol l^{-1} (b) of Bctr or βcar and cell viability was analysed with propidium iodide plus YO-PRO-1 by flow cytometry. As solvents may alter cell viability, the percentage of viable over total cells after 120 min of incubation are expressed normalized to the corresponding values in the presence of control solvents (51·18 ± 11·62, 51·88 ± 14·10 and 45·91 ± 13·08, for 0·35% ethanol (v/v), 1% ethanol (v/v) and 1% DMSO (v/v) in PBS respectively). The increments/decrements in viable, apoptotic or necrotic cells compared to each control (100%, dotted line) are shown. Dots represent three independent experiments. *Value significantly different with respect to each control without treatment ($P < 0{\cdot}05$).

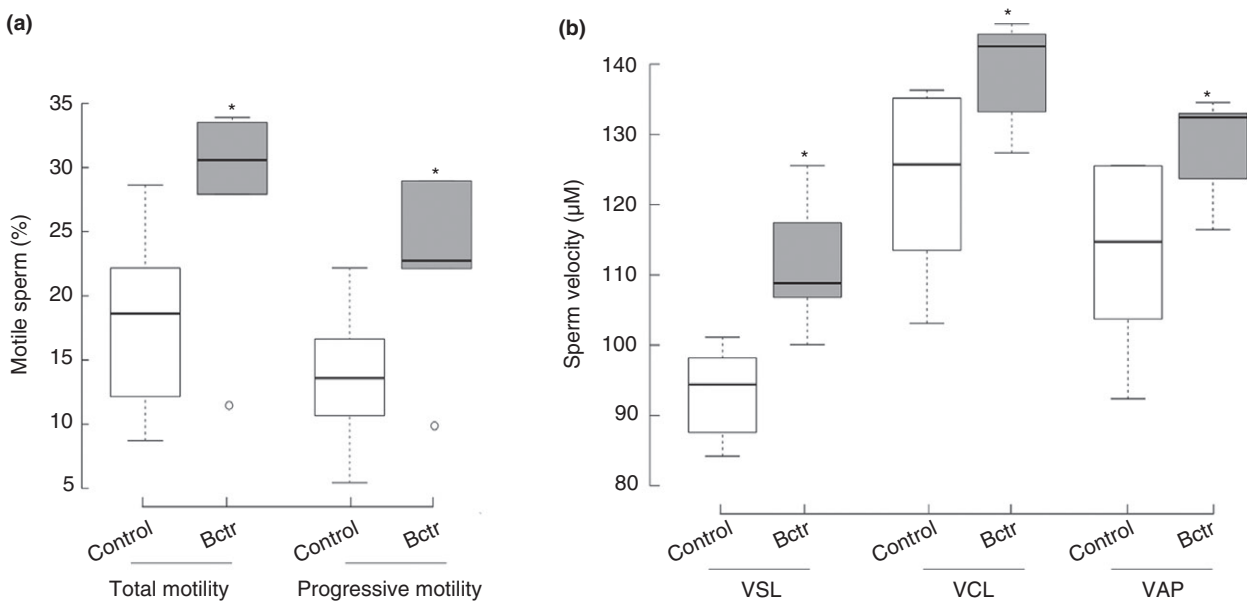

Figure 5 Effect of 7 μmol l^{-1} Bctr on cryopreserved ram sperm cell motility. Thawed sperm was incubated with or without 7 μmol l^{-1} of Bctr extract and objective motility was studied. Total and progressive (a) motility as well as kinetic parameters (velocities, b) were measured after 120 min of incubation and analysed by using a Sperm Class Analyzer software system (SCA®). For these assays, between 300 and 700 cells per independent replicate ($n = 5$) were analysed. Control was performed with the corresponding amount of the solvent according to specifications in the Materials and methods section (Experimental design section). VAP: average path velocity, VSL: straight line velocity, VCL: curvilinear velocity. *Value significantly different with respect to each control without treatment ($P < 0{\cdot}05$).

