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Influence of *Aphanizomenon flos-aquae* and two of its extracts on growth ability and antimicrobial properties of *Lactobacillus acidophilus* DDS-1

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ABSTRACT

The aim of this research was to investigate the effect of *Aphanizomenon flos-aquae* (AFA) and two of its extracts, Blue extract and Pure-PC on *Lactobacillus acidophilus* DDS-1 growth ability and antimicrobial activity. The influence of these compounds on the viability and the resistance to simulated gastrointestinal conditions of *L. acidophilus* DDS-1 was monitored by plate count agar. The antimicrobial activity of cell-free culture supernatants (CFCSs) of *L. acidophilus* grown with or without AFA, Blue extract or Pure-PC, was determined against *Escherichia coli* O157:H7 ATCC 35150 and *Candida albicans* ATCC 14053 by agar well diffusion and time kill-studies. Media supplemented with AFA, Blue extract or Pure-PC, stimulated the growth of *L. acidophilus* as compared to the standard MRS broth and enhanced the CFCSs antimicrobial effects. The highest stimulation was observed in media containing Pure-PC, that prolonged *L. acidophilus* growth up to 72 h.

Our data could suggest the use of AFA and its extracts as potential prebiotic substratum, while the combination of Pure-PC with *L. acidophilus* could be proposed to design synbiotic products playing beneficial effects on humans.

1. Introduction

The cyanophyta *Aphanizomenon flos-aquae* (AFA) is a fresh water unicellular blue-green algae that is consumed as a nutrient-dense food source and for its health-enhancing properties (Baroni et al., 2009; Scoglio et al., 2009).

In particular, AFA is a rich source of phycocyanin (PC) and of mycosporine-like amino acids (MAAs) (Benedetti et al., 2006; Scoglio et al., 2014). AFA PC is a photosynthetic protein with well documented antioxidant (Benedetti et al., 2004, 2010), anti-inflammatory (Canestrari et al., 2006) and neuroprotective properties (Sedriep et al., 2011), while, MAAs are UV-absorbing compounds which have antioxidant action and inhibitory effects towards monoamine oxidase B activity (Scoglio et al., 2014; Wada, Sakamoto, & Matsugo, 2013).

The activity of substances contained in blue-green algae on the growth of potential probiotic bacteria is almost referred to species such as *Spirulina platensis* and *Chlorella vulgaris* (Beheshtipour, Mortazavian, Haratian, & Darani, 2012; Bhowmik, Dubey, & Mehra, 2009; De Caire, Parada, Zaccaro, & de Cano, 2000), while no data are available as regards AFA.

Probiotics, "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002), mostly belong to the *Lactobacillus* and *Bifidobacterium* genera, which are health promoting bacteria of the balanced intestinal microbiota (Bergonzelli, Blum, Brussow, & Corthesy-Theulaz, 2005; Ventura et al., 2009) and are able to inhibit the growth of numerous pathogens by the production of organic acids, bacteriocins, and hydrogen peroxide (Likotrafiti et al., 2004). Beyond the assessment of probiotics and the development of

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methods to identify new probiotic microorganisms, the concept of prebiosis has become important. The main prebiotics are non-digestible food carbohydrates, such as fibers, oligosaccharides including fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS), resistant starch, as well as proteins or peptides originated by human digestion and utilized by microorganisms as a source of energy (Lupton, 2004). Moreover, there is evidence that prebiotics can help to modulate the growth of gut microbiota and stimulate bacteriocin production by probiotic strains, such as Lactic Acid Bacteria (LAB) (Kunová, Rada, Lisová, Ročková, & Vilková, 2011; Patel & Goyal, 2012). Prebiotics can influence the metabolic activity of probiotics; their combination, the synbiotic, favours the gut probiotic colonization and improves the quality of human life (Macfarlane, Steed, & Macfarlane, 2008). During the development of new synbiotic products it is very important to assess prebiotic and probiotic interactions, as well as the influence of prebiotics on probiotic growth and antibacterial activity.

To obtain new synbiotic combinations and synergistic effects, novel approaches could take into consideration the substances contained in blue-green algae, rich in carotenoids, chlorophyll, phycocyanin and many other bioactive components.

Accordingly, the aim of this study was to investigate the possible action of AFA and two of its extracts, Blue Extract and Pure-PC, as prebiotic substratum for the growth of *Lactobacillus acidophilus* DDS-1. In addition, in order to develop a new association for synbiotic products, the influence of AFA and/or one of its extracts on the expression of *Lactobacillus acidophilus* DDS-1 probiotic properties, such as resistance to gastro-intestinal conditions and "*in vitro*" antibacterial activity against the human intestinal pathogens *E. coli* O157:H7 and *C. albicans* was considered.

2. Material and methods

2.1. Aphanizomenon flos-aquae and its extracts

Aphanizomenon flos-aquae (AFA) was kindly provided by Nutratec (Urbino, Italy) in powdered formulation. To obtain the Blue Extract, the AFA powder was dissolved in 100 mM Na-phosphate buffer pH 7.4 and centrifuged at 2500 g to remove any insoluble material. The spectrophotometric analysis of the blue supernatant revealed the characteristic peaks of PC at 620 nm and of MAAs at 334 nm (Benedetti et al., 2006; Scoglio et al., 2014). Pure-PC was extracted from the Blue Extract by protein precipitation with ammonium sulphate (50% saturation). After an incubation of 60 min, the extract was centrifuged at 10000g; the clear colorless supernatant was discarded and the blue precipitate was dissolved in a small volume of 5 mM Na-phosphate buffer pH 7.0 and finally dialyzed overnight against the same buffer. PC was then purified by a single-step chromatographic run using a hydroxyapatite column as previously published (Benedetti et al., 2006). Pure-PC (ratio A620/A280 of 4.78) was stored at −20 °C.

