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Impact of cold atmospheric pressure plasma (CAPP) treatments on the prebiotic potential of *Arthrospira platensis* (Spirulina)

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ABSTRACT

Powdered *Arthrospira platensis* (Spirulina) is one of the most valuable nutraceutical products in terms of functionality and food-fortification due to its recognized prebiotic, antioxidant, and immunomodulatory potential. The present study aims to assess the quality of this matrix as a prebiotic after Cold Atmospheric Pressure Plasma (CAPP) processing. CAPP-treated Spirulina samples (1 mg/mL), at effective discharge power of 1.1, 1.7, 2.2 and 3.3 W for 5 min, were used to promote the growth of the probiotics *Limosilactobacillus reuteri* and *Lacticaseibacillus rhamnosus* GG. Chicory inulin was used as reference prebiotic material (1 mg/mL). - Microorganisms were inoculated in the different prepared media, and incubated 48 h, at 30 °C, under anaerobic conditions. The probiotics' growth rate on Spirulina (CAPP treated and untreated) dispersions, protein stability and their secondary metabolites production were evaluated. The bacterial growth (>7.0 ± 0.2 $\log_{10}$ cycles) and prebiotic indices of treated Spirulina samples (compared to untreated Spirulina) confirm the prebiotic functionality before and after CAPP processing. The proteomic and metabolomic profiles of the hydrolyzed matrix post-fermentation revealed significant differences in the nutritional values of the final fermented product, depending on the applied CAPP intensity. The present study provides first-time insight into CAPP optimization for increased bioavailability of Spirulina compounds and quality proofed nutraceutical development.

1. Introduction

Aquatic systems represent an extensive reservoir of fauna, flora, and microbiota, as well as a source of bioactive compounds with functional properties [1]. Some marine organisms, including crustaceans, fish, mollusks, sponges, bacteria, macro- and microalgae, have unique physiological adaptive capacities that allow them to produce various compounds and secondary metabolites. These include sterols, proteins, polysaccharides, antioxidants, pigments and polyunsaturated fatty acids. Marine-derived compounds have great potential as ingredient sources for application in the pharmaceutical, food, nutraceutical and cosmetic industries [1].

Spirulina (*Arthrospira platensis*) is a gram-negative filamentous cyanobacterium growing in large freshwater lakes on several continents and in the Pacific Ocean near Japan and Hawaii [2]. In 1974, during the United Nations World Food Conference, Spirulina was declared "the best food for the future", and in 1993, it was recognized by the World Health Organization (WHO) as being a protein and iron rich food that could be consumed even by children without any risk [3]. Its nutritional richness is characterised by amino acids, vitamins, beta-carotene, minerals, essential fatty acids, polysaccharides, and proteins making up to 70 % of its dry weight. In addition, it contains chlorophylls, carotenoids and phycobiliproteins, which are valuable for the food industry as natural colourants [4]. Its functional properties include antiviral, antibacterial, antifungal, antiparasitic [5], prebiotic [2], and anti-aging [4]. The prebiotic potential of Spirulina stands out due to its abundance in complex polysaccharides. The content of Spirulina in complex carbohydrates is close to 14 %. Complex carbohydrates pass through the small intestine to the lower gut where they become available for some colonic bacteria but are not utilized by the majority of the bacteria present in the

Abbreviations: APC, Allophycocyanin; CAPP, Cold Atmospheric Pressure Plasma; C-PC, C-phycocyanin; *L. rhamnosus*, *Lacticaseibacillus rhamnosus*; *L. reuteri*, *Limosilactobacillus reuteri*; MRSB, Man Rogose Sharpe Broth; W, Watts.

