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Probiotic administration improves sperm quality in asthenozoospermic human donors

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RESEARCH ARTICLE

Abstract

The objective of this study is to analyse the effect of the ingestion of two selected antioxidant probiotics strains (*Lactobacillus rhamnosus* CECT8361 and *Bifidobacterium longum* CECT7347) on sperm quality parameters in asthenozoospermic males after three and six weeks of administration. Nine asthenozoospermic men without any medical treatment under similar diet conditions participated in the study. The quality of individual sperm samples was evaluated before (previous to ingestion), during (after 3 and 6 weeks of ingestion) and after probiotic administration (3 and 6 weeks after finishing the treatment). Sperm motility was evaluated by computer-assisted sperm analysis system, DNA fragmentation by sperm chromatin structure assay, cell viability by flow cytometry and measurement of intracellular H_2O_2 (reactive oxygen species; ROS) by flow cytometry using dichloro-dihydro-fluorescein diacetate. Sperm motility was drastically improved after the treatment (approximately 6 fold change), DNA fragmentation was statistically reduced after probiotic administration from (approximately 1.2 fold change) and intracellular H_2O_2 level was decreased (approximately 3.5 fold change). Cell viability was not affected by the treatment. The significant improvement in sperm motility and the decrease in DNA fragmentation reported in this study provide preliminary evidence that probiotics could be administrated to improve motility and decrease DNA fragmentation and ROS levels in asthenozoospermic human males.

Keywords: *Lactobacillus rhamnosus, Bifidobacterium longum*, sperm motility, DNA fragmentation, ROS

1. Introduction

Nowadays, 15% of couples are affected by infertility (Hull *et al.*, 1985). It has been suggested that 40% of these events are caused by the male factor (Fleming *et al.*, 1995). World Health Organization (WHO) establishes different subtypes of sperm abnormalities: asthenozoospermia, oligozoospermia, teratozoospermia or their combinations (WHO, 2010). Asthenozoospermia pathology is defined by reduced motility or absent sperm motility in the fresh ejaculate. It is known that 1 of 5,000 men is affected by absolute asthenozoospermia (Eliasson *et al.*, 1977) and this condition involves a poor fertility prognosis. Male gamete motility is critical for spermatozoa migration in the female reproductive tract, for penetration of the oocyte, and for processes involved in fertilisation (Ortega *et al.,* 2011). Consequently, the likelihood of natural reproduction and sperm motility are strongly correlated (Beauchamp *et al.*, 1984).

Since the introduction of the intracytoplasmatic sperm injection (ICSI) the low fertilisation prognosis of these patients has improved drastically with successful gestations and live births after injection of immotile cells (Ortega *et al.,* 2011). However, new fields should be explored to try to improve sperm motility and facilitate assisted reproductive technologies (ARTs) in clinical centres or natural pregnancies.

Probiotics are defined as 'live microorganisms, which when administered in adequate amounts, confer a health benefit on the host' (FAO/WHO, 2002). The intestinal microbiome

is a complex ecosystem, which provides numerous crucial functions to the host, including: protection against pathogen invasion, carbohydrate metabolism and modulation of the immune response. The host is closely involved in the preservation of a healthy gut microbial community. This mutualism between the host and its microbiome is fundamental for maintaining the homeostasis of a healthy individual (Leser and Mølbak, 2009). Both harmful and beneficial bacteria must be, respectively, avoided by and adapted to the host in order to establish a healthy and balanced ecosystem (Gibson *et al.*, 2014). Probiotics colonise the intestinal mucus layer of the gut where they can affect the immune system, displace enteric pathogens, supply anti-mutagens and antioxidants, and many other potential effects by cell signalling processes (Kanmani *et al.*, 2013).

The most commonly used probiotics are lactic acid bacteria (LAB), an ecologically varied group of microorganisms united by the formation of lactic acid as the primary metabolite of sugar metabolism, and bifidobacteria. Recent publications demonstrated that these microorganisms could be effectively used in the treatment of diarrhoea (Chouraqui *et al.*, 2008; Gaón *et al.*, 2003), food allergies (Pohjavuori *et al.*, 2004), inflammatory bowel disease (Azcárate-Peril *et al.*, 2011; Del Carmen *et al.*, 2011) and colorectal cancer (Hirayama and Rafter, 2000; Rafter, 2002). LAB and bifidobacteria strains have also been reported to produce antioxidants (Amaretti *et al.*, 2013). These metabolic antioxidant activities may be assigned to reactive oxygen species (ROS) scavenging, enzyme inhibition, and reduction activity or inhibition of ascorbate autoxidation in the intestine by neutralising free radicals (Amaretti *et al.*, 2013).

The aim of this study was to evaluate the effect of administering a combination of two probiotic strains (*Lactobacillus rhamnosus* CECT8361 and *Bifidobacterium longum* CECT7347) with tested antioxidant activity on sperm quality (assessing sperm concentration, volume, motility, DNA fragmentation status, cell viability and intracellular H_2O_2 levels) from males suffering asthenozoospermia. In the field of human reproduction, the use of these bacteria has not been tested in males. Studies have only been carried out on the female factor using probiotic as a therapy for bacterial vaginosis, reporting positive results in clinical trials (Borges *et al.*, 2013; Mastromarino *et al.*, 2013). This is the first report evaluating the effect of probiotic ingestion on human sperm quality parameters.

2. Materials and methods

Subjects

This study included a set of nine donors under similar healthy diet conditions and with no medication. The project was approved by the Research Ethical Committee of the University of León (#15 2013). All donors gave written informed consent to take part in this study in accordance with the Declaration of Helsinki and Spanish legislation of confidentiality.

Study design

In order to assess motility, three weekly pre-analyses were performed (P1, P2, P3) prior to the beginning of the study. Seven days after pre-analysis, control sampling (C) was carried out before probiotic administration. Second sampling (T1) was made after three weeks of daily probiotic ingestion. Third sampling (T2) was taken after six weeks of daily ingestion. After the treatment, two washout samplings (W1 and W2) were performed, three and six weeks respectively after the end of the probiotic ingestion. This is an unblinded study. A schematic representation of the study design is presented in Figure 1.

