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## ***Spirulina platensis* sustainable lipid extracts in alginate-based nanocarriers: an algal approach against biofilms**

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### **Abstract**

Cyanobacteria and especially *Spirulina platensis* are recognized as abundant resources of lipids and polyunsaturated fatty acids (PUFA). The main valorization of these lipids has been the biofuel production but another approach was developed here: the investigation of *S. platensis* fatty acids as anti-biofilm agent for skin diseases. In fact, chronic wounds represent an important burden for occidental health care systems, and are usually related to the presence of a biofilm, especially of *Candida* species.

In order to develop a biomimetic approach against *Candida* biofilm formation, free fatty acids (FFA) isolated from microalgae *S. platensis* were explored. Four solvents, including three bio-sourced ones, and three extraction conditions were screened. Lipid and pigment amounts, combined with FFA profiles were analyzed to select the optimal conditions: 30 minutes of ultrasonic extraction using dimethylcarbonate or ethylacetate as extraction solvent. A vectorization using a macroalgal-alginate nanocarrier was successfully performed. Vectorized extracts were safe towards keratinocytes and thus compatible with a topical use. *Spirulina* lipid-enriched extracts showed a high anti-adhesion activity at low concentrations (about 80 % inhibition after 24h at 0.2 mg/mL). The combination of extracts in copper-alginate nanocarriers potentiated the anti-adhesion activity and exhibited a good anti-biofilm activity (about 50 % inhibition after 24h at 0.1 mg/mL). The combined approach of encapsulated *Spirulina* lipid extracts represented thus a relevant concept to develop all in one anti-biofilm weapons against *Candida* and represent a new perspective for algae biomass valorization.

**Keywords** *Spirulina platensis*, free fatty acids, alginate, sustainable extraction, *Candida albicans*, nanocarriers

## **Introduction**

Microalgae has been well known as abundant sources of lipids and especially free fatty acids (FFA) [1,2]. One major valorization of microalgal FFA was biofuel production [1,2]. But those FFA, especially polyunsaturated (PUFA), may also represent a potential sources of topical antibiotics for chronic wound healing [3].

In fact, FFA are antimicrobial agents naturally occurring on skin. They are in charge of the microbiota regulation and exhibit wide antimicrobial spectrum (antibacterial, antifungal,

antibiofilm...) depending on carbon chain length and number of double bonds [3-6]. Ruffel et al. [3] explored the antimicrobial potential of 29 FFA. Among them 15 exhibited antibacterial activity against gram positive bacteria especially *Staphylococcus aureus* and *Streptococcus pyogenes*. Lauric acid, palmitoleic acid and long chain polyunsaturated fatty acids (PUFA) were found as the most active [3]. Those data were confirmed by different studies [4-6]. Few FFA were reported as potential agent against *Candida sp.*: short chain saturated FA (C10, lauric acid mostly), monounsaturated FA (MUFA) (myristoleic and palmitoleic acids) and the PUFA linoleic acid were found to be active [7]. Those FFA were found classically in microalgae biomass. FFAs could also affect the expression of bacterial virulence factors by disrupting cell-to-cell signaling and thus could prevent initial bacterial adhesion and so biofilm formation [4].

In fact, biofilm formation is widely considered as one of the major virulence attributes of *C. albicans* and a key contributing factor to the unacceptably high mortality rates associated with candidiasis [8]. Cells in biofilms are recalcitrant to conventional antifungal therapy. Actually, sessile yeasts have been shown to become up to 1,000 fold less susceptible to conventional antifungal drugs compared to planktonic yeasts [9,10]. So, new therapeutic strategies are urgently needed to fully succeed in controlling biofilm formation and eradicating sessile *C. albicans* yeasts. In order to develop a biomimetic approach against biofilms, microalgal FFA appeared as a promising opportunity.

Among all microalgae, *Spirulina platensis*, a blue cyanobacterium, appeared as a good model. Marangoni et al. [11] recently investigated the *in vitro* antifungal activity of a water-extract of *S. platensis* against 11 strains of *C. albicans* and reported minimal inhibitory concentrations (MIC<sub>50</sub>) ranging from 0.125 to 0.5 mg/mL. Lewis Oscar et al. [12] recently investigated the anti-biofilm effect of *S. platensis* methanol extract against numerous bacteria of clinical importance, including *S. aureus* and *Escherichia coli* and reported promising results. To our

knowledge, no data is available regarding the anti-biofilm interest of *S. platensis* against *C. albicans* or *S. platensis* lipid extract as anti-adhesion agent.

The toxicity of solvents used for FFA extraction may limit the development of microalgae FFA as anti-biofilm agents at industrial scale. Considering the solvents classically used to produce FFA enriched extracts, such as chloroform/methanol mixture [13], greener alternatives should be developed to propose a sustainable process. Resulting FFA enriched extracts would be highly hydrophobic which could limit their penetration into *Candida sp.* biofilm.

Indeed, biofilms are constituted of hydrophilic molecules, mainly polysaccharides that prevent the penetration of lipophilic molecules. The encapsulation of hydrophobic active molecules in hydrophilic delivery systems could be a way to achieve a satisfactory penetration of FFA in biofilms. Several types of nano-sized drug delivery systems were recently developed targeting microbial biofilm such as polymeric or lipid-based nanocarriers [14,15]. Core-shell nanocarriers with a lipid core and a hydrophilic shell seem to be the most adapted to the administration of lipophilic molecules like FFA in biofilms. Alginate, a well known algal polysaccharide, was selected as the main component of our nanocarrier, in order to combine macro and microalgal metabolites valorization.

The work reported here was a proof of concept of the potential of *S. platensis* lipid extract and alginate-based nanocarriers combination as potential alternative therapeutic for *Candida* biofilm occurring on skin.

