

# *Lactobacillus Reuteri* grows in the Microalga *Isochrysis Galbana* Generating a Fermented Compound that reduces the AIEC Bacteria Harmfulness

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## Research Article

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# Abstract

Microalgae are currently considered as alternative sustainable resources for high-value bioproducts such as omega3 polyunsaturated fatty acids (PUFAs). Probiotics are assumed to benefit human health by their direct actions on the composition and function of gut microbiota. Aims of the study are: 1) to set up the anaerobic growth of the probiotic *Lactobacillus reuteri* (*L. reuteri*) in the omega3-rich microalga *Isochrysis galbana* (*I. galbana*); 2) to assess the potential role of the obtained fermented compound (FC) to control the harmfulness of adherent invasive *Escherichia coli* (AIEC) to intestinal epithelial cells.

*I. galbana* powder solubilized in PBS was used for the anaerobic growth of *L. reuteri*. The lipidic content of *I. galbana* and FC was analyzed by GC-MS. Colorectal adenocarcinoma cells CACO2 and the AIEC strain LF82 were used for *in vitro* experiments.

*I. galbana* is shown to be an excellent culture medium for growing *L. reuteri*. The obtained FC significantly reduces the AIEC adhesiveness and invasiveness to intestinal epithelial cells.

We show for the first time that microalgae may represent an innovative culture medium to grow probiotics in anaerobiosis. The obtained FC shows beneficial properties for human health by controlling the harmfulness of AIEC bacteria.

## Introduction

Among functional foods, microalgae are focusing the attention of the whole world thanks to their high content of elements of high value for the well-being of consumers, such as carotenoids, vitamins, essential amino acids, polyphenols and oils rich in omega [1–7]. Several studies have found that many of these components have an important impact on human health as they exhibit anti-mutagenic, anti-cancer, anti-oxidant and anti-inflammatory effects [8, 9]. Microalgae rich in polyunsaturated fatty acids (PUFA), in particular omega3-PUFA, which include eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have aroused particular interest. Indeed, these bioactive compounds appear to be strategic for their effects on the cardiovascular system and for the prevention of cognitive disorders [10–14].

Microalgae species that produces high PUFA concentrations are *Nanochloropsis gaditana*, *Isochrysis galbana*, *Phaeodactylum tricornutum*, and *Cryptocodinium cohnii* [15]. In particular, *Isochrysis galbana* (*I. galbana*) can synthesize EPA and DHA and contains vitamins, polysaccharides, sterols and carotenoids which make it a valid source for human and animal nutrition [16–18]. Interestingly, several studies have also shown that *I. galbana* has beneficial effects on human health and could be thought as a nutraceutical as well as therapeutic agent for the management of inflammatory and oxidative damage-derived disorders [19–21].

Therapeutic microbiology is expanding and beneficial bacteria are being implemented as treatment and prevention strategies for immune disorders and infectious diseases. Currently, probiotics have become highly recognized as supplements for humans and animals due to their beneficial outcome on health

improvement and well-being maintenance [22–25]. *Lactobacillus reuteri* (*L. reuteri*), a commensal-derived anaerobic probiotic, that resides in the human gastrointestinal tract, shows many beneficial effects, such as prevention and/or amelioration of diverse disorders [26–29]. Recent evidence highlights the role of *L. reuteri* in controlling the growth and survival of pathobionts correlated with infectious or chronic gastrointestinal diseases, such as the adherent-invasive *Escherichia coli* (AIEC) [30, 31].

Here, we present an innovative and low-cost method for the growth of *L. reuteri* in raw seaweed extracts from *I. galbana* instead of the conventional medium, under conditions of oxygen deprivation (anaerobiosis). We demonstrate for the first time that *I. galbana* is an excellent culture medium for the growth of *L. reuteri*. Moreover, we show that the omega-3 present in the seaweed are still available after the fermentation process and the fermented compound (FC), obtained from the growth of *L. reuteri* in *I. galbana* in anaerobiosis and thus consisting of the probiotic in a medium with available microalgae-released PUFA-omega3, slows the growth of AIEC bacteria and significantly limits their adhesiveness and invasiveness to intestinal epithelial cells.