In this study, AFA was resuspended in sterile distilled water and filtered using different pore size filters, first 200 μm and subsequently 0.45 and 0.22 μm (Millipore, Milan, Italy) with a final concentration of 6% (w/v) used in all the experiments. Blue Extract and Pure-PC, after spray drying and oven drying, were resuspended in sterile distilled water, filtered with 0.22 μm pore size membranes (Millipore) and utilized at final concentration of 2% (w/v) in all the experiments.

2.2. Bacterial strains and culture conditions

Lactobacillus acidophilus DDS-1 was kindly provided by Nutratec (Urbino, Italy) as potential probiotic strain. *L. acidophilus* DDS-1 was routinely grown in Man Rogosa and Shape agar (MRS) (Oxoid, Milan, Italy) at 37 °C for 24–48 h under microaerophilic conditions (5% O_2; 10% CO_2; 85% N_2).

Two human reference pathogens were used in this study, *E. coli* O157:H7 ATCC 35150 and *C. albicans* ATCC 14053; the strains were routinely maintained at 37 °C in Tryptic Soy Agar (TSA, Oxoid) and Sabouraud dextrose agar (SDA) (Liofilchem, Roseto degli Abruzzi, Italy) respectively. Stock cultures of each strain were keep at −80 °C in Nutrient broth (Oxoid) with 15% of glycerol.

2.3. Viability of L. acidophilus DDS-1 in liquid medium supplemented with AFA or its extracts

The effects of AFA or its extracts on *L. acidophilus* DDS-1 growth ability were determined. For this, an overnight exponential culture of *L. acidophilus* DDS-1 (about 10^6 CFU mL^{-1}) was incubated into 3 different 200 mL aliquots of MRS broth (Oxoid) with AFA, Blue Extract or Pure-PC under microaerophilic conditions for 24 h a 37 °C. *L. acidophilus* DDS-1 in MRS broth was included as control. At established time points (0, 3, 6, 9, 14, 24, 30, 48, 54, 57, 72 h), from each MRS broth culture, aliquots were aseptically removed, diluted in physiological saline solution and plated on MRS agar (Oxoid). After incubation at 37 °C for 24 h under microaerophilic conditions, plates were observed for the enumeration of colony forming unit (CFU mL^{-1}). All data were expressed as mean of three independent experiments performed in duplicate.

2.4. Resistance to artificial gastrointestinal conditions of L. acidophilus DDS-1 in liquid medium supplemented with AFA or its extracts

Acid resistance of *L. acidophilus* DDS-1 was examined in MRS broth (Oxoid) adjusted with hydrochloric acid (HCl) to a final pH of 2.5. Briefly, the strain was propagated in MRS broth under microaerophilic conditions for 24 h at 37 °C, harvested by centrifugation (3500 rpm for 10 min) and washed twice in phosphate-buffered saline (PBS), pH 7.2. Then, the bacterial suspension was inoculated (10%) into 3 different aliquots of acidified MRS broth with AFA, Blue Extract or Pure-PC and incubated at 37 °C for 1, 2 and 3 h. *L. acidophilus* DDS-1 in acidified MRS broth was included as control. At each time point, aliquots were aseptically removed, diluted in physiological saline solution, plated on MRS agar (Oxoid) and incubated for 24 h at 37 °C under microaerophilic conditions for the subsequent plate count enumeration (CFU mL^{-1}).

The same procedure was performed to test bile tolerance, inoculating *L. acidophilus* DDS-1 into MRS broth containing 0.3% (w/v) bile salts (Difco, Becton Drive, USA) with AFA, Blue Extract or Pure-PC at the above mentioned concentrations. *L. acidophilus* DDS-1 in MRS broth with 0.3% (w/v) bile salts was included as control. The incubation was carried out for 1, 2 and 3 h at 37 °C. Number of viable bacterial cells was enumerated as described above. All data were expressed as mean of three independent experiments performed in duplicate.

2.5. Preparation of cell-free culture supernatants of L. acidophilus DDS-1 in liquid medium supplemented with AFA or its extracts

L. acidophilus DDS-1 was grown in 200 mL of MRS broth (Oxoid) or MRS broth with AFA, Blue Extract or Pure-PC at 37 °C for 18 h under microaerophilic conditions. Bacterial cells from each MRS broth culture, were pelleted at 17000 rpm for 15 min at 4 °C, adjusted to pH 6.5 with 10 N NaOH and filtered (0.22 μm pore size) to remove any remaining bacteria. The cell-free culture supernatants (CFCSs) were collected and signed as follows: AFA-CFCS (from *L. acidophilus* DDS-1 grown in MRS broth with AFA 6%), Blue Extract-CFCS (from *L. acidophilus* DDS-1 grown in MRS broth with

Blue Extract, 2%), Pure-PC-CFCS (from *L. acidophilus* DDS-1 grown in MRS broth with Pure-PC, 2%); CFCS extracted from *L. acidophilus* DDS-1 grown in MRS broth (LA-CFCS) was also included. The aliquots of each CFCS were then kept at −20 °C until use.