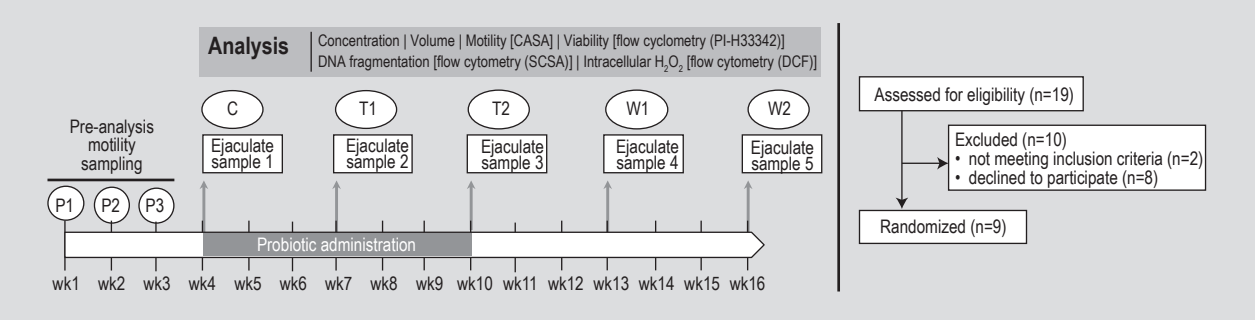

Figure 1. Experimental timeline. Capital letters represent key points along the timeline. P1, P2 and P3 represent ejaculate samples for pre-analysis motility assessment. C = control (before probiotics administration); T1 = treatment 1 (after three weeks of probiotics administration); T2 = treatment 2 (after six weeks of probiotics administration); W1 = washout 1 (three weeks after the end of probiotics administration) and W2 = washout 2 (six weeks after the end of probiotics administration).

Oxidative stress assays in *Caenorhabditis elegans*

Caenorhabditis elegans wild type strain N2 (*Caenorhabditis* Genetics Center at the University of Minnesota, USA) was used to study the *in vivo* antioxidant activity of the probiotic strains *L. rhamnosus* CECT8361 and *B. longum* CECT7347. Protocol was carried out as previously described (Grompone *et al.*, 2012; Martorell *et al.,* 2016). Age-synchronised worms were grown in NG agar medium (Nematode Growth medium: agar 17.5 g/l, NaCl 3.0 g/l, peptone 2.5 g/l, cholesterol 0.005 g/l) with a lawn of *Escherichia coli* OP50 as standard food (control media). To test the antioxidant activity of the probiotic strains, *L. rhamnosus* CECT8361 and *B. longum* CECT7347 were grown at 37 °C for 18 h on MRS-Cys (0.05%) medium, in an anaerobic atmosphere generated by means of an AnaeroGen system (Oxoid, Basingstoke, UK). Concentrated cultures (50 µl, OD=30) were then added to the NGM surface, previously seeded with *E. coli* OP50 to ensure standard nutrition conditions. NG supplemented with vitamin C (10 µg/ml) was used as positive control medium. Young adult worms were incubated at 20 °C for 5 days, transferring them to new plates every two days, to separate them from progeny. Afterwards worms were transferred to a S-Basal medium (5.85 g/l NaCl, 1 g/l K_2HPO_4, 6 g/l KH_2PO_4, 1 ml/l cholesterol (5 mg/ml in ethanol)), supplemented with 2 mM of H_2O_2 to provide an acute oxidative stress. After 5 h of incubation, worm survival was scored in each feeding condition. Worms were considered dead when they no longer responded to prodding.

Probiotic administration

L. rhamnosus CECT8361 and *B. longum* CECT7347 strains were given to volunteers in a capsule containing a combination of both microorganisms at 50%. Probiotics were supplied by the company Biopolis S.L. (Valencia, Spain). Each volunteer took a daily capsule corresponding to the administration of 10^9 cfu/day. The carrier for the lyophilised probiotics was maltodextrin. The mixture was encapsulated in hypromellose capsules.

Semen analysis

Semen samples were collected in a sterile recipient and according to the procedure recommended by the WHO (2010).

Concentration assessment

Concentration was evaluated following WHO (2010) routing counting procedure using a Neubauer chamber (VWR, Madrid, Spain).

pH evaluation

Ejaculate pH was measured with Hydrion® Brilliant pH dipsticks pH-range 6.5-13 (Sigma, Madrid, Spain).

Sperm motility evaluation

Sperm were diluted in phosphate buffered saline (PBS) to 10 to 20× 10^6 spermatozoa/ml and loaded into a Makler counting chamber (10 µm depth; Sefi Medical Instruments, Haifa, Israel) at 37 °C. The computer assisted sperm analysis (CASA) system consisted of a triocular optical phase-contrast microscope (Nikon Eclipse E400; Nikon, Tokyo, Japan) using a 10× negative phase-contrast objective, equipped with a warming stage at 37 °C and a Basler A312fc digital camera (Basler Vision Technologies, Ahrensburg, Germany). Images were captured and analysed using a computer-assisted motility analyser (ISAS; Proiser, Valencia, Spain) with specific settings to human spermatozoa. The software rendered the following global parameters: (1) percentage of motile spermatozoa; (2) percentage of progressive spermatozoa; and (3) percentage of static spermatozoa.

Cell viability

The analysis was performed with a double staining with Hoechst 33342 (H342; Sigma) and propidium iodide (PI; Sigma). Each ejaculate was diluted in 500 µl of PBS (1-2 million spermatozoa/ml) with Hoechst 33342 (5 µM) and PI (1.5 µM). After 10 min at room temperature and darkness, stained samples were evaluated by flow cytometry. Forward scatter and side scatter were used to distinguish sperm population from other events. Once spermatozoa were isolated from other events for analysis, they were classified in two cell populations: non-viable (sperm positive for IP with red fluorescence in the nucleus) and viable sperm, negative for IP. Sample acquisition was carried out using a CYAN flow cytometer (CyAn ADP, Beckman Coulter Inc., Fullerton, CA, USA) adjusted for both UV (351 nm) and blue excitation (488 nm) lines for the detection of Hoechst 33342 (450/65) and PI (670/30) fluorescence, respectively. All analyses were performed applying Weasel 3.1 free software. A total of 5,000 events were counted for each sample.

DNA fragmentation analysis

Sperm chromatin structure assay (SCSA) technique (Evenson, 2013) was performed to assess DNA fragmentation using metachromatic staining Acridine Orange (AO; Polysciences Inc., Waringtron, PA, USA). AO fluoresces in the red band when combined with denatured DNA and in the green band when combined with the undamaged double DNA helix. Ejaculates were diluted with TNE buffer (0.15 M NaCl, 0.01 M Tris-HCl, 1 mM

EDTA; pH 7.4) at a final cell concentration of approximately 1-2×10^6 cells/ml. Samples were kept immediately in liquid nitrogen until processed. Samples were thawed in a 37 °C bath and mixed with 200 µl of an acid-detergent solution (0.08 N HCl, 0.15 M NaCl, 0.15 Triton X 100; pH 1.4). After 30 s of acid detergent exposition, AO staining was performed adding 1.2 ml of stain solution containing 6 µg of AO per ml buffer (0.037 M citric acid, 0.126 M Na_2HPO_4, 0.0011 M EDTA (di-sodium), 0.15 M NaCl, pH 6.0). Just 3 min after staining, samples were analysed on a FACScalibur flow cytometer (Becton Dickinson Immunochemistry Systems; San Jose, CA, USA), equipped with standard optics and an argon laser tuned at 488 µm. Flow rates were around 200 cells/s and 5,000 events were counted for each sample. Data corresponding to the red (FL3 photodetector; 670 long pass filter) and green fluorescence (FL1 photodetector; 530/30 band pass filter) of acquired particles were recorded and analysed with Weasel 3.1 free software. The main parameter, DNA fragmentation index (DFI), corresponds to a ratio between red to total (red and green) fluorescence.