## Materials And Methods

### Microalgal and bacterial strains

*I. galbana* lyophilized (Archimede Ricerche Srl, Camporosso, Imola, Italy) was solubilized in phosphate buffered saline (PBS) (36 mg/ml) and used for bacterial growth.

The adherent-invasive AIEC strain LF82 (kindly provided by Prof. Arlette Darfeuille-Michaud, Clermont Universite, Universite d'Auvergne, Clermont-Ferrand, France) was cultured in Tryptone Soy Agar (TSA; plates Oxoid, Basingstoke, UK) for 24 hours at 37°C and then sub-cultured in Tryptone Soy Broth (TSB; Oxoid, Basingstoke, UK) with overnight incubation at 150 rpm, 37°C.

Powder of *L. reuteri* DSM17398 (BioGaia, Stockholm, Sweden) was kept at -20°C, inoculated in commercial medium De Man, Rogosa and Sharpe (MRS; Sigma-Aldrich, St. Louis, USA) and incubated overnight, 37°C without agitation.

### Cell culture

Human colorectal adenocarcinoma cell line, CACO2, was obtained from the American Type Culture Collection (ATCC, Rockville, MA, USA). Cells were grown at confluence at 37°C in Dulbecco's minimum essential medium (DMEM; Gibco, Life Technologies, Carlsbad, CA, USA), supplemented with 10% inactivated fetal bovine serum (FBS; Euroclone, Milan, Italy) and 2 mM L-glutamine, 100 U/ml penicillin and 100 g/ml streptomycin (Biochrom, Berlin, Germany).

### Anaerobic growth of *L. reuteri*

For anaerobic growth,  $2 \times 10^6$  CFU/ml of *L. reuteri* were inoculated in MRS or in 5 ml of *I. galbana* solubilized in PBS (36 mg/ml). Each vial was capped and incubated anaerobically without agitation at

37°C for 120 hours (5 days).

The bacterial growth was evaluated at different times (24, 48, 72, 96, 120 hours) by plating serially diluted samples in PBS on MRS agar plates (1,2% agarose) and incubated at 37°C for 24 hours. Resulting colonies were counted and the viability (CFU/mL) value was calculated based on the plated dilution. After 5 days, the fermentation compound (FC) contained an average concentration of  $3.5 \times 10^7$  CFU/ml.

### **Lipid extraction**

Lipids were extracted from *I. galbana* (36 mg/ml) solubilized in PBS and FC of a single experiment and the analysis was performed in duplicate.

Samples were freeze-dried for 2 days at -40°C and 60 mBar pressure by freeze-dryer (Edwards). Each sample (5 mg) was resuspended with 1 ml of dichloromethane (DCM) and 0.5 ml of methanol/sulfuric acid (MeOH/ H<sub>2</sub>SO<sub>4</sub>) and sonicated for 1 hour at 50°C, 40 KHz frequency. Hexane (1 ml) was used as extracting solvent and, after agitation, calcium carbonate (16 mg) and H<sub>2</sub>O (1 ml) were added and samples were centrifugated for 5 min at 2000 rpm. The separation of polar from apolar phase was repeated twice and finally the latter was dried with nitrogen flow (4 ml for each sample).

### **Gas-chromatography mass-spectrometry (GC-MS)**

GC-MS analysis was performed by a 7890A gas chromatograph (Agilent) with capillary columns SBP-2331 (Sigma-Aldrich) [60 m, 0.25 mm inner diameter (ID), 0.2 µm film thickness]. Helium was used as carrier gas at a linear velocity of 36.26 cm/sec and 1 µl of each sample was injected splitless. The initial column temperature was 40° and held 4 min, ramped to 140° at the rate of 20°/min, ramped to 220° at the rate of 2°/min and held 1min and then finally increased to 260° at the rate of 10°/min and kept at this temperature for 5 min. The mass spectra were recorded using a 5975C mass spectrometer (Agilent) in full scan mode from 45-450 m/z and 240°. The fatty acids concentration of each sample was determined using the software Xcalibur (Thermo Scientific, Waltham, USA) and 37 Component FAME Mix (Supelco, USA) was used as external standard for calibration.