2.6. Antimicrobial susceptibility of CFCSs by agar well diffusion method

The antimicrobial activity of AFA-CFCS, Blue Extract-CFCS and Pure-PC-CFCS was tested using the agar well diffusion method (AWDM) according to (Campana, Federici, Ciandrini, & Baffone, 2012), with several modifications. Briefly, several colonies were drawn from each plate of *E. coli* O157:H7 ATCC 35150 and *C. albicans* ATCC 14053, added to 30 mL of TSB (Oxoid) and incubated at 37 °C for 24 h. At this point, 500 μL of each pathogen culture (about 10^7 CFU mL^{-1}) was added to 20 mL of Nutrient agar (Oxoid) maintained at 50 °C, poured into petri dishes, and allowed to solidify for 20 min. Wells of 6 mm in diameter were made on the agar with sterile stainless steel cylinders and 50 μL of each CFCS were dropped into the holes; AFA, Blue Extract and Pure-PC solutions were also dropped (50 μL each) in several holes to exclude their antimicrobial activity; LA-CFCS was also included as control. After 24 h incubation at 37 °C, the diameter of the inhibition zone around each hole was measured and the antimicrobial activity was expressed as the mean of inhibition diameters produced by each CFCS. All the experiments were performed in duplicate.

2.7. Antimicrobial activity of CFCSs by killing studies

The antimicrobial activity of the different CFCSs towards *E. coli* O157:H7 ATCC 35150 and *C. albicans* ATCC 14053 was examined by time killing studies using two different experimental designs as represented in Fig. 1. In each experiment, LA-CFCS, extracted from *L. acidophilus* DDS-1 grown in MRS broth, was included as control.

In the first case, an exponential culture of each bacterial pathogen (about 10^8 CFU mL^{-1}, 500 μL) was incubated with or without 500 μL of AFA-CFCS, Blue Extract-CFCS and Pure-PC-CFCS at 37 °C. After 2, 4 and 8 h of incubation, aliquots were aseptically removed, diluted in physiological saline solution, plated on TSA (Oxoid) and SDA (Liofilchem) and incubated at 37 °C for 24 h. After the incubation period, the plates were observed and the colony forming units per milliliter (CFU mL^{-1}) for each pathogen were counted.

In the second case, *L. acidophilus* DDS-1 was grown in 200 mL of MRS broth (Oxoid) or MRS broth with Pure-PC under microaerophilic conditions for 24 h at 37 °C. At established time points (0, 4, 6, 8, 12, 24, 28, 30, 48, 54, 57, 72 h), from each broth culture aliquots were aseptically removed and the bacterial cells were pelleted at 17000 rpm for 15 min at 4 °C; then the CFCSs obtained were filtered throughout 0.22 μm pore size filters. Finally, a total of 24 CFCSs, 12 extracted from *L. acidophilus* DDS-1 grown in MRS broth and 12 in MRS broth with Pure-PC, were tested against *E. coli* O157:H7 ATCC 35150 and *C. albicans* ATCC 14053 by killing studies at 2, 4 and 8 h of incubation as described above.

All data were expressed as mean of three independent experiments performed in duplicate.

2.8. Statistical analysis

Statistical analysis was performed using Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). The assumptions for parametric test were checked prior to carry out the analysis. When the assumptions for parametric test were not respected, Mann-Whitney or Kruskall-Wallis non-parametric tests with Dunn's multiple comparison test were applied. P values of <0.05 were considered statistically significant.

3. Results

3.1. Effect of AFA and its extracts on growth ability of L. acidophilus DDS-1

The data relative to the effect of AFA (6% w/v), Blue Extract (2% w/v), and Pure-PC (2% w/v), on *L. acidophilus* DDS-1 growth ability were illustrated in Fig. 2. The results showed that culture media containing AFA or its extracts stimulated *L. acidophilus* DDS-1 growth; in fact, while in MRS broth *L. acidophilus* DDS-1 stopped growing after 48 h of incubation, in supplemented MRS broths its growth ability was prolonged up to 72 h. Interestingly, the higher growth stimulation was observed in MRS broth containing 2% Pure-PC, with CFU mL^{-1} values of 5×10^7 after 72 h.

3.2. Effect of AFA and its extracts on acid and bile tolerance of L. acidophilus DDS-1

As regards the effect of AFA, Blue Extract and Pure-PC at the mentioned concentrations on bile and acid tolerance of *L. acidophilus* DDS-1, data were illustrated in Table 1. As reported, *L. acidophilus* DDS-1 was resistant to low pH and 0.3% bile salts up to 3 h with values of 2.19×10^8 and 1.17×10^8 CFU mL^{-1}respectively. The presence of AFA or its extracts in the culture media did not affect substantially the survival of *L. acidophilus* DDS-1 to simulated gastric conditions, as demonstrated by CFU mL values quite similar to those obtained by *L. acidophilus* DDS-1 grown in presence of 0.3% bile salts or under acidic conditions without AFA or its extracts.