Cryopreservation and thawing

Sperm samples were cryopreserved following clinic protocols. 6×10^6 cells/ml were 1:1 diluted in a commercial cryoprotective medium (Sperm Freezing Medium (Irvin Scientific, Barcelona, Spain)). After 10 min of equilibration time at RT, the mixture was loaded in 0.5 ml French straws. The straws were then exposed to liquid nitrogen vapours horizontally (2 cm over the surface of liquid nitrogen) for 30 min. After that, they were plunged into liquid nitrogen and stored until used for the Intracellular H_2O_2 analysis. Thawing was carried out at RT for 5 min.

Intracellular H_2O_2 analysis

Flow cytometry

2×10^6 cells/ml from each thawed sample were incubated with 25 µM dichloro-dihydro-fluorescein diacetate (DCFH-DA) (Sigma) 40 min at RT. DAPI was used as counter stain dye. Each aliquot was analysed using a CyAn flow cytometer (ADP; Beckman Coulter, Inc.). Green fluorescence (DCF) was evaluated between 500 and 530 nm. Data were analysed with the Weasel 3.1 free software. 10,000 events were counted for each sample.

Confocal microscopy

In order to localise the presence of intracellular ROS H_2O_2 in human asthenozoospermic samples, 1-2×10^6 cells/ml were incubated with 25 µM dichloro-dihydro-fluorescein diacetate (DCFH-DA) (Sigma) 40 min at RT and 10 min at RT with 100 nM MitoTracker Deep Red (Invitrogen, Madrid, Spain). Two different aliquots were processed: one used as negative control and one used as oxidised control, exposed to 5% H_2O_2 (Sigma). A 5 µl cell suspension drop was place on a slide and immediately analysed under a LSM 800 confocal microscopy (Zeiss, Jena, Germany).

Statistical methods

Data were analysed using SPSS version 20 for Macintosh (SPSS Inc., Chicago, IL, USA). Data are presented as mean ± standard error (SE) in all cases. Mean values of each variable were compared by t-Student test for correlated variables (P<0.05).

3. Results

Antioxidant activity of *Lactobacillus rhamnosus* and *Bifidobacterium longum*

The percentage of *C. elegans* survival after the incubation with *L. rhamnosus* CECT8361 and *B. longum* CECT7347 reported higher values (58.5% and 66% respectively) than 10 µg/ml vitamin C positive control replicates confirming the antioxidant activity of both strains (Figure 2).

Concentration, pH and volume

Probiotic ingestion did not significantly modify these three parameters among the key points evaluated in the experimental design (C, T1, T2, W1 and W2). Mean concentration values (mean ± SE) were lower in control and treatment 1 sampling (73.04±11.28 and 59.07±15.56

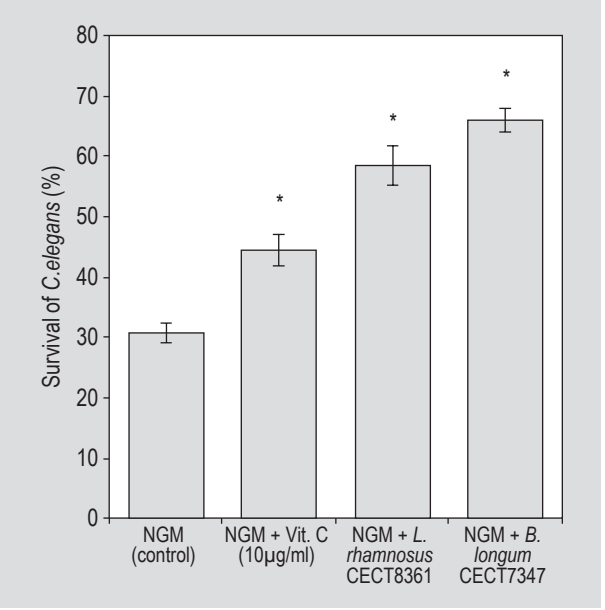

Figure 2. Survival of *Caenorhabditis elegans* N2 (wild-type strain) treated with 2 mM of H_2O_2 on NGM (control), vitamin C (positive control), and the probiotic strains *Lactobacillus rhamnosus* CECT8361 and *Bifidobacterium longum* CECT7347. * Significant at P≤0.001; ** significant at P≤0.01.**

million cells/ml, respectively) compared with treatment 2, which registered the highest mean value (179.56±54.91 million cells/ml). A decrease after stopping probiotic administration was observed in W1 and W2 (132.145±38.68 and 96.02±41.93 million cells/ml, respectively), but it was only a non-statistically significant trend (Figure 3A). Concentration variance among males was high (Table 1).

Reported pH values were the same (pH=7) in all cases for all donors throughout the experimental design. Volume data were variable among males (Table 2) without any statistically significant differences throughout the experimental procedure. The lowest registered mean volume (mean ± SE) was in the second washout, W2 (1.44±0.05 ml) similar to the control mean value (1.52±0.035 ml). The highest volume was reported after three weeks of probiotic administration, T1 (1.93±0.33 ml). However no significant differences were found after statistical analysis was performed (Figure 3B).

Motility

All donors included in this study were classified as asthenozoospermic following WHO standards (WHO, 2010) after three pre-analysis (P1, P2 and P3) motility evaluations by the computer-assisted sperm analysis (CASA) (Figure 4A). Asthenozoospermic conditions were also confirmed in the control sampling (C), prior to the beginning of the probiotic administration. Registered