## **Material and Methods**

### *1. Chemicals*

Ethyl acetate (EtOAc), chloroform (CHCl<sub>3</sub>), ethanol (EtOH) 96%, methanol (MeOH), toluene, hexane, formic acid, diethylether, glacial acetic acid, petroleum ether, sulfuric acid

96% (H<sub>2</sub>SO<sub>4</sub>) and dimethylsulfoxide (DMSO) were purchased from Carlo Erba (Val de Reuil, France). Dimethyl carbonate (DMC), (±)- $\alpha$ -tocophérol 96% (vitamin E), oleic acid, linoleic acid, palmitic acid, myristic acid, stearic acid, palmitoleic acid,  $\gamma$ -linolenic acid, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), menadione and glucose were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France). Manganese chloride (MnCl<sub>2</sub>, 4 H<sub>2</sub>O) and phosphoric acid 85 % were purchased from Merck pro analysis (Darmstadt, Germany). Labrafac® WL 1349 was purchased from Gattefossé (Saint-Priest, France). Span 80® and Tween 80® were purchased from Seppic (Castres, France). Sodium alginate was kindly provided by Setalg (Pleubian, France). Copper nitrate (Cu(NO<sub>3</sub>)<sub>2</sub>), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Dulbecco's modified Eagle medium (DMEM), Yeast Nitrogen Base (YNB), foetal bovine serum (FBS), phosphate buffer saline (PBS), solution of penicillin-streptomycin were purchased from Fisher Scientific SAS (Illkirch, France). Vanillin, acetic acid trihydrate 99+% were purchased from Acros Organics (Geel, Belgium). Water was purified using a Milli-Q system (Millipore Corporation, Bedford, MA, USA).

Lyophilized *Spirulina platensis* biomass was kindly provided by DENITRAL SA (Lamballe, France).

## 2. Extraction protocol

500 mg of *S. platensis* biomass were extracted using 10 mL of solvent. CHCl<sub>3</sub>/MeOH (2:1, v/v), EtOH, EtOAc and DMC were used as extraction solvents. Magnetic stirring (1h) and ultrasonic assisted extraction (UAE) (15 and 30 min) approaches were preliminary screened. 30 min UAE was selected to prepare all extracts. Extracts were then centrifuged at 8000×g (Rotanta 32A, Hettich) during 20 min. Supernatants were collected. This extraction step was repeated thrice. Resulting supernatants were pooled, concentrated under vacuum and then

filtered on syringe filter (PTFE, 0.45  $\mu\text{m}$ ). A scale up was performed on 20 g of biomass and 400 mL of solvent for encapsulation and biological evaluation of extracts.

### 3. Extract analysis

#### 3.1. High-Performance Thin-Layer Chromatography (HPTLC)

A CAMAG HPTLC instrumental set-up (CAMAG, Muttenz, Switzerland) was used for analyses. HPTLC Silica Gel 60 F<sub>254</sub>, 20 cm x 10 cm (Merck, Darmstadt, Germany) were employed.

##### 3.1.1. Extracts phytochemical analysis

Plates were developed with toluene/EtOAc/formic acid (25:20:2, v/v/v) [16] or petroleum ether/diethylether/formic acid (90:10:2, v/v/v) [17] as mobile phase (migration distance 8.5 mm), at room temperature. Visual inspection of plates was performed under UV (254 and 365 nm) and white light, before and after derivatization using manganese chloride reagent ( $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ) [18]. 10  $\mu\text{L}$  of standard solution were applied bandwise. Extract were solubilized with *n*-hexane/isopropanol (3:2, v/v) in order to obtain solutions at 10 mg/mL. 10  $\mu\text{L}$  of solution were applied bandwise.

##### 3.1.2. FFA quantification

Fatty acids standards (Fatty acids AGL (Sigma-Aldrich, France) were used to build up calibration curve for quantification of total FFA.

The chromatographic procedure used was adapted from Maurage et al. [19]. After the plates were derivatized and dried on a heating plate, the quantification was a function of the level intensity of grey. Visual inspection of plates was performed under white light, before and after derivatization. Plates were quantified after staining by densitometric analysis performed

using the winCATS software (Camag). FFA levels were expressed as  $\mu\text{g AGL/mg Lipid}$  extract. Full HPTLC procedure was available in Supporting Information.

### *3.2. Content in chlorophylls and carotenoids*

The protocol was adapted from Wang et al. [20]. Samples were sonicated during 5 min with hexane/acetone (60:40, v/v) and centrifuged 5 min at 1850 g using swinging-bucket centrifuge. The supernatant was then recovered, and evaporated under vacuum. Resulting solid residues were then dissolved in MeOH and absorbance was measured at 450, 645 and 663 nm using a Multiskan GO plate reader (ThermoFisher Scientific, Villebon Coustaboeuf, France) with SkanIt RE software. Mass concentration of chlorophylls and carotenoids were obtained thanks to equation described by Wang et al. [20]. Error was expressed as standard deviation.

### *3.3. Total Lipids*

The rapid total lipid assay was adapted from Park et al. [21]. Samples and reference castor oil were dissolved in  $\text{CHCl}_3$  at 1 mg/ml, evaporated at 90 °C. Solid residue was solubilized in 0.1 ml of  $\text{H}_2\text{SO}_4$  and then heated for 10 minutes at 90 °C. Samples were cooled for 5 minutes in ice and then mixed with a phosphovanillic reagent (PVR) (1.2 mg of vanillin in a mixture of 8 mL of 85%  $\text{H}_3\text{PO}_4$  and 2 mL of water). Absorbance at 530 nm was then measured using a Multiskan GO plate reader (ThermoFisher Scientific, Villebon Coustaboeuf, France) with SkanIt RE software. Results were expressed as mg of equivalent of castor oil per g of extract, error was expressed as standard deviation.

### *3.4. FFA analysis using GC-FID*

FFA were separated by one-dimensional TLC (LK5, 20 × 20 cm, Carlo Erba, Val de Reuil, France) using Hexane/Diethyl ether/Acetic acid (60:40:1, v/v/v) as mobile phase. FFA spots were scraped and collected in screw-cap glass tubes. Fatty acids were prepared as fatty acids



methyl esters before gas chromatography analysis (GC-2010plus, Shimadzu Scientific instruments, Noisiel, France) using a BPX70 capillary column (60 m, SGE, Chromoptic SAS, Courtaboeuf, France). Hydrogen was used as carrier gas with a constant pressure (120 kPa). After an on-column injection of sample at 60 °C, oven temperature increased from 60 °C to 220 °C. Fatty acids methyl esters were detected by a FID at 255 °C and identified by comparison of their retention times with commercial standards (Supelco 37, Fatty Acid Methyl Ester mix, Sigma-Aldrich, France, St Louis, MO). Fatty acids levels were expressed as the percentage of total integrated peaks area using the GC solutions software (Shimadzu, Noisiel, France). This analysis was performed once on each sample.