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colon [6]. Prebiotics have been defined as "*any ingredient selectively fermented by certain microorganisms, which promote a change in both the composition and activity of the gastrointestinal microbiota, conferring health benefits on the host*" [7]. To observe a positive health effect of their consumption, a minimum level of live microorganisms is required. This level, depend upon the strains used and the required health effect ranges between 10^8 and 10^{11} CFU/g [6]. In Spirulina, among main carbohydrates with prebiotic potential are: glucose, rhamnose, mannose, xylose and galactose. Previous studies of Asadpoor et al. [8], revealed that depending on the type and structural characteristics of oligosaccharides (low degree of polymerization and a specific sequence of monosaccharide) could be easier to ferment by gut microbiota. Specifically, certain microorganisms can grow in the presence of Spirulina (among them, *Latobacillus paracasei* ATCCSD5275, *Bifidobacterium animalis* ATCC25527, *Escherichia coli* ATCC25922, *Streptococcus thermophilus, L. casei, L. acidophilus*) [9,10], ferment protein polymers and carbohydrates into biomolecules of higher nutritional and functional value, with better intestinal absorption [11,47]. However, up to date, scarcely any information was found on the structure and prebiotic activity of the oligosaccharides from Spirulina [9]. In the food industry, Spirulina is incorporated into different recipes for bread, pasta, biscuits, snacks and yoghurts and used as a food supplement, mainly in powder form, or tabletsin the pharmaceutical sector [12]. However, powdered products are not sterile. Several studies highlight the rehydration of powdered foods (low water activity values) as a critical stage because it favors the germination of spores and the recovery of microorganisms that managed to survive on powders, and cause infectious outbreaks [13,14]. So, it is very important to explore new processes that lead to significant degree of microbial decontamination in raw dehydrated matrices, being sustainable and effective.

Cold plasma technology (CAPP) emerges as a non-thermal innovative process, effective in the rapid (minutes) inactivation of bacterial spores, at room temperature, and in maintaining the nutritional and organoleptic profile of the treated solid (including powdered) or liquid products [15,48]. The technology uses ionised gases, produced at both low and atmospheric pressure, in which neutral molecules, electrons, positive and negative ions, and electromagnetic radiation destabilize the cell membrane and/or DNA of contaminants. The effectiveness of this technique in the reduction of the bacterial load is several $\log_{10}$ units (> 5 $\log_{10}$) ($\log_{10}$ cycles calculated as the decimal logarithm of bacterial load (expressed as CFU/mL of detected cells, in controls and treated samples)), and is dependent of the energy input used, the exposure time and the type of ionised gas, such as nitrogen, helium, oxygen, air, or argon [15]. CAPP technology effectively reduces human pathogens such as *E. coli*, *Salmonella* spp., *Listeria monocytogenes*, *Staphylococcus aureus* or *Shigella* spp., and heat-resistant sporulating microorganisms that represent a risk as foodborne agents (*Bacillus cereus*, *Bacillus subtilis,* and *Geobacillus stearothermophilus*) [15]. Beyrer et al. [16] have described a 2 $\log_{10}$ cycle reduction of *B. subtilis* spores in *A. platensis* powder with a surface micro discharge-cold atmospheric pressure plasma device (SMD-CAPP).

Despite evidence of spore inactivation in both air and nitrogen plasma, several studies have shown organoleptic and nutritional alterations in processed powdered food. These alterations include loss of essential oils, impairment of antioxidant activity, variation in product colouring due to reduction of chlorophyll-a, carotenoids and phycobilins, and oxidation of proteins, fatty acids, and phospholipids [16–18]. In fact, very limited research articles have been published regarding the study of the impact of different cold plasma treatment conditions on nutritional and antinutritional factors, functional and technological properties of powder products (e.g. rice, millet, wheat flours, Spirulina powder) [16,19–21]. Considering the high lipid content of microalgae matrices, previous studies of Pina-Pérez et al. [19] demonstrated that, even at high plasma discharge power density values (> 22 mW/cm^2) applied by SMD-CAP no alternation of lipids quality was observed when common House Cricket *Acheta domesticus* powder was processed. Beyrer et al. [16] also observed in microalgae, an improvement of some quality parameters after Spirulina powder plasma processing, such as increase total phenolic content; meanwhile the antioxidant capacity of Spirulina powder was reduced when power plasma (ignited on air) increased (SMD-CAP, up to 2.2 W). To date, no studies have addressed the impact of CAPP on the prebiotic potential of Spirulina. Specifically in relation to plasma processing, Mehta et al. [22] observed that, the prebiotic activity score of CAPP-treated rice and corn bran dietary fiber, was significantly ($p < 0.05$) higher than the untreated samples when *Lactobacillus sporogenes* and *Saccharomyces boulardii* conducted the fermentation of rice bran and corn dietary fiber. These experiments showed that cold plasma treatment might improve bioavailability of sugars for bacteria through disruption of the dietary fiber sources' due to the effect of plasma ions and reactive species on upper or epidermal grain layers. Studies of Kim et al. [23], effectively propose that under cold plasma, microalgae cell wall is significantly affected by chemical reactive species, causing a complete breakage and shrinkage of the cell surface. In spite of this, the positive or negative effects of CAPP treatments on the prebiotic properties and physico-chemical and functional characteristics of powder food-grade Spirulina require further evaluation.