Discussion

In this study, we showed that the hyperpigmented *H. volcanii* strain HVLON3 produces very high levels of carotenoids enriched in Bctr (Table S1, Fig. 1), representing an excellent resource of this compound. This fact will facilitate the study of its bioactive properties as well as the implementation of potential biotechnological and

biomedical applications of Bctr and/or the Bctr-enriched extract. It has to be noted that haloarchaea grow under very stringent salt concentrations making unnecessary sterilization procedures and they are nonpathogenic for animals and humans thus, in this regard, haloarchaea show an advantage over other micro-organisms of biotechnological relevance, especially when applied as treatment to samples of animal origin. Examination of the HVLON3 extracts by GC-MS showed that, in addition to Bctr, they contain dihydrofarnesol, a sesquiterpenoid (C_{15}) related to precursors of Bctr (Fig. 2) (Rodrigo-Baños *et al.* 2015). This compound was also present in the nonpigmented *H. volcanii* strain, at a lower concentration based on the GC-MS analysis. It is likely that dihydrofarnesol may correspond to the common HPLC-peaks with Rt around 3·2 min and maximal absorbance at 270 nm. However, this issue needs to be confirmed. The predominance of Bctr in *H. volcanii* HVLON3 was consistent with the composition of carotenoids extracted from *H. volcanii* wt (Fig. 1) (Ronnekleiv 1995; Biswas *et al.* 2016; Hou and Cui 2018), *Halobacterium salinarum*, *Halococcus morruhae*, *Haloterrigena turkmenica*, *Halorubrum* sp *and Haloarcula japonica* (see Table S1 for detailed references).

HVLON3 Bctr-rich extracts were a more effective antioxidant than βcar (threefold) (Fig. 3). Comparison of the antioxidant capacities of extracts from HVLON3 and the non-pigmented strain measured using DPPH and EPR analysis, demonstrated that Bctr was mainly responsible for this activity (Fig. 3). Consistent with our results, it has been shown that *trans, trans*-farnesol has poor to mild antioxidant capacity (IC_{50} for DPPH could not be determined; IC_{50} = 1·8 mmol l^{-1} for hydroxyl radicals; Vinholes *et al.* 2014). However, it is interesting to note that these authors observed that sesquiterpenic compounds (including *trans, trans*-farnesol) show cytotoxic and antiproliferative activity *in vitro* against cancer cell lines. On the other hand, 2,3,dihydrofarnesol (R-DHF) and its isomers inhibited the growth of Dermatophytes, causal agents of human skin infections (Brasch *et al.* 2013). This means that the HVLON3 extract contains other bioactive compounds, in addition to Bctr, with beneficial effects to human health. 2,3, dihydrofarnesol is produced by plants as a volatile compound of flowers (Brasch *et al.* 2013) and it is used as a pheromone in male bees (Brabcová *et al.* 2015). In *Candida albicans*, farnesol is involved in quorum sensing signalling (Padder *et al.* 2018), however, its biological relevance in microorganisms is scarcely known.

The higher antioxidant potential of the HVLON3-Bctr extracts *vs* βcar is in agreement with the values determined for Bctr purified from *H. japonica TR-1* (Yatsunami *et al.* 2014). Similar DPHH radical scavenging activity was also reported for the carotenoids extracted from various haloarchaea, including *H. volcanii* (Hou and Cui 2018). The higher antioxidant activity observed in the Bctr-rich extracts from the strain HVLON3 compared to βcar was not surprising and it is consistent with the fact that the ability to scavenge free radicals increases with the conjugated double bonds (Bctr has C_{50} and 13 CDB *vs* C_{40} and 9 CDB of βcar) (Saito *et al.* 1997; Miller *et al.* 1996; Jiménez-Escrig *et al.* 2000).

As a natural bioactive, the HVLON3-Bctr extract improved the population of viable sperm (Fig. 4) and their motility (Figs 5 and 6). Our results are consistent with those reported by Souza *et al.* (2017) which showed a clear positive effect on ram sperm motility after 2 h post-thawing with similar concentrations of canthaxanthin supplemented to the freezing medium. Similar to our study, Sapanidou *et al.* (2015) detected a significant improvement in bovine sperm motility and viability with crocin added post-thawing at concentrations in the mmol l^{-1} range after 2 h of incubation.

The fact that Bctr extracts exerted a positive effect on sperm cells increasing the viable pool and reducing the apoptotic and necrotic population is a relevant issue since Bctr-treated thawed sperm could survive longer times into the female tract after insemination occurs. Together with the improvement of motility, a higher number of viable spermatozoa would be more competitive to reach the egg and fertilize it.

In this study, extracts bearing concentrations up to 20 μmol l^{-1} Bctr or similar concentrations of βcar applied on ram sperm after thawing neither improved the degree of lipid oxidation, nor diminished the intracellular ROS content as evaluated by flow cytometry (data not shown). Results of ROS production observed by other authors when carotenoids were added post-thawing are controversial (Sapanidou *et al.* 2015; Souza *et al.* 2017). Even under conditions of induced oxidative stress where the effect of a ROS scavenger should be more evident, the reports in sperm cells are not consistent among different authors who did (Domínguez-Rebolledo *et al.* 2010) or did not find (Anel-Lopez *et al.* 2016) a positive effect on ROS content. In this context, the failure of the Bctr extract to reduce ROS in thawed ram sperm was not surprising and may be attributed to an insufficient Bctr extract concentration, a short incubation time and/or a combination of both.