### *3.5. Statistical analysis*

ANOVA Tukey test was performed using MiniTab software.

## *4. Nanocarriers*

### *4.1. Preparation of extract-loaded copper alginate-based nanocarriers (ANC)*

ANC were prepared using ultrasound oil-in-water emulsification followed by surface gelation with cupric ions inspired by Nguyen et al. [22]. Appropriate quantities of DMC-based or EtOAc-based *S. platensis* dry lipid extracts were dissolved in Labrafac ® WL 1349 (6 mg/mL). This solution was mixed with sorbitan monooleate to constitute the oil phase. It was incorporated into an aqueous phase made of polysorbate 80 and sodium alginate solution, using an ultrasonic probe (Vibra-cell ultrasonic processor, Sonics, 20 kHz). A nanoemulsion was obtained, in which the oil droplets were surrounded by sodium alginate chains. Still under probe sonication, a solution of copper nitrate was added to obtain stable copper alginate-based nanocarriers (n=3 for each extract). For the biological studies, ANC concentrations were expressed in dry matter (g/L). These concentrations were obtained after freeze-drying of the samples.

#### 4.2. Physicochemical characterization of alginate-based nanocarriers

*Cryo-Transmission electron microscopy (cryo-TEM):* Specimens were prepared using a “cryoplunge” cryo-fixation device (Gatan, USA). The samples were observed under low dose conditions ( $< 10 \text{ e-/A}^2$ ), at  $-178 \text{ }^\circ\text{C}$ , using a JEM 1230 'Cryo' microscope (Jeol, Japan) operated at 80 kV and equipped with a LaB6 filament. All micrographs were recorded on a Gatan 1.35 K x 1.04 K x 12 bit ES500W CCD camera. Cryo-TEM images were processed using the ImageJ software to enhance the contrast.

*Size:* The hydrodynamic diameter (HD) and polydispersity index (PdI) were assessed using a dynamic light scattering (DLS) instrument (NanoZS, Malvern Instruments, UK). Each sample was diluted 1/50 in ultra-pure water before measurements in triplicate at  $25^\circ\text{C}$ .

*Surface charge:* zeta potential was determined on a 1/50 dilution of the ANC suspension with a NanoZS zetameter (Malvern Instruments, UK) in triplicate at  $25^\circ\text{C}$ , with the detection angle at  $13^\circ$  and a 633 nm red laser.

*Stability:* ANC were stored at  $4^\circ\text{C}$  as suspension in water. Size and surface charge were measured after one month of storage and repeated every month during three months.

#### 5. *In vitro* toxicity on human HaCaT cells

The toxicity of extract-loaded ANC was explored *in vitro* on HaCaT (human keratinocytes) cells in culture to determine safe concentrations of use. Cells were grown in DMEM enriched with 10 % FBS and 1% penicillin-streptomycin, at  $37^\circ\text{C}$  and 5 %  $\text{CO}_2$ . The MTT assay was performed in 96 multiwell plates. Cells were seeded at 5000 cells per well and grown for 24h in complete culture medium. Cells were then treated by increasing concentrations of ANC (from 0.01 to 10 % w/w, i.e. 0.015 to 15g/L of dry matter) dispersed in culture medium for 24h. Control cells were treated by the same amount of culture medium. Cell viability was determined using a multiwell-scanning spectrophotometer plate reader at 540 nm (ELx800,

Bio-Tek, France). Cell viability was calculated as a percentage referring to untreated cells. A Kruskal-Wallis test was conducted to determine differences among the test groups.

## 6. *Biofilm of Candida albicans*

### 6.1. *Stock solution preparation*

Stock solutions of *S. platensis* biomass extracts were prepared at 10 mg/mL in DMSO. Each extract stock solution was diluted extemporaneously in YNB medium, supplemented with 50 mM glucose (YNB-Glc) to obtain extract solutions at 0.4 mg/mL or 0.2 mg/mL. In these conditions, final DMSO concentrations did not exceed 2% of the overall volume in wells over the next experiments.

### 6.2. *Yeast preparation*

*C. albicans* ATCC 28367 yeasts were grown for 24 h on Sabouraud agar slants. Four loopfuls of this culture were carefully transferred to 30 mL of YNB-Glc and incubated overnight at 37 °C without shaking. Obtained blastospores were harvested, washed twice in 0.1 M phosphate-buffered saline (PBS, pH 7.2) and adjusted to  $10^7$  or  $2 \cdot 10^7$  blastospores/mL in YNB-Glc.

### 6.3. *Anti-biofilm growth*

Untreated 96-well tissue culture plates received 100  $\mu$ L of the yeast suspension ( $2 \cdot 10^7$  cells/mL) per well and 100  $\mu$ L of a test-solution: extract solution at 0.4 mg/mL, or extract-loaded ANC stock solutions at 0.2 mg/mL (extract concentration in nanocarriers). Control wells received 100  $\mu$ L of the yeast suspension ( $2 \cdot 10^7$  cells/mL) per well and 100  $\mu$ L of PBS (control corresponding to ANC-LE) or DMSO in PBS (final concentration 2 %) (control corresponding to LE). After 24 h or 48h at 37 °C, non-adherent microorganisms were removed by aspiration. Wells were then washed with PBS and observed under classical inverted optical microscope (IX51® inverted microscope, Olympus) for visual inspection

prior to biofilm quantification using a previously described metabolic assay based on the reduction of a tetrazolium salt (XTT) [26, 27]. Briefly, 300 mg/L XTT and 0.13 mM menadione were added to 200  $\mu$ L of PBS in each well. Plates were incubated for 3 h at 37°C without shaking, then gently agitated and XTT formazan was measured colorimetrically at 450 nm (Tecan Sunrise microplate reader). Background formazan values were determined with plates containing PBS only or containing PBS, XTT, and menadione; these values did not exceed 0.005 absorbance units and were therefore considered not significant. Each condition was repeated 10 times in two separate experiments (20 results).