The present study aims to evaluate the impact of CAPP technology on the prebiotic potential of Spirulina treated in powder form through the growth-stimulating capacity of two probiotic strains, the lactic acid bacteria (LAB) *Lacticaseibacillus reuteri* and *Lacticaseibacillus rhamnosus* GG, on Spirulina dispersions. Additionally, the protein and metabolic profiles of fermented substrates were tested.

2. Materials and methods

2.1. Spirulina, media and microorganisms

The commercial preparation of Spirulina powder, *A. platensis* (purity >80 %), was purchased from Phytopharma S.A. (Grandvillard, Switzerland). The selective medium for *Lactobacilli*, Man Rogose Sharpe Broth (MRSB) and solid medium (MRSA) were purchased from Scharlab S.L. (Barcelona, Spain). Chicory inulin was purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). *Lactobacillus* sp. (*L. reuteri* CECT 925 and L. *rhamnosus* GG CECT 288), recognized as probiotics [24], were provided by the *Colección Española de Cultivos Tipo* (CECT, Paterna, Spain).

2.2. Pre-culture and microbial stocks

L. *reuteri* and *L. rhamnosus* were reactivated according to CECT instructions. Briefly, the lyophilised bacteria were resuspended in 20 mL of sterile MRSB and incubated at 30 °C for 48 h, under anaerobic conditions, using 2.5 L Jars (Scharlab, Barcelona, Spain) with AnaeroGen™ 2.5 L anaerobiosis bags (Thermo Scientific™, Basingstoke, UK). Subsequently, the bacterial suspension was transferred to a flask with 700 mL of MRSB, which was kept under agitation and appropriate incubation conditions at 30 °C for 48 h under anaerobiosis. The grown cell culture was collected by centrifugation (2000 rpm, 10 min) and washed three times with 20 mL of MRSB, discarding the supernatant and collecting the pellet. Finally, a stock was prepared in 20 % *v*/v glycerol and stored at −80 °C for further use (initial concentration under study $\approx 2 \times 10^8$ CFU/mL).

2.3. Cold Atmospheric Plasma Pressure (CAPP) treatments

Spirulina powder samples were treated by CAPP using a surface microdischarge (SMD) – cold atmospheric pressure plasma device developed by the Institute of Systems Engineering in collaboration with the Institute of Life Technologies of HES-SO Valais-Wallis, Sion (Switzerland) [16]. Air at atmospheric pressure was used as the working gas. The equipment comprises a flat mesh electrode (mesh size = 9.8′ 9.4 mm) fed at high voltage, compacted with a Teflon dielectric barrier.

The upper electrode is connected to a cooling system. The surface area of the treatment chamber is 149.76 cm^2. The Spirulina powder samples were placed at 6 mm from the mesh electrode. Filamentary discharges of a few millimetres between the dielectric and the flat electrode led to the ignition of the gas (air) and activated the plasma cloud that act on the samples. Plasma obtained by the SMD-CAPP emits violet light dominated by the excitation of nitrogen molecules from ambient air [16,25]). Spirulina powder samples are exposed to the plasma in thin layers on sterile glass slides (5 mg/cm^2). The samples were treated for 5 min at an effective discharge power of 1.1, 1.7, 2.2 and 3.3 W. This plasma discharge power values have been selected according to the previous work of Beyrer et al. [16]. These researchers demonstrated that CAPP discharge power in between 1.1 and 2.2 W was effective in *B. subtilis* spores reduction by 3 log_{10} cycles (effective as decontamination method) The temperature was kept below 50 °C for all treatments applied. Samples were processed in triplicate for each condition. After treatment, powder samples were stored in Eppendorf tubes at −20 °C until analysis (1 week).