3.3. Antimicrobial activity of L. acidophilus DDS-1 CFCSs

The antimicrobial activity of AFA-CFCS, Blue Extract-CFCS and Pure-PC-CFCS was tested against *E. coli* O157:H7 ATCC 35150 and *C. albicans* ATCC 14053 by AWDM and killing studies using, in the last case, two different experimental designs.

As regard AWDM, remarkable zones of inhibition were observed for all the tested CFCSs against *E. coli* O157:H7 ATCC 35150 and *C. albicans* ATCC 14053. The presence of AFA or its extracts in the culture media of *L. acidophilus* DDS-1 enhanced the antimicrobial effect of the relative extracted CFCSs (12 mm of inhibition diameters). In our experiments, no inhibition zones were observed around the holes filled with AFA, Blue Extract or Pure-PC solutions, respectively (Table 2).

Results of the first experimental design of killing studies were illustrated in Fig. 3 (a–b). Data showed that the presence of AFA, Blue Extract or Pure-PC in the culture media maximized the antimicrobial efficacy of *L. acidophilus* DDS-1 CFCSs against *E. coli* O157:H7 ATCC 35150 (Fig. 3a) as demonstrated by the reported CFU mL^{-1} values, which were lesser than those obtained with the control LA-CFCS. In particular, after 8 h of incubation with Pure-PC-CFCS, CFU mL^{-1} reached values of 7.70×10^6 in comparison to 2.50×10^7 CFU mL^{-1} obtained with LA-CFCS. Analogously, when *C. albicans* ATCC 14053 was incubated with AFA-CFCS, Blue Extract-CFCS and Pure-PC-CFCS, a positive effect on antimicrobial properties of *L. acidophilus* DDS-1 was observed (Fig. 3b). In detail, after 8 h of incubation with Pure-PC-CFCS a decrease up to 4.97×10^6 CFU mL^{-1} was evidenced, in comparison to 1.70×10^7 CFU mL^{-1} obtained with LA-CFCS.

Results of the second experimental design of killing studies, expressed as logarithmic reduction of pathogen growth, were illustrated in Table 3. In this case, since Pure-PC showed the most interesting results in stimulating the growth and antimicrobial activity of *L. acidophilus* DDS-1, a total of 24 CFCSs (12 extracted from *L. acidophilus* DDS-1 grown in MRS broth and 12 in MRS broth

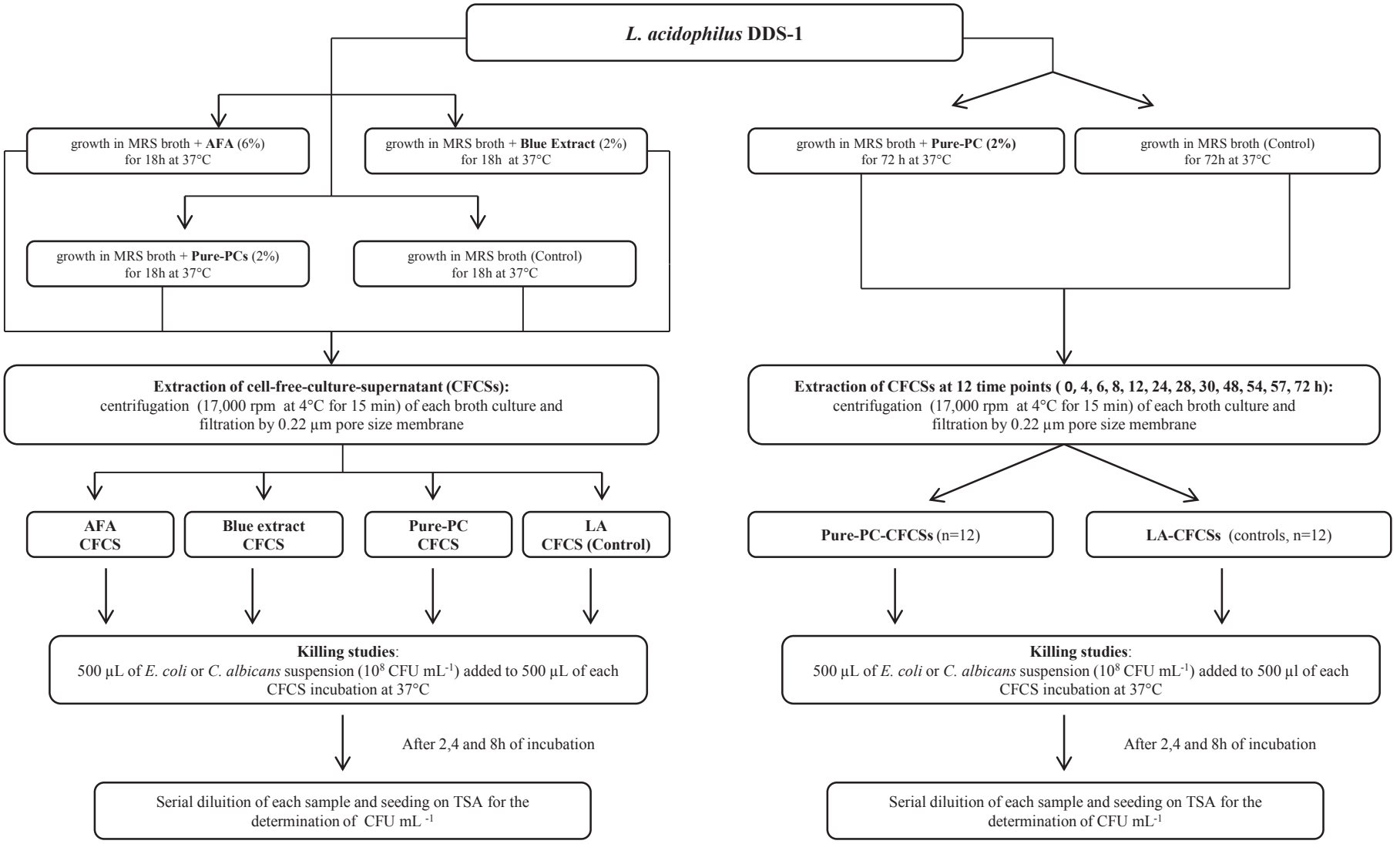
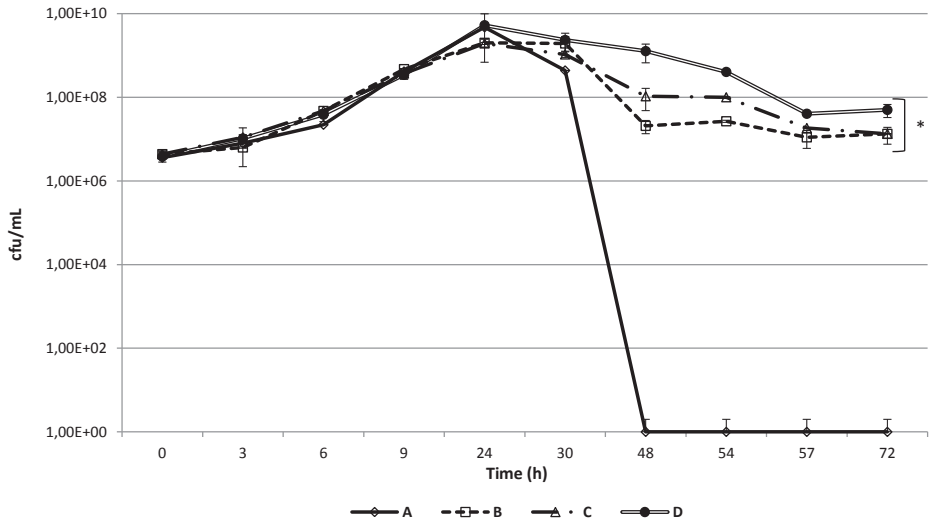