Statistical analysis was conducted thanks to Mann-Whitney test to determine differences among the test groups.

#### *6.4. Anti-biofilm tests*

Untreated 96-well tissue culture plates received 200  $\mu$ L of the yeast suspension ( $10^7$  cells/mL) per well. After 24h of incubation at 37°C, the non-adherent microorganisms were removed by aspiration and wells were washed with PBS. Then, 200  $\mu$ L of extract solutions at 0.1 or 0.2 mg/mL or extract-loaded ANC at 0.1 mg/mL (extract concentration in nanocarriers) were added to each well and plates were incubated for 24h at 37°C. Control wells received 200  $\mu$ L of PBS (control of ANC-LE) or DMSO 2% (control of LE). Wells were then washed with PBS and observed under inverted optical microscope prior to biofilm quantification XTT assay [23, 24]. Each condition was repeated 10 times in two separate experiments (20 results).

Statistical analysis was conducted thanks to Mann-Whitney test to determine differences among the test groups.

## **Results and discussion**

### *1. Spirulina platensis FFA extraction optimization*

The first part of the work aimed to optimize the microalgae FFA extraction process. Microalgae lipids extraction was one of the main bottleneck for their valorization. Most of the processes described used highly toxic solvents, like chloroform or hexane, not compatible with a sustainable production. Biosourced solvent, like ethanol and ester solvents, recently appeared as interesting solvents to enhance FFA content in lipid extracts from yeast [25]. Thus, four extraction solvents were selected here and screened to compare their performance: CHCl<sub>3</sub>/MeOH (2:1, v/v) mixture as reference solvent (Lipid extract (LE1)), and three bio-based solvents, two of which were ester solvents: EtOH (LE2), EtOAc (LE3) and DMC (LE4). The main objective was to produce FFA-enriched extracts with lipophilic pigments' rates as low as possible. In fact, chlorophylls and carotenoids were usually co-extracted with lipids due to similar lipophilicity.

Different extract processes were also screened (1h magnetic stirring, 15 and 30 min UAE) and 30 min of UAE was selected as the best compromise between productivity and yield.

All LEs obtained were then analyzed to determine total lipids, chlorophylls and carotenoids' rates (Table 1). Considering lipids, FFA content was also quantified using HPTLC-densitometry protocol [19] (Table 1) and the FFA profile was obtained using GC-FID (Figure 1).

*Insert here table 1 and figure 1*

LE1 and LE2 showed very similar results ( $p > 0.05$ ), for all parameters: chlorophylls about 60 mg/g of LE, carotenoids about 15 mg/g of LE and total lipids about 350 mg of equivalent castor oil/g of LE. Interestingly, LE3 and LE4 differed from those previous extracts. They were especially twice more enriched in total lipids, indicating a higher selectivity of EtOAc and DMC towards lipids. Looking at FFA's rate in extract, LE3 and 4 were 8-fold more concentrated than LE1 and 2, highlighting selectivity towards FFA. This was also noticeable

on HPTLC plate (Figure 1A) by more intense FFA blue spot. Considering lipophilic pigment rates, LE3 was enriched in chlorophylls and carotenoids. On the other hand, DMC exhibited a very high selectivity for lipids since LE4 exhibited very low (about 2 mg/g) chlorophyll and carotenoid rates.

Considering FFA profiles (Figure 1B) obtained by GC-FID, again LE1 and 2 were very close. Saturated FFA occurred in majority, mainly palmitic and stearic acids. Mono-unsaturated (mainly oleic acid) and  $\omega$  6-PUFA (linoleic and  $\gamma$ -linolenic acids) accounted each for almost 15 % and 30 % of all FFA respectively. Unidentified FFA, *i.e.* not included in our standards list (See Supporting information), represented about 6 %. Those FFA were probably short chain saturated FFA, like lauric acid, or long chain unsaturated FFA (more than 22 carbons) or uncommon unsaturated FFA, like  $\omega$  9, occurring in microalgae biomass [9]. Unidentified FFA might be interesting, especially short chain FFA that were well known for antibacterial and antifungal activity [3,4].

LE3 and LE4 exhibited distinct FFA profiles. In LE3, saturated FFA were important (31 %) but  $\omega$  6-PUFA were the majority (almost 50 %). In LE4,  $\omega$  6-PUFA and unidentified FFA represented here almost 75 % (53 and 19 % respectively) of the FFA fraction, and saturated FFA decreased to less than 20 %. LE4 exhibited then the highest rate of unidentified FFA. Nevertheless,  $\gamma$ -linolenic and linoleic acids were the major FFA found in both extracts.

*Insert here Table 2*

To conclude on extraction optimization process, two of the bio-based solvents exhibited a good selectivity towards lipids and especially FFA: EtOAc and DMC. Resulting LE3 and 4 extracts were 8-fold FFA enriched comparing to reference  $\text{CHCl}_3/\text{MeOH}$  mixture. Moreover, DMC could discriminate lipids and lipophilic pigments. For encapsulation and biological

evaluation, only LE3 and LE4 were selected according to their higher rates of FFA and to their high MUFA and PUFA contents, compared to LE1 and 2.

## *2. Extracts encapsulation in alginate-based nanocarriers*

### *2.1. Physico-chemical characteristics of extract-loaded ANC*

To obtain a potential synergetic effect against biofilms, FFA enriched *S. platensis* green lipid extracts were encapsulated in alginate-based nanocarriers (ANC), previously described by Nguyen et al [24]. ANC are core-shell nanosystems (see Figure 2A) adapted for the topical administration of pharmaceutical or cosmetic active ingredients [22, 26].

