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Preliminary evaluation of *Spirulina maxima* and *Ascophyllum nodosum* effect on 3 different bacterial strains

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Aim. **We evaluated the effect of *Spirulina maxima* and *Ascophyllum nodosum* on the growth of one *lactobacillus strain* (*Lactobacillus Paracasei subsp. Paracasei* F19) and two enterococci (*Enterococcus casseliflavus* IM416K1, *Enterococcus faecalis* IM388C).**
Methods. **Bacterial growths were evaluated by plating all strains into a 96-well microplate in triplicate and then reading the O.D. values at a wavelength of 595 nm at 0, 2, 4, 6, 8, 20, 26 and 28 hours by means of a microplate reader.**
Results. **A growth inhibition occurred both for *Enterococcus casseliflavus* IM416K1 and *Enterococcus faecalis* IM388C (P<0.01) in presence of any *Ascophyllum nodosum* concentration, whereas a growth enhancement was observable at lower concentrations of *Spirulina maxima* and *Ascophyllum nodosum* (0.2, 0.4 and 0.6 mg mL^{-1}) for *Lactobacillus Paracasei subsp. Paracasei* F19 (P<0.0001) and at lower concentrations of *Spirulina maxima* for *Enterococcus casseliflavus* IM416K1 and *Enterococcus faecalis* IM388C (P<0.0001).**
Conclusion. **In conclusion, these preliminary experiments demonstrated that bacterial growth enhancement or inhibition may be influenced by *Spirulina maxima* and *Ascophyllum nodosum* concentration.**

KEY WORDS: Enterococcus - Growth - Lactobacillus - *Spirulina.*

Spirulina is a filamentous, multicellular, blue-green microalga which belongs to two genera, *Spirulina* and *Arthrospira.*[1] Its chemical analysis has pointed out a high content protein (50-70%) of dry (15-25%), vitamins, essential aminoacids, dietary minerals (2.2-4.8%), and essential fatty acids (1.5-2% of the total lipid content) [1] which make this alga a useful support against malnutrition,[2] hyperlipidemia,[3, 4] obesity [5] and diabetes.[6]

Further, *Spirulina* is endowed of anticancer (mainly due to the presence of phycocyanobilin and chlorophyllin with potent anti-proliferative activity),[7] chelating,[8] antioxidant (due to the high content of vitamin C) [9] and immunestimulating (due to the presence of lipopolysaccharides) [10] and prebiotic [11, 12] properties.

Another widely studied alga is *Ascophyllum nodosum,* a brown seaweed that belongs to the *Fucaceae* family of the macroalgae.[13] As for *Spirulina*, *Ascophyllum nodosum contains* bioactive compounds such as polysaccharides, phlorotannins, amino acids, amino acids, vitamin and carotenoids.[14, 15] Moreover it shares the same metal adsorption capacity of *Spirulina* [16] as literature reports have shown its adsorption capacity for Pb, Zn, Cd, Cr, Cu and Ni [17, 18] probably regulated by ionic-exchange processes.[19] Literature reports have also evidenced a direct antibacterial activity from an extract of *Ascophyllum nodosum* against Gram-positive and Gram-negative bacteria [20] as well as its prebiotic,[21, 22] scavenger [23] and antihyperglycemic [24] activity, due to the pres-

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ence of polysaccharides and the high content of phenolic compounds.

The purpose of our study was to evaluate the effect of *Spirulina maxima* and *Ascophyllum nodosum* on the growth of one *lactobacillus* strain (*Lactobacillus Paracasei subsp. Paracasei* F19) and two enterococci (*Enterococcus casseliflavus* IM416K1, *Enterococcus faecalis* IM388C).