### **AIEC adhesion and invasion assay**

#### *Adhesion assay*

CACO2 cells were grown on 24-well plates at confluence ( $3 \times 10^5$  cells) and infected with LF82 ( $3 \times 10^6$  CFU), or LF82+ *L. reuteri* ( $3.5 \times 10^6$  CFU), or LF82 + *I. galbana* (100 µl), or LF82 + FC (100 µl) at 37 °C for 3 hours. To quantify the adherence of LF82, we followed the protocol of A. Darfeuille-Michaud et al. [32]. Briefly, infected cells were washed twice in PBS and lysed for 10 minutes with 0.5 ml of 0.1% Triton X-100 in PBS buffer. Adherent bacteria were recovered and plated on LB agar plates. The latter were incubated at 37°C overnight and then the colonies were counted for statistical analysis.

## *Invasion assay*

CACO2 cells were infected and incubated as above. For invasion assay, we followed the protocol of A. Darfeuille-Michaud et al [32]. Briefly, after incubation, cells were washed twice in sterile PBS and then incubated in DMEM and McCoy's medium, respectively with 0.1 mg/ml gentamicin for 1 hour to kill the extracellular bacteria. Cells were washed twice in sterile PBS. Lysis, incubation and counts were performed as in the adhesion assay. To ensure maximum reproducibility, accuracy and statistical significance, adhesion and invasion assays were carried out simultaneously in triplicates. To obtain an accurate count of adhesive bacteria, the number of invasive colonies was subtracted from the number of the adhesive ones.

## Statistics

Data are given as mean  $\pm$  standard deviation. All experiments were repeated three times. Comparison between groups was performed by a two-tailed Student t-test (significance taken as  $P < 0.05$ ).

## Results

### **The unicellular microalga *I. galbana* is an excellent culture medium for the growth of *L. reuteri***

*I. galbana* extracts were obtained from dehydrated whole seaweed. *L. reuteri* was inoculated at a concentration of  $2 \times 10^6$  CFU/ml in physiological solution containing *I. galbana* (36 mg/ml) or commercial medium (MRS) and placed at 37°C. The growth was followed for 5 days, that is the time necessary for the bioavailable sugars to guarantee the growth of the lactobacillus in anaerobiosis. Results showed that *L. reuteri* grows in the microalga medium similarly as in MRS reaching the final concentration of  $3.5 \times 10^7$  and  $1.9 \times 10^7$ , respectively (Fig. 1).

### **The PUFA-omega3 content of the microalgae *I. galbana* are still mostly available after the fermentation by *L. reuteri***

The GC-MS lipidomic analysis of *I. galbana* confirmed that the microalga is rich in omega3, especially DHA. More interestingly, the analysis showed that the availability of DHA and EPA is similar before and after fermentation by *L. reuteri*. Indeed, the amount of EPA is unchanged, while alpha-linoleic acid (ALA) and DHA undergoes a small variation between 15 and 20% less after fermentation (Fig. 2). Therefore, the FC, in its fullness, contains the probiotic but is also rich in omega3 and, thus, might represent a very innovative and beneficial compound for the human health.

### ***I. galbana* favors the growth of the probiotic *L. reuteri* respect to that of the pathobiont AIEC LF82**

The AIEC bacteria, known as potent pro-inflammatory microorganisms, strongly increase in the gut of people with intestinal inflammatory disorders and represent a significant challenge for clinicians. Then, we investigated whether *I. galbana* could also represent an optimal culture medium for the growth of harmful bacteria. Then, *L. reuteri* and the AIEC prototype, LF82, were grown together in the microalgae.

Interestingly, although LF82 had been inoculated at a concentration of 1 log higher, in order to mimic the selective advantage this bacterial group has in the inflamed environment, however, the growth curve of LF82 decreases after the first 24 hours while that of *L. reuteri* improves and at the end of fermentation the two species are present at roughly the same concentration ( $9.9 \times 10^6$  and  $6 \times 10^6$ , respectively), clearly suggesting that the probiotic has an advantage in the microalgae culture medium (Fig. 3).