Fig. 1. Procedures of the two experimental designs used for killing studies. LA, *L. acidophilus* DDS-1; AFA, *Aphanizomenon flos-aquae*; CFCS, cell-free-culture-supernatant.



Asterisk represented values statistically significant (*P<0.05) in comparison to the control group (*L. acidophilus* DDS-1 in MRS broth), (Kruskall-Wallis non parametric test).

Fig. 2. *L. acidophilus* DDS-1 growth ability in MRS broth (A) and in MRS broth with AFA 6% (B), Blue Extract 2% (C) or Pure-PC 2% (D), as determined by agar plate counts. Values represent CFU mL^{-1} (mean ± standard deviation) of three independent experiments performed in duplicate.

Table 1
Resistance of L. *acidophilus* DDS-1 to simulated gastro-intestinal conditions (pH 2.5 and 0.3% Bile Salts) in MRS broth and in MRS broth supplemented with AFA, Blue Extract and Pure-PC, as determined by agar plate counts.

Culture media	Mean CFU mL^{-1} (±sd) after		
	1 h	2 h	3 h
MRS broth pH 2.5	5.01×10^8 ($\pm 1.74 \times 10^8$)	5.04×10^8 ($\pm 2.0 \times 10^8$)	2.19×10^8 ($\pm 2.29 \times 10^7$)
MRS broth pH 2.5 + 6% AFA	7.08×10^8 ($\pm 1.15 \times 10^8$)	5.37×10^8 ($\pm 1.32 \times 10^8$)	3.09×10^8 ($\pm 3.47 \times 10^7$)
MRS broth pH 2.5 + 2% Blue Extract	5.25×10^8 ($\pm 5.75 \times 10^7$)	6.46×10^8 ($\pm 3.02 \times 10^7$)	6.61×10^8 ($\pm 2.88 \times 10^7$)
MRS broth pH 2.5 + 2% Pure-PC	4.47×10^8 ($\pm 1.95 \times 10^8$)	4.37×10^8 ($\pm 1.32 \times 10^8$)	2.09×10^8 ($\pm 2.40 \times 10^7$)
MRS broth 0.3% bile salt	3.24×10^8 ($\pm 1.66 \times 10^8$)	2.69×10^8 ($\pm 1.82 \times 10^8$)	1.17×10^8 ($\pm 5.62 \times 10^7$)
MRS broth 0.3% bile salts + 6% AFA	5.62×10^8 ($\pm 5.75 \times 10^7$)	7.24×10^8 ($\pm 3.72 \times 10^7$)	8.32×10^8 ($\pm 1.10 \times 10^8$)
MRS broth 0.3% bile salts + 2% Blue Extract	4.57×10^8 ($\pm 5.74 \times 10^7$)	2.95×10^8 ($\pm 3.89 \times 10^7$)	2.29×10^8 ($\pm 1.82 \times 10^7$)
MRS broth 0.3% bile salts + 2% Pure-PC	3.31×10^8 ($\pm 1.74 \times 10^8$)	2.51×10^8 ($\pm 4.47 \times 10^7$)	1.15×10^8 ($\pm 3.89 \times 10^7$)

Table 2
Antimicrobial activity of *L. acidophilus* DDS-1 cell-free supernatants (CFCSs), extracted from MRS broth containing AFA, Blue Extract or Pure-PC, toward *E. coli* O157:H7 ATCC 35150 and *C. albicans* ATCC 14053 performed by agar well diffusion method.