Beyond the fact that enterococci can be considered as part of the lactic acid bacteria [25] and are generally considered as low pathogenic [26] they also share some physiological properties such as growth conditions and catalase activity [27] and can be found in the human gastrointestinal tract [27, 28] and food.[29] *Enterococcus casseliflavus* IM416K1 and *Enterococcus faecalis* IM388C were previously isolated from naturally fermented Italian sausages.[30] Both enterococci presented a high antibacterial activity, in particular *Enterococcus casseliflavus* IM416K1 showed a very strong activity against *Listeria monocytogenes probably* due to the presence of a bacteriocin which seemed more resistant to both chemical and physical parameters variation. It is worth noting that in some cases enterococci may lead to severe diseases such as bacteremias, endocarditis, wound and urinary tract infections in immunocompromised individuals.[26, 31]

Lactobacillus Paracasei subsp. Paracasei F19, which belongs to the homofermentative family of lactic acid bacteria able to convert almost quantitatively glucose to lactic acid,[32] has shown a great ability to bind human and bovine gastric mucin, collagen I and III and fibronectin, to express high surface hydrophobicity as well as to survive to pH 2.5 for 1 hour and 20% bile for 2 hours.[33] This *lactobacillus* has been also extensively studied for its genetic stability,[34, 35] which has warranted its use among consumers, for its ability to strongly induce Th1 and repress Th2 cytokines, for its efficacy and safety in patients with IBS [36] and its stimulating activity towards the lipoprotein lipase inhibitor ANGPTL4 (involved in the triglyceride deposition control into adipocytes) and peroxisome proliferator activated receptors (PPARγ and PPARα) for the treatment of type 2 diabetes and dyslipidaemia.[37] Zampieri *et al.* reported the use of *Lactobacillus Paracasei subsp. Paracasei* F19 in necrotizing enterocolitis Bell's stage 2 management by preventing the clinical progression to stage 3.[38] Recently, it has been also clearly demonstrated that low concentrations of hyaluronic acid (HA) concentrations were able to induce an increased bacterial strains growth within 24 hours, suggesting a possible protective role of HA towards F19 and possibly promoting the *in vivo* proliferation and engraftment after oral administration.[39]

Materials and methods

Tryptic Soy Broth, TSB (Oxoid LTD, Basingstoke, Hampshire, UK) was employed for bacterial strains growth, maintenance and viable count assessment. *Spirulina maxima* was kindly provided by Apulia Kundi, Bari, Italy; whereas *Enterococcus casseliflavus* IM416K1 and *Enterococcus faecalis* IM388C were previously isolated from naturally fermented Italian sausages and *Lactobacillus Paracasei subsp. Paracasei* F19 (Genefilus F19) was provided by Siffra Farmaceutici Florence, Italy. All bacterial strains were maintained in MRS broth medium.

Evaluation of minimal inhibitory concentration for Spirulina maxima and Ascophyllum nodosum

Dilutions for *Spirulina maxima* and *Ascophyllum nodosum* MIC determination were performed in sterile deionized water with concentrations ranging from 0.1 up to 4 mg mL^{-1} for a total of 8 levels of exposure. Then, 50 µl of each dilution were loaded into their respective spots in MRS agar plates seeded with strains. pH values of *Spirulina maxima* and *Ascophyllum nodosum* solutions were evaluated by means of pH-meter (Beckman PHI43).

Assessment of Spirulina maxima and Ascophyllum nodosum effect on Lactobacillus Paracasei subsp. Paracasei F19, Enterococcus casseliflavus IM416K1 and Enterococcus faecalis IM388C

The effects of *Spirulina maxima* and *Ascophyllum nodosum* were evaluated on *Lactobacillus Paracasei subsp. Paracasei* F19, *Enterococcus casseliflavus* IM416K1 and *Enterococcus faecalis* IM388C. The assay was performed in 96-well microplates (Corning Inc., NY, USA).