### **The FC derived from the 5 days-growth of *L. reuteri* in *I. galbana* strongly limits the adhesiveness and invasion of LF82 to intestinal epithelial cells**

The human epithelial colorectal adenocarcinoma cells, CACO2, are a recognized *in vitro* model of intestinal epithelial barrier. Hence, we used confluent CACO2 cells to assess the ability of the FC, administered as such, to control the adhesiveness and invasiveness of AIEC LF82, better than the probiotic alone. Confluent CACO2 were exposed for 3 hours to LF82 alone ( $3 \times 10^6$  CFU) or LF82 + *L. reuteri* ( $3.5 \times 10^6$  CFU) or LF82 + *I. galbana* (100  $\mu$ l) or LF82 + FC (100  $\mu$ l).

Results showed that the FC significantly reduces the adhesion ( $P = 0.002$ ) and invasion ( $P = 0.0002$ ) of LF82 compared to the probiotic alone. Surprisingly, *I. galbana* shows a very good capacity to decrease AIEC harmfulness even if lower than that of the FC (Fig. 4).

## **Discussion**

To date, probiotic production has almost exclusively been carried out using conventional batch fermentation and suspended cultures, but there is an emerging interest from the scientific community and increasing demand from the business world to explore and set up innovative fermentation technologies.

Here, we present a new method to grow the probiotic *L. reuteri* in the microalga *I. galbana*, under anaerobiosis condition. The advantages of this protocol are several. First, the cost is low. Besides, the probiotic can be administered without being previously purified from its culture medium, which, on the contrary, being made up of an omega3-rich microalga, could show beneficial properties for the host organism. Finally, since probiotics must colonize an oxygen-deprived gut environment, the fermentation of the microalga in anaerobiosis can be considered a form of pre-adaptation of probiotics that could improve their survival in the bowel.

Interestingly, our results show for the first time that the microalgae *I. galbana* is an excellent medium to grow the probiotic *L. reuteri*, which in fact lives and proliferates in the microalgae as in the conventional medium.

Recent data demonstrate that adding the microalgae *Chorella vulgaris* to the *Lactobacillus* spp. growth medium accelerates the growth and metabolic activity of bacteria, suggesting that the combination allows for the creation of innovative, functional products which confer favorable properties to the final product [33].

In addition to proposing the microalgae as a growth medium for Lactic acid bacteria, we also support the idea that the microalgae-probiotic combination shows great potential for generating a novel functional product. Actually, we show that, after fermentation, the PUFA-omega3, of which *Isochrysis* is rich, are not consumed by *L. reuteri*, who instead prefers the consumption of sugars, and remain almost all available in the FC, giving the latter beneficial properties.

Accordingly, we aimed to investigate whether the FC had the ability to impair AIEC-induced inflammation with greater effectiveness than the probiotic alone. AIEC bacteria represent a particular pathotype of *Escherichia coli* abnormally colonizing the intestinal mucosa of patients with chronic intestinal inflammation, such as those affected by Crohn's disease [34]. Recent evidence suggests that perturbation of the microbial community (dysbiosis) favors the emergence of opportunistic pathogens, in particular AIEC, that can increase the incidence and severity of gut inflammation, since they strongly adhere to and invade intestinal epithelial cells, inducing inflammatory cytokine secretion [35]. Using an in vitro model of gut epithelial barrier, we first confirmed previous evidence that *L. reuteri* is able to reduce the pathogenicity of enteroinvasive *Escherichia coli* [36, 37], including AIEC [31]. More interestingly, we showed that the treatment with FC prevented the AIEC adhesiveness and invasiveness to epithelial cells more effectively than the probiotic alone. We speculate that this improved effect is due to the omega3 present in the compound that act in combination with the probiotic.

Furthermore, we showed for the first time that *I. galbana* itself significantly reduces the adhesiveness and invasiveness of AIEC LF82 to epithelial cells. Although previous authors demonstrated that several microalgae, including *I. galbana*, are prospective candidates to inhibit the growth of gram-positive bacteria [38], however, to our knowledge, their potential in controlling pathobionts has never been reported yet. It is worth noting that in our experimental set up, *I. galbana* had already proven to be a deterrent for the AIEC group, hampering their growth while favoring that of *L. reuteri*.