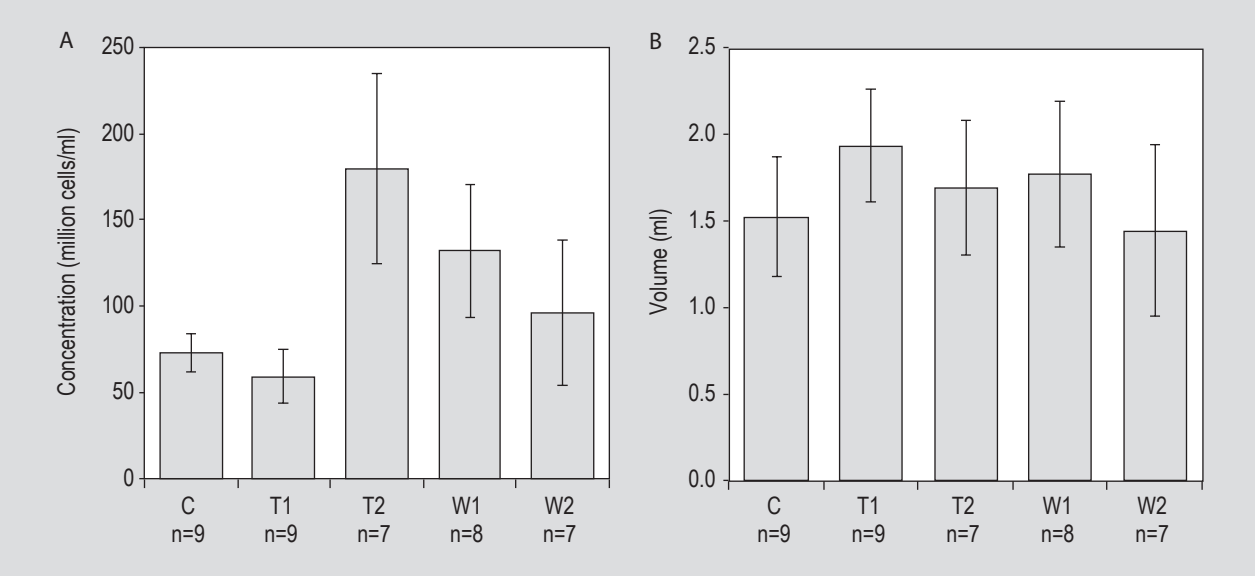

Figure 3. (A) Spermatozoa concentration (sperm counts/ml) variation along the experimental design. (B) Ejaculate volume (ml) variation along the experimental design. Capital letters represent key points along the timeline (C = control (before probiotics administration); T1 = treatment 1 (after three weeks of probiotics administration); T2 = treatment 2 (after six weeks of probiotics administration); W1 = washout 1 (three weeks after the end of probiotics administration) and W2 = washout 2 (six weeks after the end of probiotics administration)). No significant differences were found after statistical analysis.

Table 1. Concentration data for each individual in each key point of the experimental design.

	Concentration (sperm counts/ml)				
	Control	**Treatment 1**	**Treatment 2**	**Washout 1**	**Washout 2**
Individual 1	51.00	144.83	–	159.67	326.67
Individual 2	98.17	22.85	9.22	126.33	44.67
Individual 3	52.43	21.50	91.98	–	–
Individual 4	58.90	30.88	69.64	87.20	–
Individual 5	121.00	25.38	285.70	105.75	0.39
Individual 6	99.83	49.00	–	74.87	58.67
Individual 7	106.80	38.75	391.40	90.00	85.50
Individual 8	47.20	128.33	305.75	385.71	136.00
Individual 9	22.00	70.13	103.27	27.63	20.25

Table 2. Volume data for each individual in each key point of the experimental design.

	Volume (ml)				
	Control	Treatment 1	Treatment 2	Washout 1	Washout 2
Individual 1	0.20	0.90	–	1.20	0.50
Individual 2	2.50	1.30	0.50	1.70	2.00
Individual 3	0.90	1.30	1.30	–	–
Individual 4	1.00	1.80	0.75	1.50	–
Individual 5	1.00	1.50	2.50	2.00	0.60
Individual 6	1.40	2.00	–	0.80	0.50
Individual 7	3.00	4.00	3.50	4.00	4.00
Individual 8	3.00	3.00	1.50	2.80	1.90
Individual 9	0.70	1.60	1.80	0.15	0.60

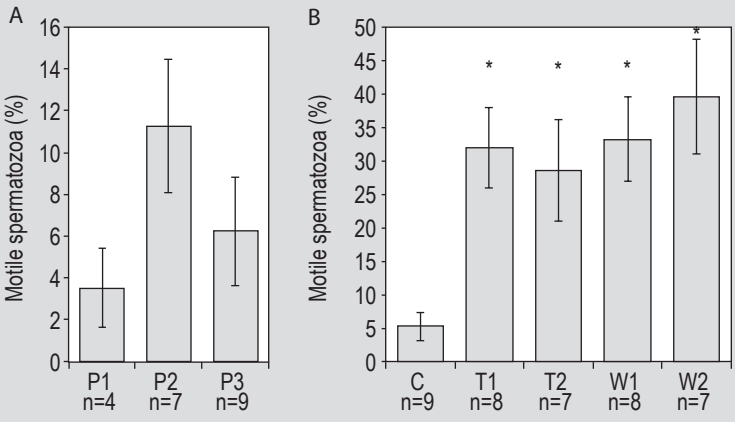

Figure 4. (A) Motile spermatozoa (%) variation along the pre-analysis motility assessment. (B) Motile spermatozoa (%) variation along the experimental design. Values with superscript asterisks are significantly different to the control after t-Student test for correlated variables (*P*<0.05). Capital letters represent key points along the timeline (P1, P2 and P3 represent ejaculate samples for pre-analysis motility assessment; C = control (before probiotics administration); T1 = treatment 1 (after three weeks of probiotics administration); T2 = treatment 2 (after six weeks of probiotics administration); W1 = washout 1 (three weeks after the end of probiotics administration) and W2 = washout 2 (six weeks after the end of probiotics administration).

motile cells percentages in P1, P2 and P3 were: 3.5±1.89; 11.29±3.20 and 6.22±2.59%, respectively (mean value ± SE).

Control sampling reported a motility percentage of 6.43±2.63% (mean value ± SE) in accordance with the pre-analysis results. After three weeks of probiotic administration (T1), motile spermatozoa percentage increased by around six fold, compared to the previous samplings, reaching 31.88±6.01% (mean value ± SE). This increment was maintained after six weeks of probiotic exposure (T2), as well as both washouts performed after the end of the ingestion of the probiotic (W1 and W2). Reported data were 28.57±7.58; 33.25±6.38 and 39.57±8.52%, respectively (Figure 4B). Individual data (static, motile and progressive cell percentages) of each donor are presented in Table 3.

Sperm chromatin structure analysis

The DFI obtained for each volunteer can be found in Table 4. Figure 5 shows the mean percentage values obtained in each key point throughout the experimental design. Control reported the highest DFI with 25.74±0.59% ratio. During the six weeks of probiotic administration this fraction was reduced in both samplings 21.11±1.00 and 21.58±0.93% (T1 and T2, respectively). After the washout, the recorded improvement seen during probiotic administration started to change. W1 and W2 acquired a higher DFI comparing T1 and T2 with a DFI ratio of 21.64±1.73 and 23.09±1.27%, respectively, changing the trend.

Table 3. Motility parameters for each individual in the three pre-analysis and in each of the key points of the experimental design.