Firstly, 150 mg of *Spirulina maxima* were weighed (CPA225D, Sartorius S.P.A., Florence, Italy) and then placed into a 50 ml Falcon tube (Corning Inc., NY, USA). Then 200 µl of *Spirulina maxima* or *Ascophyllum nodosum* + MRS (2, 1, 0.8, 0.6, 0.4, 0.2 and 0.1 mg mL^{-1}) were added in duplicate in each plate. Then 50 µl of *Lactobacillus Paracasei subsp. Paracasei* F19, *Enterococcus casseliflavus* IM416K1

or *Enterococcus faecalis* IM388C (working concentrations of about 1 x 10^5 CFU ml^{-1}) in sterile saline solution were added. Non-inoculated TSB was used as negative control whereas TSB + *Lactobacillus Paracasei subsp. Paracasei* F19, *Enterococcus casseliflavus* IM416K1 or *Enterococcus faecalis* IM388C was used as positive control. Microplates were incubated at 37°C in an incubator (Ekort 1500, Angelantoni industrie, Milano, Italy). The O.D. values were measured at a wavelength of 595 nm at 0, 2, 4, 6, 8, 20, 24, 26 and 28 hours by means of a microplate reader (Tecan, Austria).

Effect of Spirulina maxima and Ascophyllum nodosum on Lactobacillus Paracasei subsp. Paracasei F19 tolerance to simulated gastric juice

Spirulina maxima and *Ascophyllum nodosum* effect on *Lactobacillus Paracasei subsp. Paracasei* F19 tolerance to simulated gastric juice for 150 minutes was evaluated according to the procedure proposed by Michida *et al*. Shortly, F19 was harvested by centrifugation (4000 g, 10 min), washed twice and then resuspended with a sterile saline (0.5%, w/v). The preparation of simulated gastric juice consisted in suspending pepsin (1:10 000, ICN) in sterile saline (0.5%, w/v) to a final concentration of 3 g L^{-1} and adjusting the pH to 2.00 with concentrated HCl using a pH meter. Aliquots (200 µL) of the cell suspensions were transferred to a 2 mL Eppendorf tube (Eppendorf s.r.l., Milan, Italy), mixed with 300 µL of sterile water solutions of *Spirulina maxima* and *Ascophyllum nodosum* (2, 1, 0.8, 0.6, 0.4, 0.2 and 0.1 mg mL^{-1}) and finally mixed with 1 ml of simulated gastric. *Lactobacillus Paracasei subsp. Paracasei* F19 viability was assessed according to the drop counting method after incubation at 37°C for 150 min on MRS agar plates (incubation for 72 h at 30 °C).[40]

Statistical analysis

Data obtained from the O.D. readings were used to draw charts where O.D. was expressed as a function of time. Each point of the curves is the average value of two replicates (subtracted of the blank) performed in the same experimental conditions. At each time, two-way analysis of variance (ANOVA) and Bonferroni *post hoc* test were carried out to assess overall differences in O.D. readings obtained from different strains in relation to the control.

Results and Discussion

The antimicrobial effect of *Spirulina maxima* and *Ascophillum nodosum,* which was evaluated by means of MIC test in MRS agar, revealed that all strains were completly inhibited above 4 mg mL^{-1}. Conversely, below this concentration there was a strong inhibition on *Enterococcus casseliflavus* IM416K1 or *Enterococcus faecalis* IM388C by *Ascophyllum nodosum* but not on *Lactobacillus Paracasei subsp. Paracasei* F19 (data not shown). pH values of *Spirulina maxima* and *Ascophyllum nodosum* dilutions ranged from 4.5 to 11.0, depending on both the alga and its concentration.

As to *Lactobacillus Paracasei subsp. Paracasei* F19, it is a probiotic strain with a documented ability to survive during passage in the GI up to 2 hours [41] and this was the reason of testing its potential ability to possibly survive for a longer period (150 min) in presence of increasing concentration of *Spirulina maxima* and *Ascophyllum nodosum* (0.1-2 mg ml^{-1}) to simulated gastric juice. A weak positive effect on probiotic survival appeared directly correlated to *Spirulina maxima* and *Ascophyllum nodosum* concentration: 1) at 2 and 0.8 mg mL^{-1} a five Log of reduction (from 7 to 2 CFU ml^{-1}) was recorded; 2) at 0.6 and 0.2 mg mL^{-1} a 5.5 Log reduction (from 7 to 1.5 CFU ml^{-1}) was recorded; 3) at concentration ≤0.1 mg mL^{-1} no strain survival was detected. At the used concentrations, *Spirulina maxima* and *Ascophyllum nodosum* were not able to enhance the survival of *Lactobacillus Paracasei subsp. Paracasei* F19 during a 150 minutes long exposition to simulated gastric juice, but further studies would be useful to understand if results may be improved by considering higher concentration of these three algae.