## Conclusions

Current evidence indicates that microalgae have the potential to become a novel source of bioactive molecules, especially for those who might wish to enhance the nutritional and functional quality of foods. Even further, we show that microalgae may represent an innovative culture medium to grow probiotics in anaerobiosis and that the obtained FC should be administered as such without separating the probiotic from the culture medium which, indeed, by selecting a proper microalga, could be a useful resource of beneficial bioactive molecules such as omega3. Really, we demonstrate that the FC has beneficial properties by decreasing the harmfulness of AIEC bacteria to gut epithelial cells.

These findings are important in the development of novel tools for the production of probiotics, whose market is growing dynamically, as well as for the supply of novel fermented products that should represent in the future safe therapeutic agents to be utilized for the management of oxidative damage-derived or inflammatory disorders.

Finally, the *L. reuteri* grown in *I. galbana* should be considered a true novel vegetarian probiotic since free from all animal-derived ingredients differently from probiotics grown in the traditional culture medium.

## Declarations

### Data Availability

The data that support the findings of this study are available from the corresponding author [L. S.], upon reasonable request.

### Author contributions

S.L. and C.V. conceived and designed the experiments; C.E., S.G. and L.B. conducted the experiments; L.I., V.R., N.A. and P.F. analyzed the data and performed the statistical analysis; S.L. wrote the manuscript. All authors read and approved the final manuscript.