These nanocapsules were made of a triglyceride core in which the extract can be dissolved, and a shell of alginate gel, a polysaccharide extracted from brown algae, which interest has been already demonstrated as components of wound dressings [27-29]. ANC were obtained by ultrasound oil-in-water emulsification, followed by a surface gelation via a divalent cation. The presence of surfactant molecules in order to obtain a nanoemulsion may have an interest against biofilms. On the other hand, cupric ions we used to obtain the final surface gelation are able to further increase the effect on biofilms. Indeed, cupric ions have a strong capacity to form gels with alginate [28,30] and have an intrinsic antimicrobial activity [31]. Moreover, copper-polysaccharide nanocomposites have already shown interesting antimicrobial activity [32].

*Insert here figure 2*

The activity of different nanosystems against planktonic and/or sessile *C. albicans* yeasts has already been studied [33-38], but no data were reported concerning those nanocarriers.

Encapsulating complex natural extract was also a challenge, and few data were reported in the literature [39-41]. Most authors reported the encapsulation of polyphenols extracts in various nanocarriers based on chitosan. During the first step of ANC formulation, LE3 extract in

EtOAc or LE4 extract in DMC were dried then dissolved in medium chain triglycerides (Labrafac® WL1349) at a concentration of 6 mg/g. The second step consisted in encapsulating these extracts in alginate-based nanocapsules by a nanoemulsification-surface gelation method with  $\text{Cu}^{2+}$  ions.

The obtained systems, further referred to as ANC-LE3 and ANC-LE4, showed a spherical shape with a rough gelled surface (Figure 2B) as determined by cryo-TEM. The physico-chemical characteristics of  $\text{Cu}^{2+}$ -stabilized ANC were very close to the ones of  $\text{Ca}^{2+}$ -stabilized ANC described by Nguyen et al [22]: (i) their size was around 230 nm; (ii) their polydispersity index (PdI) was inferior to 0.2, confirming a low polydispersity of the suspensions, and (iii) their zeta potential was negative. No significant difference was noticeable between the nanoparticles loaded with the two types of extracts, neither in size nor in charge of the ANC. The extract-loaded ANC were stable during 1 month of storage at 4°C (see Figure 2C): no change was noticed neither for the size nor surface potential of the nanocarriers, whatever the extract encapsulated. Also the integrity of the encapsulated extract was ensured, showing no degradation during nanocarrier preparation.

## *2.2. In vitro toxicity of extract-loaded ANC on skin cells*

As ANC-LE systems were intended for topical application on skin or wound-associated biofilms and their immediate surroundings, their toxicity against the main cells of the epidermis was determined *in vitro* by the MTT test [42] (Figure 3).

*Insert here figure 3*

ANC show no toxicity (viability >70%) up to a concentration of 1.5g/L. An important toxicity is observed for the highest concentrations (10g/L). A Kruskal-Wallis test performed simultaneously on the toxicity profiles did not permit to highlight a significant difference between both extracts or between blank and LE-loaded ANC. The observed toxicity could be



mainly attributed to the components of the nanocarriers [22]. With an IC<sub>50</sub> of respectively 5.7, 5.1 and 4.5 g/L, blank ANC, ANC-LE3 and ANC-LE4 showed a lower or similar toxicity on keratinocytes compared to other core-shell systems used to deliver active molecules to skin or for wound healing reported in literature, like lipid nanocapsules or SLN [22, 26, 43, 44]. ANC enriched in *S. platensis* lipid extracts can then be considered as skin-compatible at doses up to 1.5 g/L. Free and vectorized extracts were then investigated on *C. albicans* biofilms.

### *3. Anti-biofilm activity against Candida albicans*

#### *3.1 Prevention of biofilm growth of the selected S. platensis lipid extracts and encapsulated lipid extract*

##### *3.1.1 Selected S. platensis lipid extracts*

FFA, especially MUFA and PUFA, were known to disturb the first step of bacterial biofilm formation: adhesion [4]. Thus LE3 and LE4 were investigated as potential preventive agent against *C. albicans* biofilm growth. Those investigations were done with free and encapsulated LE. Results were summarized in Figure 4.

*Insert here Figure 4*

LE3 and LE4 showed a high anti-adhesion activity after 24h (percentage of inhibition of LE3: 76 %; LE4: 80 %;  $p < 0.0001$  for both). This activity was extended even after 48h (LE3: 71 %; LE4: 78 %;  $p < 0.0001$  for both). Those extracts were inactive on planktonic *C. albicans*, since their MIC<sub>50</sub> were found to be  $> 0.2$  mg/mL (data not shown), highlighting a specific activity against biofilm.

It was interesting to note that both extracts exhibited a similar activity even if their composition was different. LE3 was enriched in both lipids and lipophilic pigments, whereas LE4 was only enriched in lipids. Those data were in favor of a limited impact of chlorophylls

and carotenoids on anti-biofilm growth activity. The FFA profile of LE3 and LE4 extracts exhibited similarities as MUFA and PUFA represented 66 and 62 % of total FFA respectively, which may confirm a key role of those metabolites in anti-adhesion activity. More precisely,  $\gamma$ -linolenic acid and linoleic acid were found in large amount in the two extracts but in different ratios (see Table 2).

In fact, the disturbing potential of microalgae's lipid extract on biofilm growth was highlighted here for the first time for fungal strain. This confirmed the potential of *S. platensis* FFA as biofilm preventive agent.

### 3.1.2 Encapsulated lipid extracts

The anti-biofilm growth activity of ANC-LE3 and ANC-LE4 was measured (Figure 4).

Interestingly, the encapsulation did not reduce the activity of the extracts. The inhibition rate of nanosystems at 0.1 mg/mL reached: 82 % (24h) and 68 % (48h) for the ANC-LE3 and 70 % (24h) and 61 % (48h) for the ANC-LE4. These values were similar to or higher than that described in the literature for extract-loaded nanocarriers of comparable size tested on *C. albicans*. For nanoemulsions loaded with *Eucalyptus globulus* oil, the 68 to 86 % inhibitory potential was reported with 100-fold higher extract concentrations (11.25 - 22.5 mg/mL) than those tested in our study [45]. Similarly, for nanostructured lipid nanocarriers loaded with *Melaleuca alternifolia* essential oil, the inhibition of biofilm formation was close to 70 % using 31% (w/w) loading, whereas we obtained the close activity using ANC-LE3 and ANC-LE4 with loading of 2.5 % (w/w) and 5 % (w/w) respectively [46].