The beneficial effect of the Bctr extracts on sperm cells was not through its antioxidant activity at the concentrations assayed. It must be considered that polar carotenoids are capable of stabilizing biological membranes by integrating onto the bilayer and by the formation of hydrogen bonds between the terminal hydroxyl groups and the opposite polar regions of the lipid bilayers

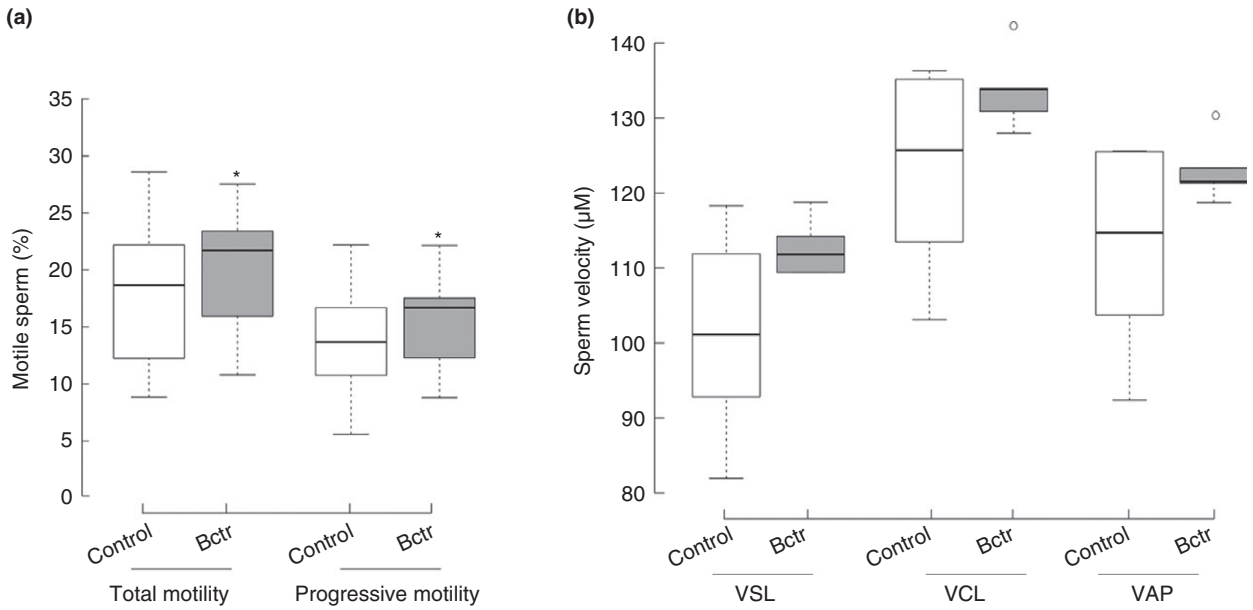

Figure 6 Effect of 20 μmol l^{-1} Bctr on cryopreserved ram sperm cell motility. Thawed sperm was incubated with or without 20 μmol l^{-1} of Bctr extract and objective motility was studied. Total and progressive (a) motility as well as kinetic parameters (velocities, b) were measured after 120 min of incubation and analysed by using a Sperm Class Analyzer software system (SCA®). For these assays, between 300 and 700 cells per independent replicate (n = 5) were analysed. Control was performed with the corresponding amount of the solvent according to specifications in the Materials and methods section (Experimental design section). VAP: average path velocity, VSL: straight line velocity, VCL: curvilinear velocity. *Value significantly different with respect to each control without treatment ($P < 0{\cdot}05$).

(Havaux 1998; Grudzinski *et al.* 2017). Thus, we hypothesize that it might be through stabilizing membranes as described for other polar carotenoids. Consistent with our hypothesis, a role of Bctr in reinforcement of reconstituted lipid membranes was previously reported for the archaeon *Halobacterium* sp (Ronnekleiv 1995).

On the other hand, DNA damage and mitochondrial activity were not affected by any of the carotenoid treatments (data not shown). Chromatin breakdown usually is a consequence of ROS increase. However, the fact that high levels of DNA damage were only observed after treatment with DNase (data not shown) indicates that spontaneous DNA damage was not evident after freezing and thawing. It is possible that the overcondensation of the DNA in cryopreserved sperm (Anzar *et al.* 2002) made the 3′OH ends inaccessible to the deoxynucleotidyl transferase (component of the Tunnel reagent). In this regard, Anel-Lopez *et al.* (2016) only observed a protective effect on DNA fragmentation when sperms were first exposed to oxidative stress and then treated with an antioxidant.