### Additional information

**Correspondence** and requests for materials should be addressed to S.L.

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## Figures

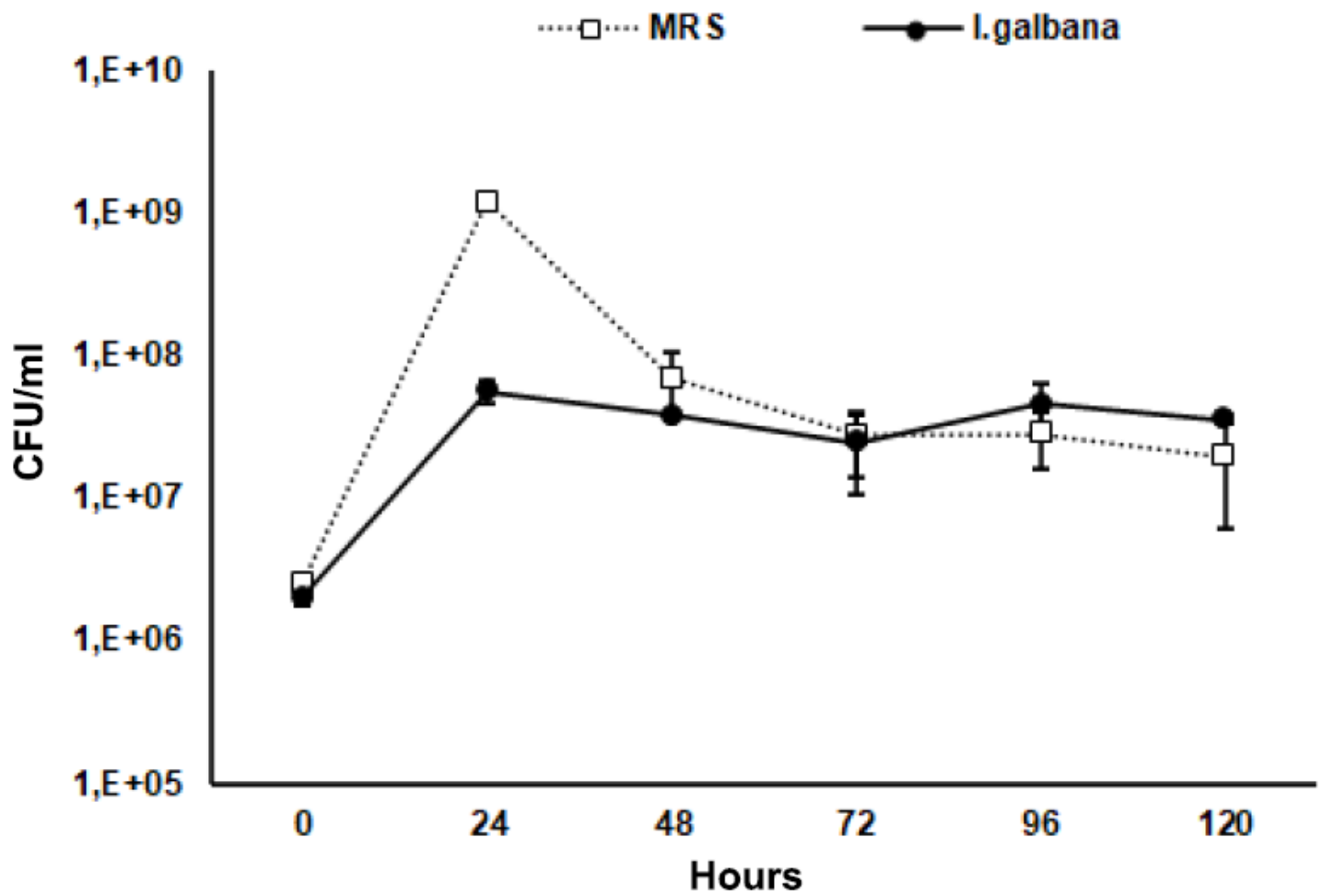


Figure 1

*L. reuteri* grows well in the microalga *I. galbana*. *L. reuteri* reaches the same concentration in *I. galbana* (●) and MRS (□) after 5 days of fermentation. *L. reuteri*: *Lactobacillus reuteri*; *I. galbana*: *Isochrysis galbana*; MRS: commercial medium