	Motility (%)								
	P1 Motile	P2 Motile	P3 Motile	Control			Treatment 1		
				Static	Motile	Progressive	Static	Motile	Progressive
Individual 1			15.00	86.00	14.00	9.00	68.00	32.00	27.00
Individual 2			7.00	93.00	7.00	4.00	69.00	33.00	25.00
Individual 3	3.00	12.00	23.00	82.00	18.00	16.00	56.00	44.00	41.00
Individual 4		5.00	2.00	99.00	1.00	-	99.00	1.00	-
Individual 5		12.00	4.00	97.00	3.00	2.00			
Individual 6	1.00	1.00	2.00	99.00	1.00	-	42.00	58.00	53.00
Individual 7		28.00	1.00	99.00	1.00	-	65.00	35.00	30.00
Individual 8	9.00	12.00	2.00	98.00	2.00	-	83.00	17.00	14.00
Individual 9	1.00	9.00	-	100.00	-	-	65.00	35.00	30.00
	Treatment 2			Washout 1			Washout 2		
	Static	Motile	Progressive	Static	Motile	Progressive	Static	Motile	Progressive
Individual 1				52.00	48.00	43.00	61.00	39.00	33.00
Individual 2	84.00	16.00	8.00	64.00	36.00	32.00	80.00	20.00	17.00
Individual 3	48.00	52.00	50.00						
Individual 4	94.00	6.00	3.00	95.00	5.00	2.00			
Individual 5	78.00	22.00	19.00	57.00	43.00	38.00	97.00	3.00	2.00
Individual 6				43.00	57.00	52.00	46.00	54.00	50.00
Individual 7	45.00	55.00	48.00	61.00	39.00	34.00	35.00	65.00	61.00
Individual 8	61.00	39.00	30.00	72.00	28.00	23.00	65.00	35.00	26.00
Individual 9	90.00	10.00	5.00	90.00	10.00	10.00	39.00	61.00	55.00

Table 4. DNA fragmentation index (DFI) for each individual in each of the key points of the experimental design.

	DNA fragmentation (%)				
	Control	Treatment 1	Treatment 2	Washout 1	Washout 2
Individual 1	27.92	22.57	–	21.82	23.20
Individual 2	26.32	22.03	23.13	24.88	28.93
Individual 3	24.89	18.40	18.29	–	–
Individual 4	28.28	27.34	25.67	31.79	–
Individual 5	24.54	17.11	21.85	15.87	–
Individual 6	23.04	18.73	–	20.54	20.35
Individual 7	27.26	21.29	22.51	17.97	22.33
Individual 8	24.77	22.20	20.00	19.11	23.29
Individual 9	24.71	20.39	19.54	21.15	20.49

Viability

The percentage of live cells obtained after flow cytometry in each sampling was homogeneous during the experimental procedure (Table 5). No statistically significant difference was established. Live cells mean percentages were over 55% in all samplings (Figure 6). No correlation between viability and the ingestion of probiotics could be found.

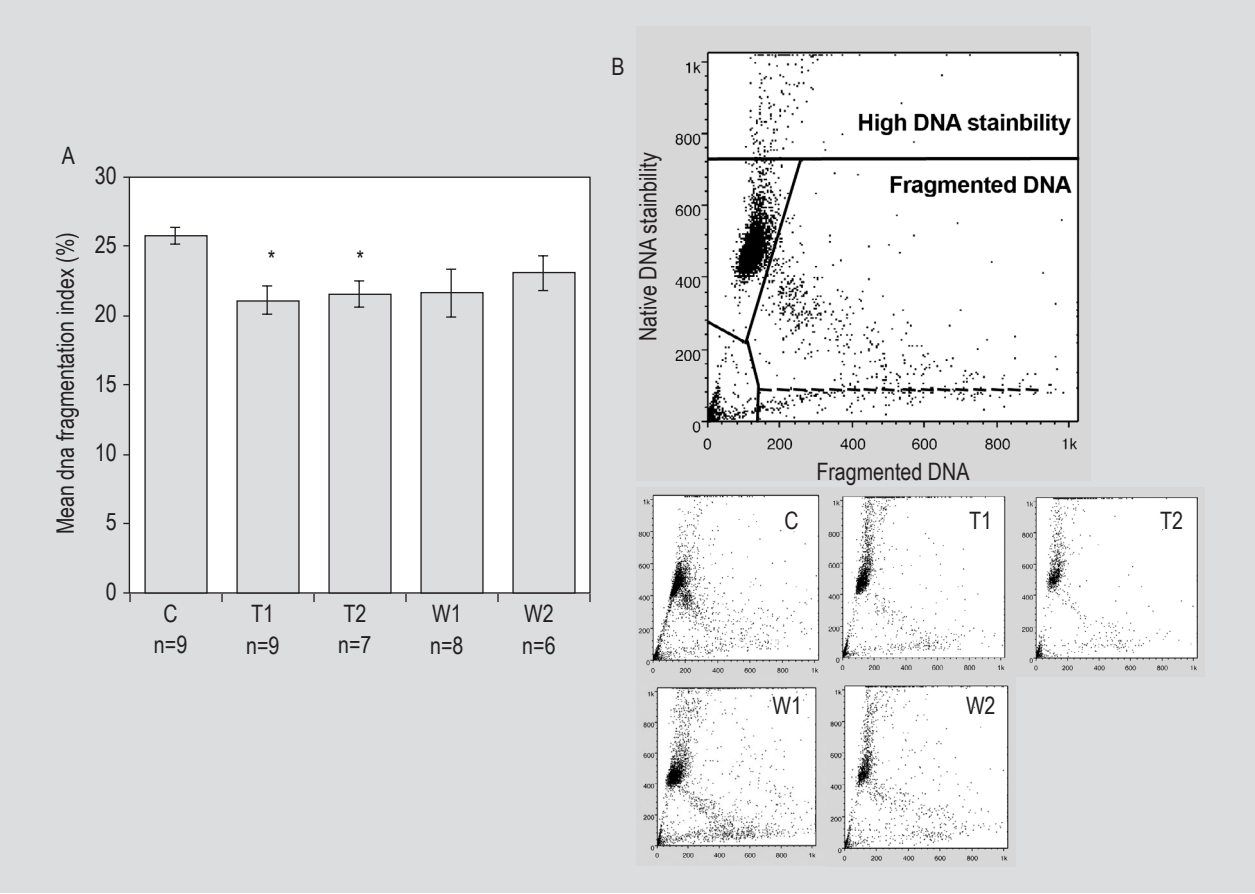

Figure 5. (A) Mean DNA Fragmentation Index (%) variation throughout the experimental design. Values with superscript asterisks are significantly different to the control after t-Student test for correlated variables (*P*<0.05). (B) Sperm chromatin structure assay (SCSA)-derived cytograms. They show green (normal double stranded DNA) versus red (fragmented DNA, denatured single-stranded DNA) fluorescence. Capital letters represent: S = cytogram example and key points along the timeline (C = control (before probiotics administration); T1 = treatment 1 (after three weeks of probiotics administration); T2 = treatment 2 (after six weeks of probiotics administration); W1 = washout 1 (three weeks after the end of probiotics administration) and W2 = washout 2 (six weeks after the end of probiotics administration).

Table 5. Viable and dead cell population percentage for each individual in each of the key points of the experimental design.