CFCSs	Zone of inhibition	
	E. coli O157:H7 ATCC 35150	*C. albicans* ATCC 14053
AFA- CFCS (MRS Broth with 6% AFA)	++	+++
Blue Extract -CFCS (MRS Broth with 2% Blue Extract)	++	++
Pure-PC-CFCS (MRS Broth with 2% Pure-PC)	++	++
LA-CFCS (MRS Broth)	+	+
AFA (6%)	–	–
Blue Extract (2%)	–	–
Pure-PC (2%)	–	–

Antimicrobial activity was detected as zone of inhibition with widths of (+) 8 mm; (++) 10–12 mm; (+++) > 12 mm; (–) no effect.

with Pure-PC) extracted at different time points (0, 4, 6, 8, 12, 24, 28, 30, 48, 54, 57, 72 h), were examined by killing studies after 2, 4 and 8 h of incubation against *E. coli* O157: H7 ATCC 35150 and *C. albicans* ATCC 14053.

As regards *E. coli* O157: H7 ATCC 35150 (Table 3a), a logarithmic reduction of 2.56 was observed after 2 h of incubation with Pure-PC-CFCS extracted at time point 24 h, while a less reduction (1.69) was evidenced using the correspondent control LA-CFCS. Similarly, after 4 and 8 h of incubation with Pure-PC-CFCS extracted at time point 24 h, increased logarithmic reductions of 3.8 and 4.43 were evidenced, respectively. The antimicrobial activity of Pure-PC-CFCSs extracted at the time points from 26 to 57 h was still evident, with logarithmic reductions higher than the corresponding LA-CFCSs. In particular, a logarithmic reduction of 2.96 was observed after 4 h of incubation with Pure-PC-CFCS extracted at time point 57 h compared to 1.77 log reduction of the correspondent LA-CFCS. Similarly, a logarithmic reduction of 3.65 was obtained after 8 h of incubation with Pure-PC-CFCS extracted at time point 57 h in comparison to 1.39 log reduction observed with the correspondent LA-CFCS.

As regards *C. albicans* ATCC 14053, relative data were summarized in Table 3b. As observed for *E. coli* O157: H7 ATCC 35150, after 2 h of incubation with Pure-PC-CFCS extracted at time point 24 h a logarithmic reduction of 3.74 was registered, higher than 1.60 log obtained with the correspondent LA-CFCS. Similarly, after 4 and 8 h of incubation with Pure-PC-CFCS extracted at time point 48 h, logarithmic reductions of 3.34 and 3.23 were observed, respectively. Pure-PC-CFCSs extracted at the later time points (from 54 to