### 3.2. Activity against already formed biofilm

As we could expect, *S. platensis* LE did not show any activity on pre-formed biofilm ( $p > 0.2$ , Figure 5), certainly due to their hydrophobicity. However, their encapsulation may enhance

their penetration in the biofilm. So the anti-biofilm activity of ANC-LE3 and ANC-LE4 was tested on 24h-old pre-formed *C. albicans* biofilms (Figure 5).

*Insert here figure 5*

Compared to untreated biofilm control ( $p= 0.0004$ ), the growth of the biofilm treated during 24h with 0.1 mg/mL in extract-loaded ANC was inhibited: of 34% for LE4 and of 52 % for LE3. The difference between ANC-LE3 and ANC-LE4 could be explained by their different oily core composition. As we can notice in the Table 2, LE3 and LE4 exhibited similar FFA ratios except for linoleic acid which was 5 % higher in LE3. A potential role of carotenoids or chlorophyll could not be excluded this time, since they were found in high rates in LE3. As we could expect blank ANC exhibited a proper anti-biofilm activity, similar to loaded extract-ANC. This highlighted new perspective for other extracts vectorization against biofilm. This result did not exclude in fact a contribution of LE extracts to antibiofilm activity of ANC-LE3 and ANC-LE4, since the interactions between nanocarriers component with the oily core were complex.

Nevertheless, this result is promising compared to similar nanocarriers described in the literature. Nanoemulsions of *E. globulus* oil at a concentration around 22 mg/mL induced a decrease of the metabolic activity of cells in the preformed biofilm of 84% [45]. In another study chitosan nanocarriers encapsulating ferrulic acid decreased the metabolic activity of *C. albicans* in biofilms of only 22.5% but still at a higher concentration, whereas their positive surface charge could have been favorable to the penetration in the negatively charged biofilm [47]. Melaleuca oil-loaded nanostructured lipid carriers incubated in the same conditions but at the concentration of 31 % (w/w) led to a destruction of almost 70% of a formed biofilm [46]. The results obtained with ANC-LE3 and ANC-LE4 suggest that extracts and anti-biofilm components of the capsules, surfactant molecules and copper ions, could efficiently reach *C. albicans* cells through the biofilm. We can conclude that the nanovectorization of the

extracts using ANC-LE3 and ANC-LE4 permitted an enhanced anti-biofilm activity that can be assigned to its combined activity with the components of the capsule.

## **Conclusion**

Bio-sourced solvents, EtOAc and DMC, were found to be sustainable alternatives to produce lipid and FFA enriched extracts from *Spirulina* microalgal biomass. DMC demonstrated a good selectivity towards lipids and FFA especially  $\omega$ 6-PUFA.

The resulting lipid extracts were successfully vectorized in Cu-alginate based nanocarriers, ANC, which have physico-chemical properties suitable for a topical application. The extract-loaded ANC appear compatible with a topical use, since they demonstrated a good safety towards keratinocyte cultures.

On one hand, microalgal lipid extracts obtained with green solvents EtOAc and DMC both demonstrated promising anti-adhesion activity towards *Candida* cultures, both in 24 and 48h. The microalgae FFA anti-adhesion activity towards fungal strain was reported here for the first time. On the other hand, ANC loaded with low concentrations of the extracts showed an interesting potential against already formed *C. albicans* biofilms. These results established the proof of concept of the combination of lipid extract with alginate-nanocarrier as potential anti-biofilm agent.

In conclusion, the combination of the *S. platensis* lipid extracts and copper alginate-based nanocapsules allowed thus the development of an all in one active ingredient, exhibiting both anti-adhesion and anti-biofilm activity. This combination could be a prime candidate to both prevent and cure biofilm-related infections such as chronic wounds. Thus a new valorization opportunity of *S. platensis* FFA and macroalgal alginate was highlighted in this work.

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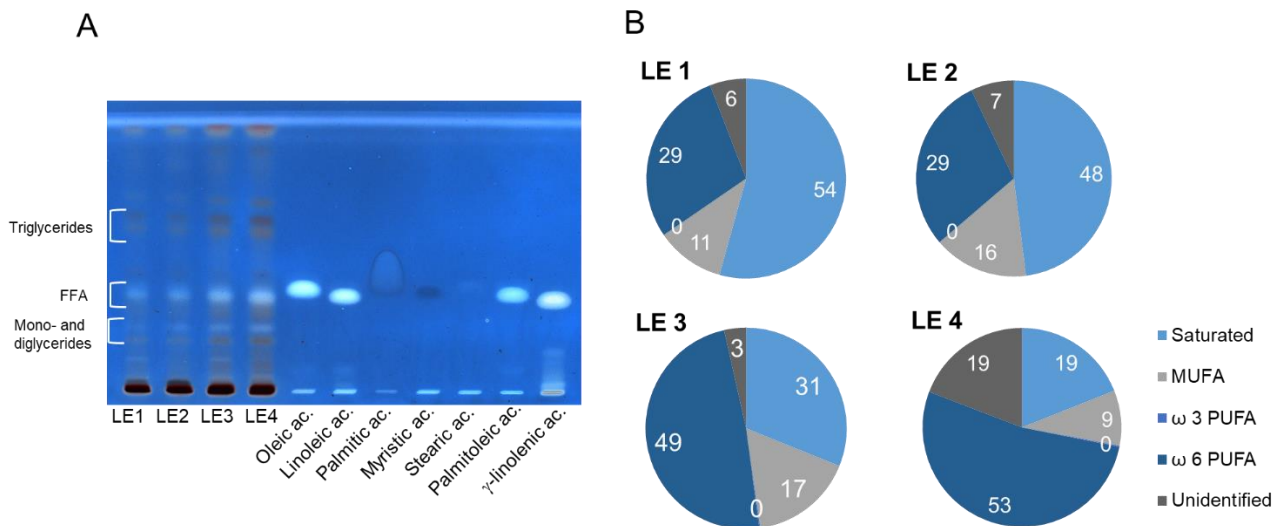
Table 1: Composition of different lipid extracts (LE) vs *S. platensis* biomass