We used the innovative method of microplate reader, based on the old concept of dynamic light scattering, in order to perform simultaneous evaluations of several *Spirulina maxima* and *Ascophyllum nodosum* doses. By means of this approach comparable kinetic curves of *Lactobacillus Paracasei subsp. Paracasei* F19, *Enterococcus casseliflavus* IM416K1 and *Enterococcus faecalis* IM388C were obtained in presence of growing concentrations of *Spirulina maxima* and *Ascophyllum nodosum* until 28 hours.

As shown in Figure 1, a recurrent trend in the O.D. kinetics of each strain was observed. In detail, curve profiles raised until 22 h and, after that, kept a plateau trend until 28 hours. However, it is worth

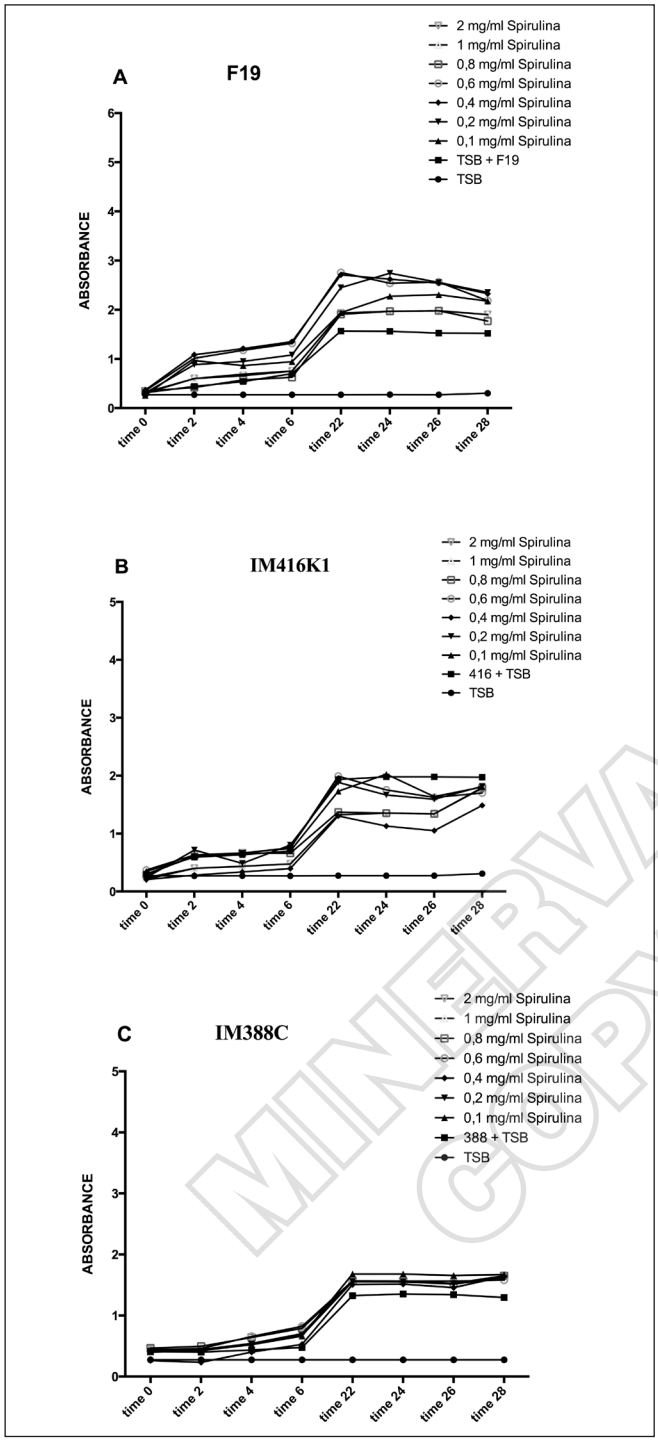

Figure 1.—Effects of *Spirulina maxima* on *Lactobacillus Paracasei subsp. Paracasei* F19 (A), *Enterococcus casseliflavus* IM416K1 (B) or *Enterococcus faecalis* IM388C (C) until 28 h. Bacteria were employed at a starting concentration of 1 x 10^5 CFU mL^{-1}. ****P<0.0001.

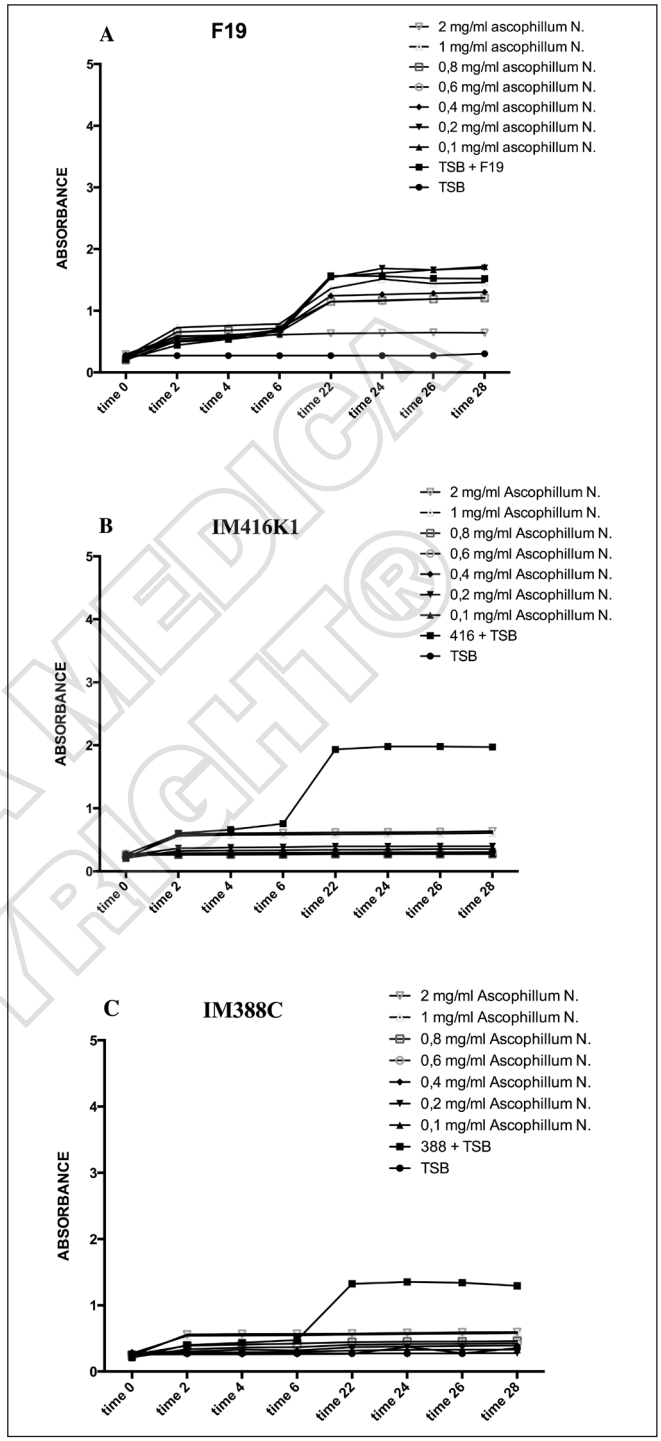

Figure 2.—Effects of *Ascophyllum nodosum* on *Lactobacillus Paracasei subsp. Paracasei* F19 (****P<0.0001), *Enterococcus casseliflavus* IM416K1 or *Enterococcus faecalis* IM388C (**P<0.01) until 28 h. Bacteria were employed at a starting concentration of 1 x 10^5 CFU mL^{-1}.