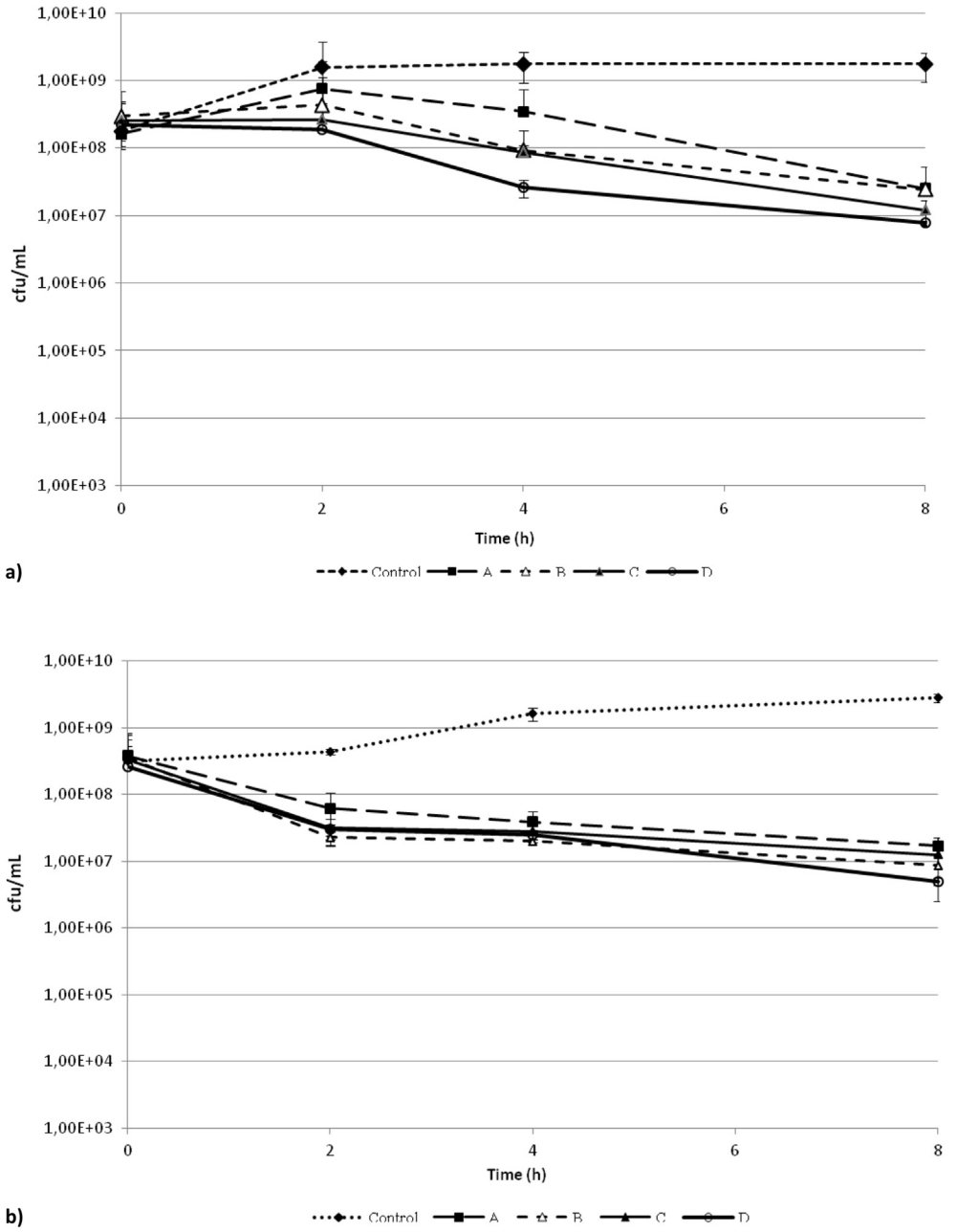


Fig. 3. Time kill studies of LA-CFCS (A), AFA-CFCS (B), Blue Extract-CFCS (C), Pure- PC-CFCS (D) obtained by *L. acidophilus* DDS-1 against (a) *E. coli* O157:H7 ATCC 35150 and (b) *C. albicans* ATCC 14053. Values represent CFU mL^{-1} (mean ± standard deviation) of three independent experiments performed in duplicate.

72 h) showed gradually decreased logarithmic reductions, remaining, in any case, higher compared to the corresponding LA-CFCSs.

4. Discussion

Probiotics represent an area of increasing scientific interest in the field of so-called functional foods or nutraceuticals. The main problems associated with probiotics are their low resistance to production conditions (post-acidification during storage in fermented products, hydrogen peroxide production, oxygen toxicity and storage temperatures) (Klayraung, Viernstein, & Okanogi, 2009) and to the obstacles encountered during gastrointestinal transit (pH, enzymes, bile salts, etc.), that limited the survival and functionality of probiotics and, finally, their health benefits after intake. For this reason, increased attention has been paid to the use of prebiotic substances, natural compounds, that improve probiotic survival. Microalgae provide higher nutritious and stimulatory media for lactic acid bacteria and stimulate their growth and activity. In fact, spray-dried microalgal biomasses contain 3%–7% moisture, 46%–63% proteins, 8%–17% carbohydrates, 4%–22% lipids, 2%–4% nucleic acid, 7%–10% ash, and a wide range of vitamins and biologically active substances (Gyenis, Szigeti, Molnár, & Varga, 2005). In this work, the influence of *Aphanizomenon flos-aquae* (AFA) and two of its extracts, Blue Extract and Pure-PC, on the growth ability of *L. acidophilus* DDS-1 was evaluated. Blue Extract is a hydrophilic extract mainly containing the previously characterized bioactive molecules PC and MAAs (Benedetti et al., 2006;

Table 3
Logarithmic reduction of *E. coli* O157:H7 ATCC 35150 (a) and *C. albicans* ATCC 14053 (b) in time killing studies (2, 4 and 8 h) performed with LA-CFCS (obtained from *L. acidophilus* DDS-1 grown in MRS broth) and Pure-PC-CFCS (obtained from *L. acidophilus* DDS-1 grown in with Pure-PC) extracted at different time points (0–72 h).

a)

Time points:	2 h		4 h		8 h	
	LA-CFCS	Pure-PC-CFCS	LA-CFCS	Pure-PC-CFCS	LA-CFCS	Pure-PC-CFCS
0 h	0.01	0.02	0.05	0.07	0.05	0.07
4 h	0.80	0.85	0.64	0.82	0.80	0.89
6 h	0.85	0.89	0.69	0.86	0.84	0.93
8 h	1.75	1.78	0.76	1.40	0.92	1.06
12 h	1.58	1.59	1.12	1.89	1.80	2.03
24 h	1.69	2.56	1.73	**3.80**	**4.16**	**4.43**
28 h	1.62	2.55	2.23	**3.84**	**3.13**	**3.73**
30 h	1.64	2.34	2.19	**3.89**	2.23	**3.46**
48 h	1.64	2.25	2.15	**3.32**	2.14	**3.36**
54 h	1.69	1.87	2.19	**3.30**	2.07	**3.38**
57 h	1.75	1.76	1.77	2.96	1.39	**3.65**
72 h	1.25	1.75	1.29	2.41	1.15	2.57

b)