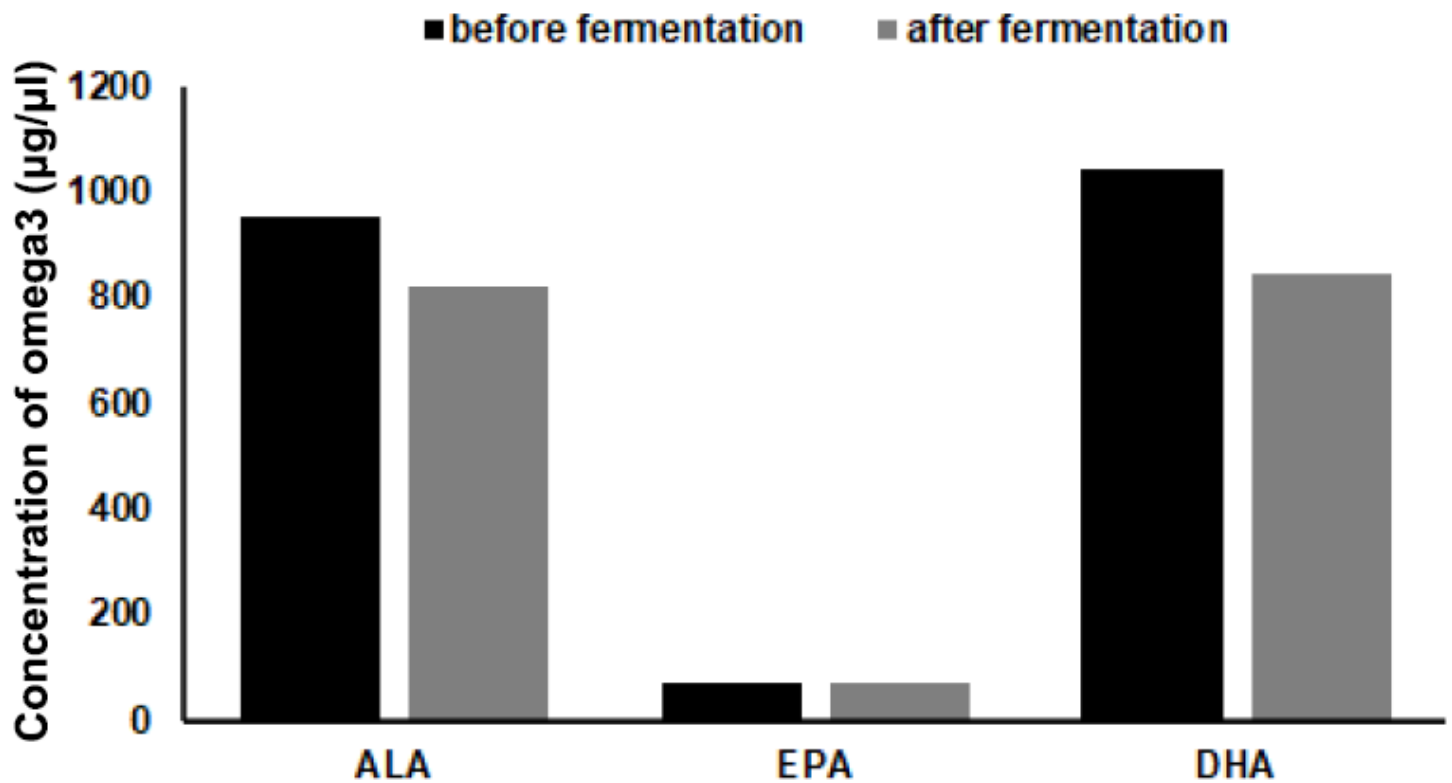


Figure 2

Microalgal omega3 are still available after the probiotic fermentation. Lipidomic analysis by GC-MS of *I. galbana* shows that ALA and DHA are slightly reduced, while EPA is unchanged after fermentation. ALA: alpha-linoleic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid.