noting that there were some differences regarding the *Spirulina maxima* concentration effect on each strain growth trend. For instance, *Lactobacillus Paracasei subsp. Paracasei* F19 growth resulted significantly increased at low concentrations of *Spirulina maxima* (0.2, 0.4 and 0.6 mg ml^{-1}, respectively) with respect to higher concentrations (0.8, 1 and 2 mg mL^{-1}, respectively) (P<0.0001). Conversely, *Enterococcus casseliflavus* IM416K1 growth resulted significantly lowered at 0.4, 0.8, 1 and 2 mg mL^{-1} with respect to 0.1, 0.2 and 0.6 mg mL^{-1} (P<0.0001). As to *Enterococcus faecalis* IM388C, its growth was significantly lowered at 0.4 mg mL^{-1} and enhanced at 0.1 mg ml^{-1} (P<0.0001) whereas the other concentrations had almost the same effect.

This might be explained by the fact that all strains could use *Spirulina maxima* as a source of carbohydrates which are transported and phosphorylated by the phosphoenolpyruvate: carbohydrate phosphotransferase system (PTS) with a production of lactate,[41] and also a growth.[42] Thus, once the strains have reached the maximum amount of carbohydrates to growth, which probably are present at lower concentrations of *Spirulina maxima*, a plateau state is reached thus meaning that the strains do not require any supplementary amount, naturally present at higher concentrations *Spirulina maxima*.

The same strains were even employed for the same experiment previously described, but in presence of *Ascophyllum nodosum* instead of *Spirulina maxima*. According to obtained data (Figure 2), strains displayed a completely different behavior: *Enterococcus faecalis* IM388C (P<0.01) and *Enterococcus casseliflavus* IM416K1 exhibited an overall absence of O.D. increase, indicating a bacterial growth inhibition. Conversely, *Lactobacillus Paracasei subsp. Paracasei* F19 displayed a behavior similar to that observed for *Spirulina maxima,* meaning a possible prebiotic activity of both algae (P<0.0001).

In light of the results achieved the inhibitory effect exerted by *Ascophyllum nodosum,* both on *Enterococcus casseliflavus* IM416K1 and *Enterococcus faecalis* IM388C growth, might be considered a valuable alternative in the treatment of some nosocomial infections [43] due to lack of antimicrobial agents and the increasing number of antibiotic resistant bacteria.[44] On the other hand the prebiotic activity of *Ascophyllum nodosum* and *Spirulina maxima* may pave the way for a new era of prebiotics with either stimulating, in terms of bacterial growth, and energizing products.

The possible prebiotic and inhibitory effect of *Spirulina maxima* and *Ascophyllum nodosum*has never been evaluated before. In this study, the effect of *Spirulina maxima* and *Ascophillum nodosum*, on two enterococci and one probiotic *lactobacillus* strain was assessed.

In conclusion, these preliminary experiments, demonstrated that bacterial growth enhancement or inhibition may be influenced by *Spirulina maxima* and *Ascophyllum nodosum* concentration, Our method indicated that a bacterial growth inhibition occurred both for *Enterococcus casseliflavus* IM416K1 and *Enterococcus faecalis* IM388C in presence of any *Ascophyllum nodosum* concentration, whereas a bacterial enhancement was observable at lower concentrations of *Spirulina maxima* and *Ascophyllum nodosum* (0.2, 0.4 and 0.6 mg mL^{-1}) for *Lactobacillus Paracasei subsp. Paracasei* F19 and at lower concentrations of *Spirulina maxima* for *Enterococcus casseliflavus* IM416K1 and *Enterococcus faecalis* IM388C.

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Conflicts of interest.—The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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