	Viability (%)									
	Control		Treatment 1		Treatment 2		Washout 1		Washout 2	
	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead
Individual 1	84.12	15.88	62	38	–	–	76.96	23.04	67.96	32.04
Individual 2	54.64	45.36	66.08	33.92	53.26	46.74	58.29	41.71	49.85	50.15
Individual 3	81.13	18.87	66.68	33.32	59.86	40.14	–	–	–	–
Individual 4	37.27	62.73	38.16	61.84	36.95	63.05	47.34	52.66	–	–
Individual 5	43.46	56.54	56.94	43.06	57	43	60.69	39.31	75.54	24.46
Individual 6	45.8	54.2	56.96	43.04	–	–	67.9	32.1	76.67	23.33
Individual 7	73.97	26.03	63.89	36.11	72.68	27.32	71.56	28.44	71.43	28.57
Individual 8	45.81	54.19	55	45	69.96	30.04	72.9	27.1	75.8	24.2
Individual 9	69.07	30.93	39.22	60.78	38.46	61.54	59.03	40.97	81.03	18.97

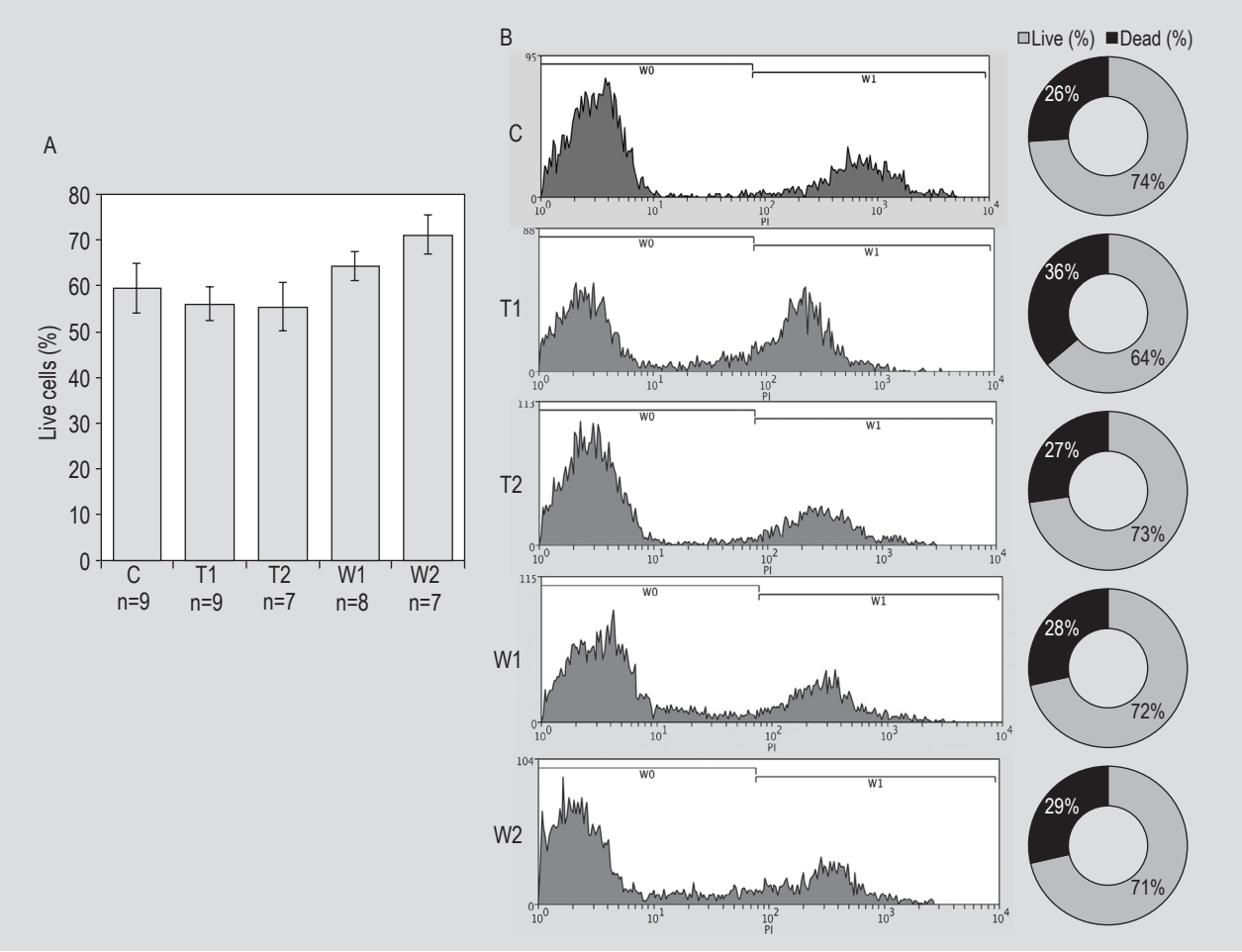

Figure 6. (A) Live cells (%) variation along the experimental timeline. No significant differences were found after statistical analysis. (B) Representative histograms obtained after propidium iodide-Hoeschst 33342 obtained in one case of study. Rings show percentages of cells in each histograms. Capital letters represent key points along the timeline (C = control (before probiotics administration); T1 = treatment 1 (after three weeks of probiotics administration); T2 = treatment 2 (after six weeks of probiotics administration); W1 = washout 1 (three weeks after the end of probiotics administration) and W2 = washout 2 (six weeks after the end of probiotics administration).

Intracellular H_2O_2 levels

The percentage of dichloro-dihydro-fluorescein (DCF) positive cells obtained after flow cytometry reported a statistically significant difference between control replicates prior to probiotic ingestion (16.57±3.34%) and T1 (5.02±0.93%), T2 (6.2±1.77%), W1 (7.27±2.37%) and W2 (7.9±1.36%) (Figure 7).

Intracellular H_2O_2 localisation

Confocal microscopy showed the co-localisation in the sperm cell (Figure 8A) of intracellular H_2O_2 with mitochondria in the middle piece in both negative and oxidised controls (Figure 8B and C). Moreover, cells exposed to 5% H_2O_2 showed a high amount of ROS also in the nucleus (Figure 8C).

4. Discussion

Spermatic dysfunction is the most common origin of infertility cases recorded by clinics working in ARTs. Asthenozoospermia is one of the male subfertility pathologies defined by the WHO (2010) as a condition in which the percentage of progressively motile sperm is abnormally low. Nowadays, ICSI is widely used in these cases with male-factor infertility, and it is clear that this technique has grown as a key revolutionary tool in ARTs (De Mendoza *et al.*, 2000). However, with immotile spermatozoa, the positive pregnancy rates are still low (Liu *et al.*, 1995, 1997), mainly due to the struggle to discriminate non-viable from viable immotile spermatozoa. The identification of new ways to increase sperm quality could be very useful to improve reproductive performance of individuals and in reproductive clinics prior to insemination, *in vitro* fertilisation or ICSI. The present study analyses the effect