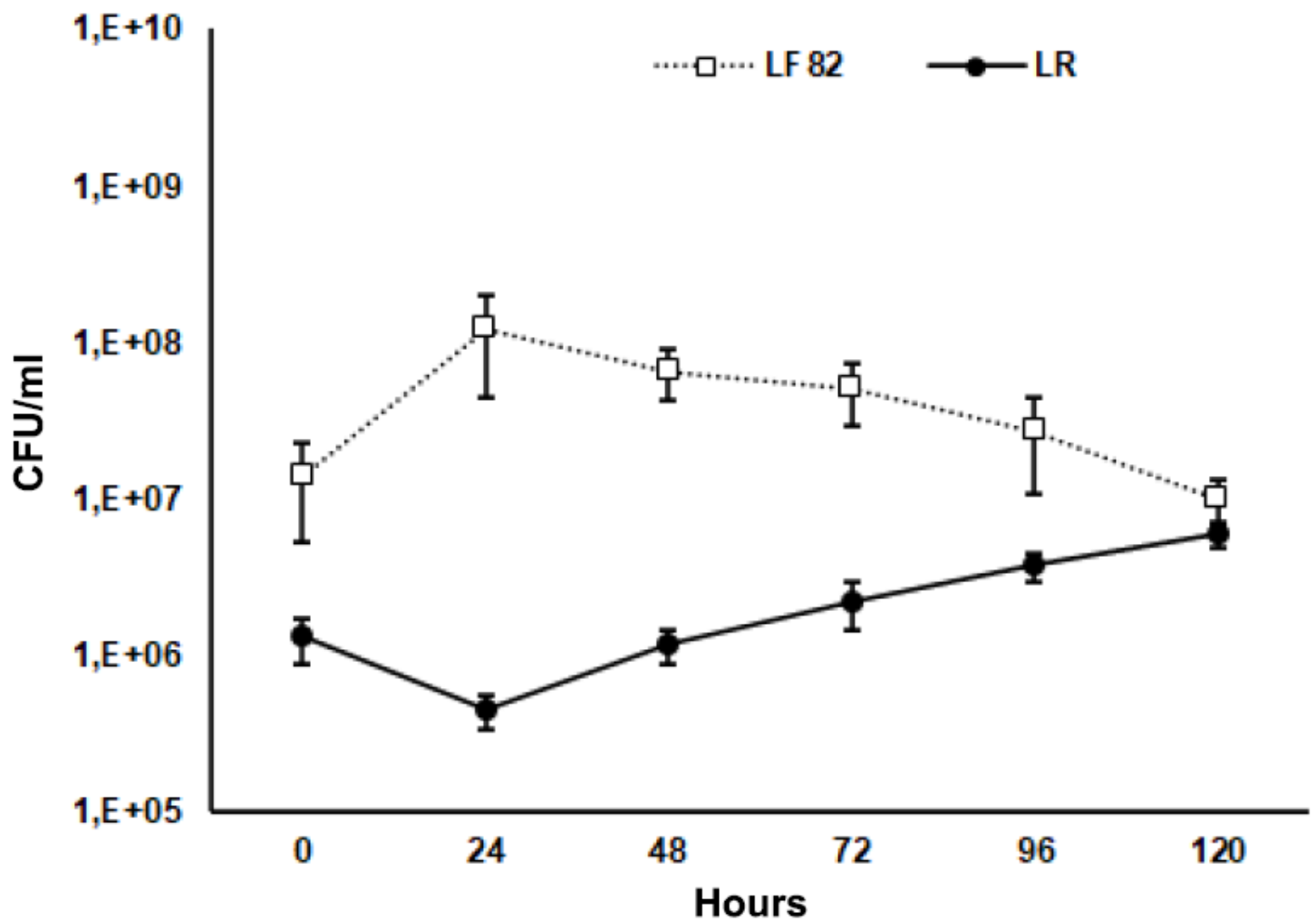
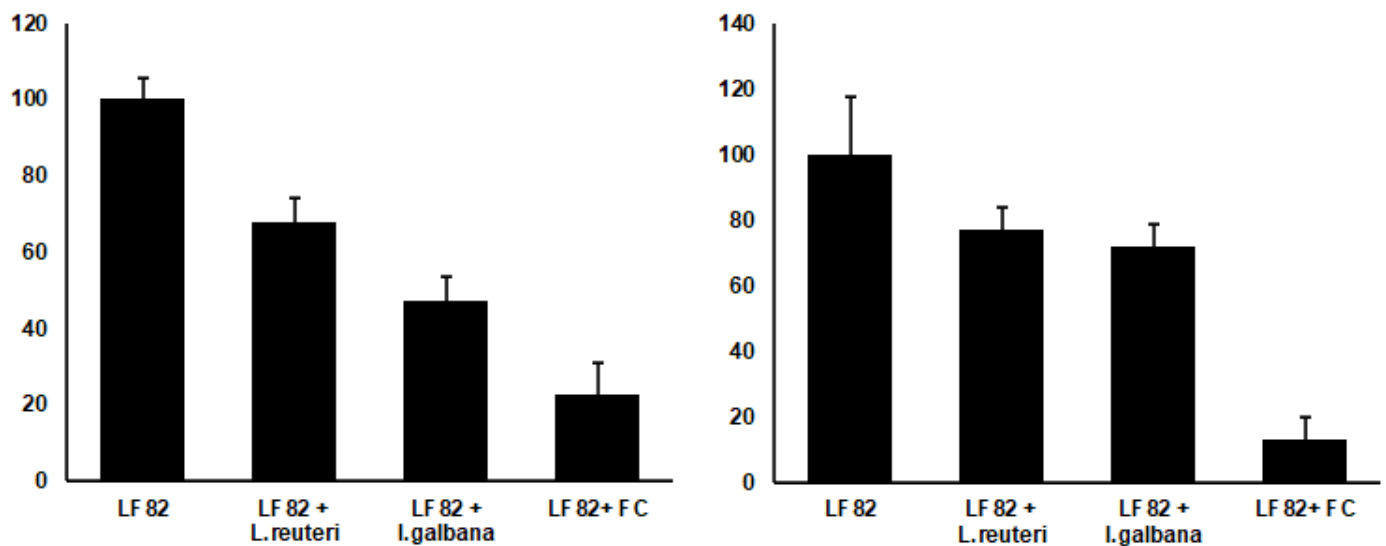


Figure 3

*I. galbana* favors the growth of *L. reuteri* respect to AIEC LF82. After 24 hours of growth, LF82 decreases (□) while *L. reuteri* increases (●) reaching the same concentration of LF82 after 5 days of fermentation.



## Figure 4

The FC reduces the adhesiveness and invasiveness of AIEC LF82 to CACO2 cells. The FC significantly reduces the adhesion and invasion of LF82 compared to the probiotic and microalgae alone. AIEC: adherent invasive *Escherichia coli*; FC: fermented compound. \*/ $\Delta$   $P < 0.05$ ; \*\*/ $\Delta\Delta$   $P < 0.01$ ; \*\*\*/ $\Delta\Delta\Delta$   $P < 0.001$ .