As mentioned above, the most relevant positive effect of HVLON3 Bctr extracts was observed on both the percentage of motile sperm and on the speed of progressive cells (Fig. 5). Under this condition, it was unexpected that mitochondrial membrane potential was not affected considering the known correlation with functional sperm parameters such as motility and fertilization ability (Amaral *et al.* 2013). In support of our results, there is evidence suggesting that mitochondria-derived ATP is not essential for progressive motility but rather glycolysis may be the main ATP resource (Amaral *et al.* 2013; Tourmente *et al.* 2015).

Taken these results together, the high yields of Bctr produced by HVLON3 combined with the strong antioxidant activity of the Bctr-rich extracts makes this haloarchaeal strain an excellent source of natural microbial C_{50} carotenoids (bioproduct) with potential applicability in the Biotechnology (e.g. Veterinary) and Biomedical fields. Specifically, this study shows that HVLON3 Bctr extracts improved the quality of cryopreserved ram sperm cells probably by stabilizing cell membranes after thawing, thus, they could be applied to increase insemination yields.

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Ethical approval

All applicable international, national and/or institutional guidelines for the care and use of animals were followed. Animal handling was performed in accordance with local regulation (Spanish Animal Protection Regulation, RD 1201/2005, which conforms to European Union Regulation 2010/63 or Ethics Committee of the National University of Mar del Plata RD 150/15).

Conflict of Interest

The authors declare that they have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Set of points defining a spermatozoon head path and the velocities (μm s^{-1}) measured over it: curvilinear (dotted line), average (dashed line) and straight line (solid line). Curvilinear velocity (VCL): velocity over the total distance moved in the path length; average path velocity (VAP): velocity over a calculated smoothed path; straight line velocity (VSL): velocity calculated using the straight line distance between the beginning and end of the sperm track.

Figure S2 Time course of sperm progressive motility. Thawed sperm (without treatment) were incubated for 180 min in Sp-TALP medium at 37°C. Samples at 0, 30, 60, 90, 120, 150 and 180 min were analysed by using a Sperm Class Analyzer software system. The percentage of progressive motility over total sperm for each independent replicate was calculated (n = 5, 300–700 cells analysed per replicate in each time). The downtrend line throughout the time is indicated (dotted line).

Figure S3 HPLC analysis of carotenoid extracts from H26 (wt), HVLON3 and the non-pigmented *Halofrax volcanii* Δ*crtB strains*. Carotenoids extracts were prepared from equal amounts of biomass (stationary phase cells) using acetone: methanol (50 : 50, v/v). Carotenoids were eluted with an acetonitrile : methanol gradient (40 : 60 at t = 0; 20 : 80 at t = 4·5 min; 20 : 80 at t = 10 min) with a flow rate of 1 ml min^{-1}. Chromatograms were examined at 370 nm. The vertical line points to the

Rt = 3·2 min (left panels) and shows the presence of compounds with maximal absorbance at 270 nm in all the strain (right panels).

Figure S4 Effect of ethanol on sperm viability. Sperm was incubated without (0%) solvents or with 0·125% (v/v), 0·35% (v/v), 0·75% (v/v) or 1% (v/v) ethanol or with 1% (v/v) DMSO in PBS (final concentrations), for 120 min. Cell viability was analysed with propidium iodide plus YO-PRO-1 by flow cytometry. Percentage of viable cells are expressed over total cells for each condition showing that ethanol was not cytotoxic at any of the concentrations assayed. Data represent three independent experiments.

Table S1 Summary of the production, chemical composition and bioactive properties of carotenoids isolated from haloarchaea. Carotenoids were extracted from HVLON3 (four independent replicate cultures) using acetone : methanol (1 : 1). BR yield was calculated as indicated using $\varepsilon^{490} = 2600$ mol l^{-1} cm^{-1}. Ac: acetone; M: methanol; Hex: hexane; EA: ethyl acetate. BR: bacterioruberin; MABR: monoanhydrobacterioruberin; BABR: bisnahydrobacterioruberin; TABR: triasnhydrobacterioruberin; IDR: isopentenyldehydrorodopin. ND: not determined. *Calculation of *Haloferax volcanii* HVLON3 cell dry weight per litre of culture (DW per l) was based on data reported for bacteria and archaea (including *H. volcanii*) which on average is 0·5 g l^{-1} ($OD_{600} = 1$) (De Poorter *et al.* 2007; Hamidi *et al.* 2014; Biswas *et al.* 2016).