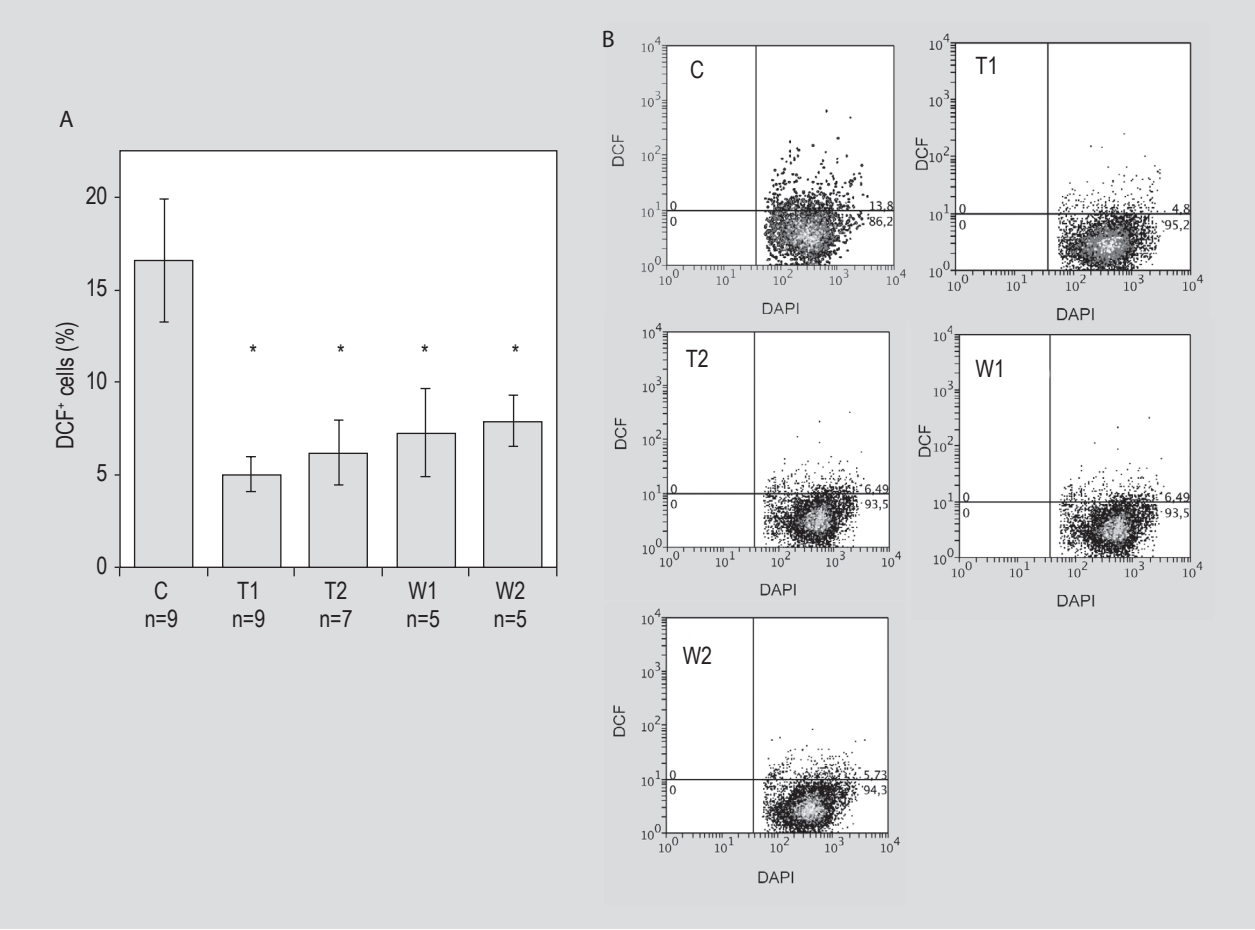

Figure 7. Intracellular H_2O_2 (% DCFH-DA$^+$ cells) along the experimental timeline. Control value is significantly different (asterisk) to the rest of values after one-way ANOVA test (*P*<0.05). Capital letters represent key points along the timeline (C = control (before probiotics administration); T1 = treatment 1 (after three weeks of probiotics administration); T2 = treatment 2 (after six weeks of probiotics administration); W1 = washout 1(three weeks after the end of probiotics administration) and W2 = washout 2 (six weeks after the end of probiotics administration)).

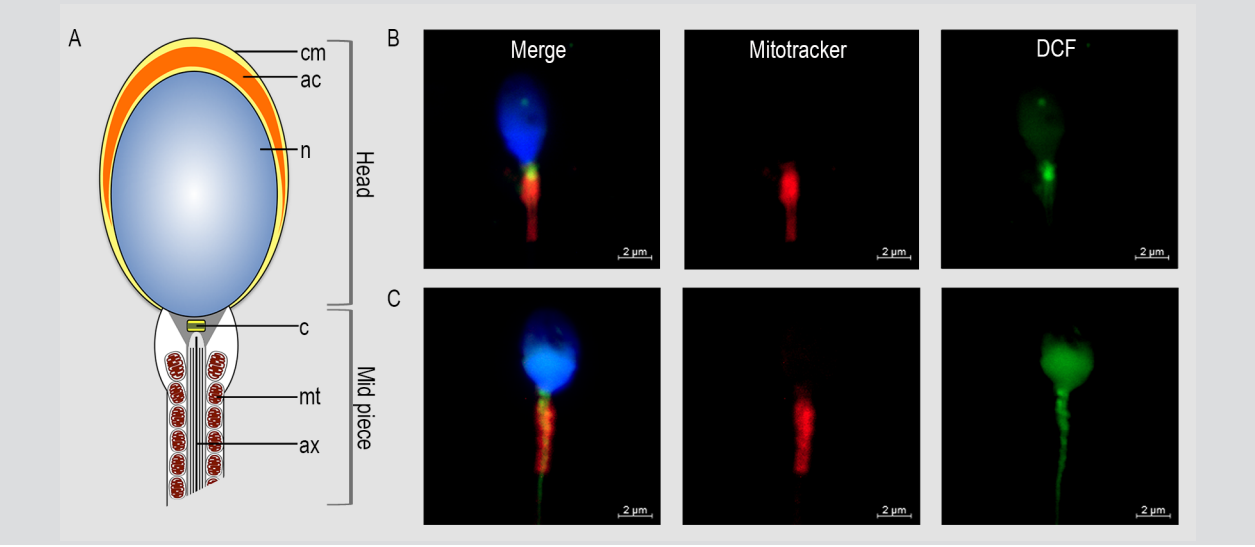

Figure 8. Intracellular H_2O_2 localisation in spermatozoa. (A) Diagram of the head and mid piece of a human spermatozoa. Cm = cell membrane; ac = acrosome; n = nucleus; c = centriole; mt = mitochondrial sheath; ax = axoneme. Confocal microscopy images. DNA stained with DAPI, mitochondria stained with Mitotracker Deep Red, intracellular H_2O_2 stained with DCFH-DA. (B) Control cell. (C) 5% H_2O_2 exposed cell.

of oral administration of a combination of two strains of probiotic bacteria with tested antioxidant effect on sperm quality from asthenozoospermic donors.