	<b>Solvent</b>	<b>Chlorophylls (mg/g of LE)*</b>	<b>Carotenoids (mg/g of LE)*</b>	<b>Total lipids (mg of equiv. castor oil/g of LE)*</b>	<b>FFA (mg/g LE)</b>
<b>Biomass</b>		2.4±0.45	1.7±0.1	39.7±3.1	4.0
<b>Lipid extract 1 (LE1)</b>	CHCl <sub>3</sub> /MeOH (2:1,v/v)	57.4±3.4 <sup>a</sup>	14.8±0.6 <sup>d</sup>	387.4±67.4 <sup>s</sup>	3.8
<b>Lipid extract 2 (LE2)</b>	EtOH	68.5±1.9 <sup>a</sup>	15.9±1.0 <sup>d</sup>	326.1±45.9 <sup>s</sup>	3.5
<b>Lipid extract 3 (LE3)</b>	EtOAc	101.2±1.1 <sup>b</sup>	30.0±1.3 <sup>e</sup>	700.6±89.6 <sup>h</sup>	25.1
<b>Lipid extract 4 (LE4)</b>	DMC	2.4±0.4 <sup>c</sup>	1.7±0.1 <sup>f</sup>	762.9±70.8 <sup>h</sup>	26.2

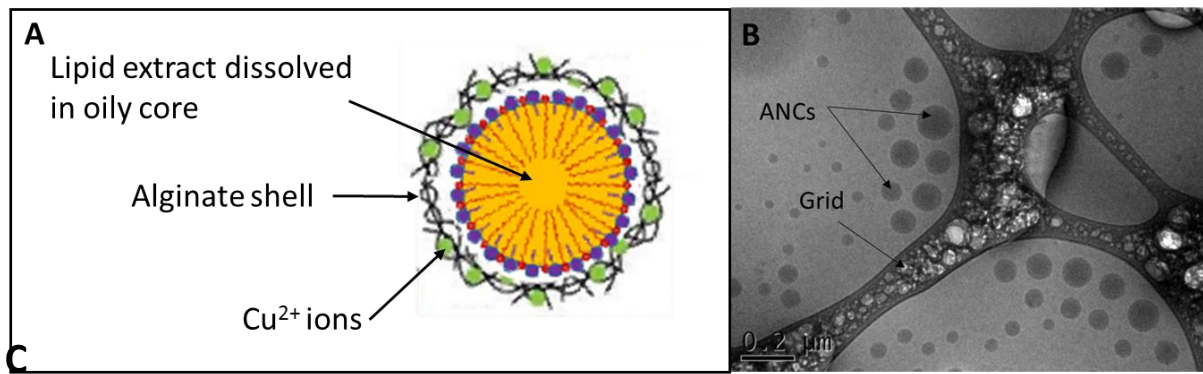
\* extracts which don't share any letter were significantly different ( $p < 0.05$ )

Table 2: FFA ratios in LE3 and LE4, in relative percentage of total FFA

	<b>LE3</b>	<b>LE4</b>
<i>Saturated</i>		
<b>Myristic acid</b>	0.3	0.6
<b>Palmitic acid</b>	14,8	6,9
<b>Stearic acid</b>	15,1	1,4
<i>MUFA</i>		
<b>Myristoleic acid</b>	0.1	0.1
<b>Palmitoleic acid</b>	0.9	1.3
<b>Oleic acid</b>	13,7	5,6
<i>PUFA</i>		
<b>Linoleic acid</b>	21,8	14,6
<b>γ-linolenic acid</b>	24,8	36,9

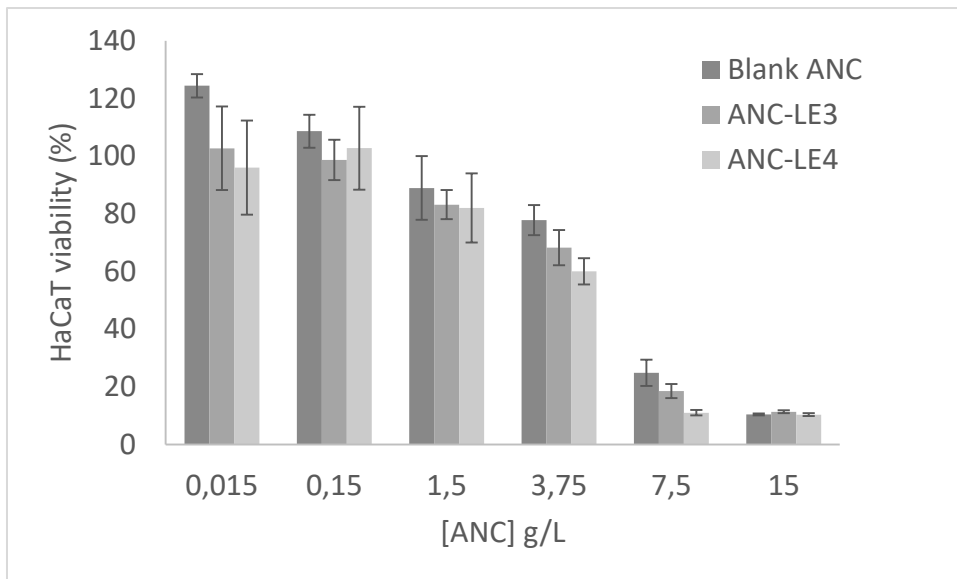


*Figure 1: (A) HPTLC plate of lipid extracts analysis versus FFA standards, mobile phase Petroleum ether/Diethylether/Formic acid (90:10:2, v/v/v), derivatized with  $MnCl_2 \cdot 4H_2O/H_2O/MeOH/H_2SO_4$  at 366 nm; (B) FFA relative percentages of lipid extracts obtained by GC-FID.*