Time points:	2 h		4 h		8 h	
	LA-CFCS	Pure-PC-CFCS	LA-CFCS	Pure-PC-CFCS	LA-CFCS	Pure-PC-CFCS
0 h	0.03	0.04	0.04	0.04	0.06	0.07
4 h	0.48	0.74	0.52	0.66	0.53	0.71
6 h	0.49	0.52	0.53	0.74	0.53	0.72
8 h	0.70	1.68	1.25	1.99	1.23	1.71
12 h	1.84	1.96	1.74	1.96	1.84	1.93
24 h	1.60	**3.74**	1.84	**3.19**	1.94	**3.59**
28 h	1.73	**3.67**	1.31	**3.57**	2.09	**3.78**
30 h	1.71	**3.26**	1.57	**3.74**	2.29	**3.67**
48 h	1.55	**3.16**	1.36	**3.34**	1.59	**3.23**
54 h	1.77	2.87	1.30	2.72	2.15	2.20
57 h	1.62	2.60	1.36	2.32	1.50	2.29
72 h	1.24	2.20	1.28	2.13	1.43	2.10

In bold are presented the logarithmic reductions greater than 3.

Scoglio et al., 2014); however, the presence of other unidentified water-soluble compounds cannot be ruled out. On the contrary, Pure-PC is an extract containing exclusively the purified protein (Benedetti et al., 2006).

Our results demonstrated that culture media supplemented with AFA, Blue Extract or Pure-PC have a positive effect over time on the growth of *L. acidophilus* DDS-1 in comparison to the standard MRS broth, and let us suppose that substrates contained in AFA may influence the metabolic features of *L. acidophilus* DDS-1. To our knowledge, this is the first report on the possible effect of AFA as prebiotic substratum, and, therefore, a comparison of the obtained data results very difficult. However, our findings are in agreement with those reported by Bhowmik et al. (2009) which evidenced the positive effect of *Spirulina platensis* added to MRS broth on the growth of several probiotic strains.

Considering that once ingested, probiotic bacteria have to pass through the entire human digestive tract, strains able to tolerate low pH, bile salts, and digestive enzymes, are strongly preferred to be used as probiotics. In this context, we reported that *L. acidophilus* DDS-1 is tolerant to simulated gastric conditions in the presence of AFA, Blue Extract or Pure-PC in culture media. Therefore, the preparation of prebiotic formulations specifically enriched with substances derived from algae, should gain considerable attention, consistent with their potential use as food vehicled-probiotics, as reported for *Spirulina* and *Chlorella* genera in fermented dairy products (Beheshtipour et al., 2012).

In literature data relative to different species of cyanobacteria have been reported; *Spirulina* spp. is the most investigated cyanobacterium and its biological activities, such as anti-cardiotoxic, antiviral and antibacterial activities, are well documented (El-Sheekh, Daboor, Swelim, & Mohamed, 2014; Hernandez-Corona, Nieves, Meckes, Chamorro, & Barron, 2002; Khan et al., 2005). As regards the antimicrobial activity of microalgae, it has been attributed to compounds belonging to several chemical classes including indoles, terpenes, acetogenins, phenols, fatty acids and volatile halogenated hydrocarbons (Cardozo et al., 2007). Microalgal cell-free extracts are already being tested as additives for food and feed formulations, in attempt to replace the synthetic antimicrobial compounds currently in use (Tramper et al., 2003).

Clearly, the development of suitable vehicles for friendly microorganisms might even utilize their probiotic performances, in particular the antimicrobial activity against intestinal pathogens. In this study, the antimicrobial activity of *L. acidophilus* DDS-1 toward *E. coli* O157:H7 and *C. albicans* was enhanced by AFA and its extracts, as shown by time-kill studies. In addition, in the experiments performed using CFCSs of *L. acidophilus* DDS-1, extracted at different time points during the incubation in MRS broth with phycocyanin Pure-PC, the antimicrobial properties of *L. acidophilus* DDS-1 were maximized over time. This effect was probably related to the prolongation of viability observed in *L. acidophilus* DDS-1 grown in MRS broth supplemented with 2% of Pure-PC.

AFA and its extracts used in our study can be considered prebiotics as substratum for new synbiotic combinations able to maximize over time the antimicrobial activity of *L. acidophilus* DDS-1. AFA and its extracts could be proposed as ingredients for functional foods since, beside the well known nutrition capacity, they could also improve the health state reducing the risk of infectious diseases. Further studies on the effects of this alga on other probiotic strains and on their possible addition in various food matrices are suggested.

5. Conclusion

The presented results could offer the possibility to create synbiotics on the basis of probiotics and prebiotics, herein represented by *Lactobacillus acidophilus* DDS-1 and AFA, Blue Extract or Pure-PC respectively, which could regulate not only the growth but also the efficiency of the antibacterial activity. Obviously, because food processing, such as freezing or drying, and storing could modify the chemical characteristics and biological activities of green-blue algae and their derivatives, it becomes crucial to better investigate the link between substrate, manufacture processes and storage, to select the best conditions to improve the positive effects of such compounds.

Conflict of interest

The authors declare no conflict of interest.

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