Probiotics consumption is increasing worldwide as a therapy for many different diseases and disorders. The scientific community is focusing on isolating bacterial strains able to improve human health and describing the biological molecular basis of probiotic function. *Lactobacillus* and *Bifidobacterium* were selected in this study because they are common genera of the endogenous intestinal tracts of mammals (Guarner and Malagelada, 2003), they have been widely used as probiotics and they are able to stabilise the intestinal microbiome, inducing host immunomodulation and reducing the symptoms of a wide range of gastrointestinal disorders (Manley *et al.*, 2007; Pant *et al.*, 2007). *C. elegans* is a soil nematode that feeds on bacteria. This nematode is an extremely powerful and well-studied biological system, which has been used as a model to study aging and oxidative stress. In addition, this nematode has become an excellent model to evaluate ingredients and probiotics exerting antioxidant and anti-inflammatory effects (Grompone *et al.*, 2012; Martorell *et al.*, 2011, 2016). The two selected strains used in this experiment have a clear antioxidant activity as demonstrated in *C. elegans*. The use of antioxidants in semen quality studies is not new. Over the last decades clinical studies have been carried out to establish the possible beneficial effects of treatment (Ross *et al.*, 2010) with antioxidants in the improvement of sperm parameters in men, as well as fertilisation or pregnancy rates in their partners. The most commonly studied antioxidants are vitamin C, vitamin E, selenium, glutathione, zinc, N-acetyl-cysteine and L-carnitine (Ross *et al.*, 2010). Some of these studies evaluate the effect of *in vitro* antioxidants (added as supplements in the solution with cryoprotectants) and they conclude a beneficial effect of these additives in protecting spermatozoa from exogenous oxidative molecules and freezing-thawing protocols (Gharagozloo and Aitken, 2011). However, regarding the question as to whether oral administration of antioxidant compounds is effective in improving sperm quality, Bejarano and coworkers (2014) observed that when males were treated with melatonin, it increased their seminal total antioxidant capacity and improved sperm quality. Other studies evaluated the effect of oral antioxidant administration on sperm parameters. In 2010, Ross reviewed the beneficial effects of antioxidants ingestion (Ross *et al.*, 2010). In this work, 75% of the analysed trials showed an improvement in sperm in at least one sperm parameter: 63% improvement in motility, 33% improvement in sperm concentration, 17% improvement in sperm morphology. In the female factor, several studies have reported beneficial effects of the use of probiotic strains in reproductive biology, focusing only on its properties as therapy against bacterial vaginosis (Borges *et al.*, 2013; Mastromarino *et al.*, 2013). Until now, our results are the first report describing the effect of probiotic ingestion on human male germ cells, taking into account the significant effect of ingested antioxidants on human sperm quality. It is reasonable to believe that the antioxidant properties of the probiotic strains used in the present study could be potentially beneficial for sperm quality.

Participants in the present study were defined as asthenozoospermic men following WHO criteria. Six-week probiotics consumption of *L. rhamnosus* CECT8361 and *B. longum* CECT7347 did not affect parameters, such as concentration, volume or pH. Following the same trend, viability was not modified throughout the experimental design and was considered normal following WHO criteria. However, a significant improvement was registered in sperm motility comparing control (C) and treatment samplings (T1 and T2) after 3 and 6 weeks respectively, revealing a clear effect of the probiotics on this parameter. Unpredictably, the increase was maintained after the end of the exposure in both washouts (W1 and W2). Thus, probiotic administration significantly improved sperm quality attending to motility parameters.

Concomitantly, an analysis of sperm DNA fragmentation was performed. It is well known that there is a correlation between poor motility and high levels of DNA damage (Aitken *et al.,* 2014). The test used was SCSA (Evenson, 2013). This protocol measures the percentage of sperm with a high vulnerability to low pH-induced DNA denaturation and is expressed as the DFI%. This percentage is a highly accurate, repeatable measure of DNA quality that is proportional to the level of DNA strand breaks (Aravindan *et al.*, 1997; Sailer *et al.,* 1995). In our study, control sampling showed the highest DNA Fragmentation Index throughout the whole experimental design (25.74±0.59%). Treatment samplings values were lower in both cases, correlating to the improvement of the motile cells percentages. Following the same trend as motility, registered fragmentation reduction was maintained after washout although only statistical differences were found during probiotic administration samplings. The DFI percentage threshold establishing male subfertility is variable depending on different publications: ≥20% (Boe-Hansen *et al.*, 2006), ≥27% (Larson-Cook *et al.*, 2003) and ≥30% (Payne *et al.*, 2005; Zini *et al.*, 2005). However, it is clear that a higher fragmentation level is correlated with poor motility and worse fertility success although high levels of DNA fragmentation are compatible with on-going pregnancy using ART (Boe-Hansen *et al.*, 2006). ROS are considered an important cause of DNA fragmentation in human spermatozoa (Lopes *et al.*, 1998) and intracellular H_2O_2 is one of the most common forms of ROS. H_2O_2 levels were measured in order to confirm if the observed effect of probiotic ingestion on preventing DNA fragmentation was produced by a decrease in ROS due to their antioxidant properties. DCFH-DA was used as probe due to its confirmed ability to measure H_2O_2

(Guthrie, 2006; Mahfouz *et al.*, 2009). Reported intracellular H_2O_2 levels in the control group (16.57±3.34%) are similar to previous Mahfouz results, around 20% in mature sperm (Mahfouz *et al.*, 2009). The probiotic ingestion showed the same effect in this parameter, following the previously described trend: a positive reduction after the beginning of the administration which is maintained until sampling washout. Therefore, the decrease in intracellular H_2O_2 levels supports our hypothesis, suggesting that antioxidant capacity of probiotic strains could decrease intracellular ROS preventing DNA from fragmentation. The reduction in DNA fragmentation could also explain the improvement in sperm motility.

The antioxidant effect of the LAB bacteria and bifidobacteria has been widely studied (Guo *et al.*, 2013; Lin and Chang, 2000). This effect could cause DNA to be less influenced by ROS and this would explain the registered improvement in DNA Fragmentation Index. Sperm cells are rich in mitochondria because they need a constant resource of energy for their motility. The formation of superoxide in the electron transfer chain in these organelles and, in a supplementary way, NADPH oxidase are the central sources of ROS in sperm (Koppers *et al.*, 2010; Kothari *et al.*, 2010). Specific probes for mitochondria-produced ROS (mROS) shows that unnecessary production results in membrane peroxidation and loss of motility (Aitken *et al.*, 2012; Koppers *et al.*, 2008). Furthermore, a higher content of unsaturated fatty acid on sperm is also associated to an increase in mROS again leading to motility loss and DNA damage (Koppers *et al.*, 2010). Our results could be explained considering that the administration of antioxidant probiotic bacteria to asthenozoospermic males act as a defence for the sperm mitochondria against ROS and, as consequence, motility is improved.

In conclusion, the results of our study demonstrated that *L. rhamnosus* CECT8361 and *B. longum* CECT7347 administration as an antioxidant additive improves sperm motility, reduces DNA fragmentation, and decreases intracellular H_2O_2 sperm levels. The specific molecular mechanisms underlying probiotic-mediated stimulation of sperm motility are unknown and further studies are needed in this respect. This work opens new avenues on male infertility therapy, using probiotic strains prior to ARTs or natural reproduction.

Conflict of interest

Authors from Biopolis S.L. provided the probiotic strains and the evidence of their antioxidant properties in *C. elegans,* but they had no other role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

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