Sample		Size (nm)	Pdl	zeta potential (mV)
Blank ANC	initial	235 ± 11	0.174 ± 0.009	-19.0 ± 0.4
	1 month of storage (4°C)	239 ± 1	0.165 ± 0.007	-19.3 ± 0.9
ANC-LE3	initial	231 ± 8	0.177 ± 0.018	-19.5 ± 1.0
	1 month of storage (4°C)	228 ± 15	0.177 ± 0.024	-21.8 ± 1.2
ANC-LE4	initial	237 ± 8	0.196 ± 0.018	-25.1 ± 1.0
	1 month of storage (4°C)	233 ± 6	0.197 ± 0.016	-23.8 ± 0.7

Figure 2: constitution (A), typical cryo-TEM image (B) and (C) physico-chemical characteristics of blank and extract-loaded alginate nanocapsules



*Figure 3: Viability of HaCaT cells after a 24 h contact with blank and extract-loaded alginate nanocarriers at various concentrations, expressed in dry matter, as determined by the MTT assay.*



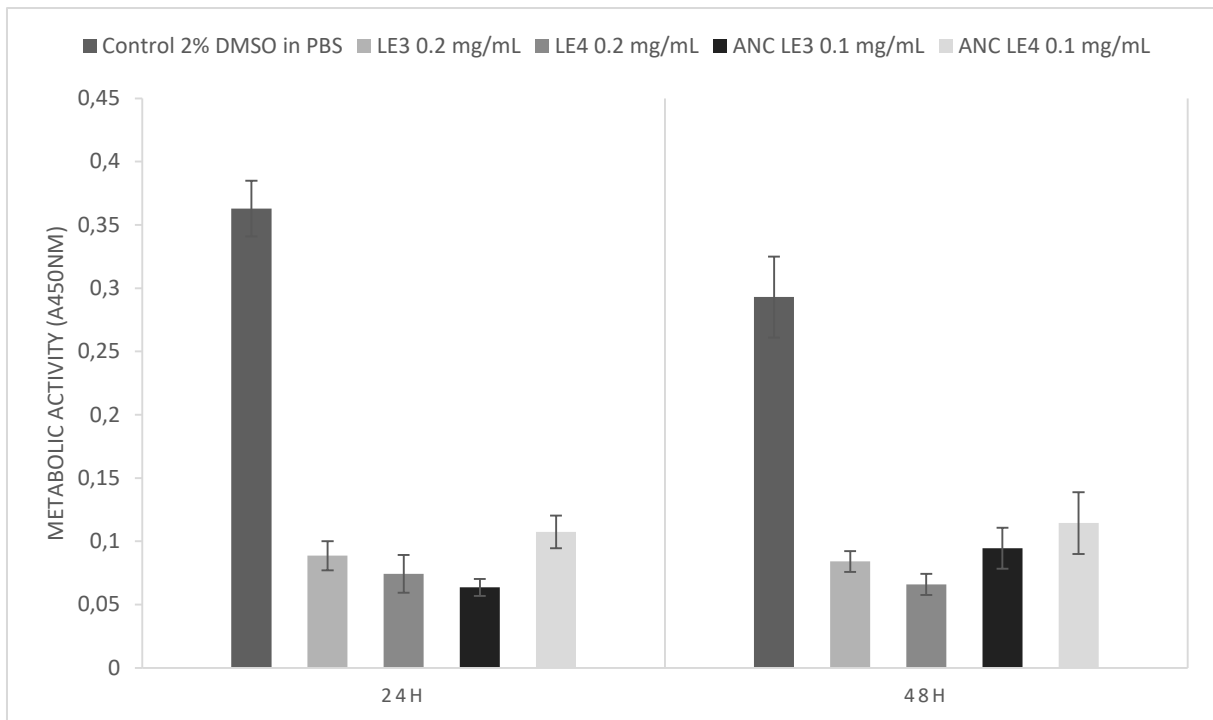


Figure 4: Anti-adherent activity of extracts and extract-loaded nanocarriers. The adhesion phase was for 24h or 48h in the different conditions before removing planktonic yeasts

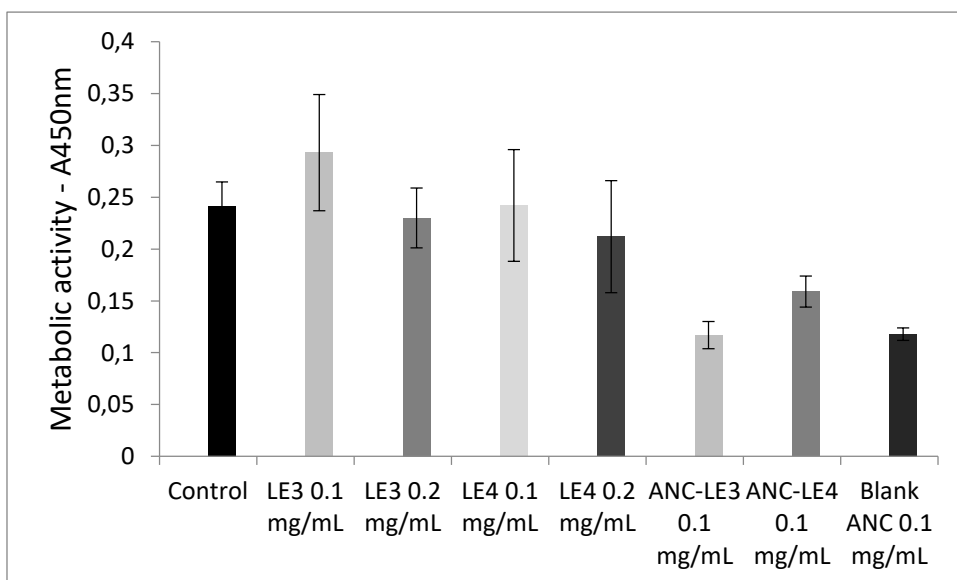


Figure 5: Activity of lipid- extracts and nanocarriers against a 24h old *C. albicans* biofilm (XTT assay)