2.4. Evaluation of the prebiotic effect of Spirulina

For the prebiotic analysis, treated and untreated Spirulina powders were dispersed in sterile MRSB, to prepare a final concentration of 50 mg/mL. Inulin was used as a reference prebiotic material added at a concentration of 1 mg/mL in MRSB, according to Li et al. [26]. Inulin has been selected as reference because of (i) is the most studied prebiotic that has undergone extensive testing on its resistance in the upper intestinal tract and on its specific fermentability by LAB; (ii) with demonstrated effects on weight regulation, improvement of metabolic parameters, decreasing serum cholesterol and triglyceride rates, reducing hepatic lipogenesis and with positive effects on satiety and body weight [6].

Aliquots of both, inulin (as reference) and Spirulina (treated and untreated) (300 μL) were taken and dispensed into falcon MRSB tubes to a final concentration of 1 mg/mL.

The prebiotic capacity of Spirulina was assessed following the protocol by Rubel et al. [27] with minor adjustments, where MRSB was introduced as the control in place of glucose. Studied substrates (MRSB + inulin; MRSB + CAPP treated Spirulina; and MRSB + untreated Spirulina) were inoculated with 200 μL of a precultured (≈ 10^9 CFU/mL in MRSB at 30 °C) L. *reuteri* and L. *rhamnosus* achieving an initial load in fermentation process close to 1 × 10^5 CFU/mL. *L. reuteri* and *L. rhamnosus* grown in MRSB without Spirulina supplementation were used as negative controls. In total, 18 falcon tubes were prepared: two replicates for testing growth for each microorganism as control in MRSB; two replicates for the reference prebiotic in MRSB + inulin for each microorganism; two replicates for controls with untreated Spirulina for each microorganism in MRSB; and three replicates for each Spirulina sample treated at different effective CAPP power discharge (1.1, 1.7, 2.2 and 3.3 W – 5 min). The growth of both probiotics on the different substrates was recorded during 48 h of incubation at anaerobic conditions and 37 °C. Aliquots of the liquid cultures were extracted with a pipette (volume 1 mL) after 0, 18, 24, 41 and 48 h, and serial decimal dilutions (1 × 10^{-2} to 1 × 10^{-8}) were carried out in buffered peptone water, which was plated in duplicate on MRSA plates (results expressed as CFU/mL). Each experiment was repeated in independent sessions in triplicate. In parallel to the growth study, pH was recorded at 0, 19, 24, 24, 43 and 48 h. Additionally, aliquots of all treatments were collected and frozen at −80 °C for subsequent proteomic and metabolomic analysis. The results obtained in growth were used to estimate the relative growth of each bacteria, in each media (MRSB and MRSB supplemented with Spirulina, according to the studies of Rubel et al. [27], being N_f the count (CFU/mL) for each time and N_0 the count at the initial time:

$$Prebiotic\ index = \frac{\left[log_{10}\ N_f - log_{10}\ N_0\right]_{MRSB+Spirulina}}{\left[log_{10} N_f - log_{10}\ N_0\right]_{MRSB+inulin}} \quad (1)$$

2.5. Protein fragmentation using SDS–PAGE

For sample preparation, Laemmli Sample Buffer (4′) with β-mercaptoethanol (4 μl) was added to 15 μL of homogenised material by stirring. The samples were denatured at 95 °C for 5 min. Afterwards, gel separation and staining were done. The electrophoresis was performed using a 12 % precast gel (Bio-Rad) at 200 V for 30 min. The gel was fixed with 40 % ethanol/10 % acetic acid for 1 h and was stained with colloidal Coomassie (Bio-Rad) for 1 h. Subsequently, the gel was washed with H_2O milliQ to remove the unbound colourant. Finally, the gel was scanned with an Image Scanner (GE). In-gel digestion: Gel bands were cut and treated with sequencing-grade trypsin. An LC-MS/MS system composed of an Ekspert nanoLC 425 (Eksigent) and a mass spectrometer nanoESI qQTOF (6600plus TripleTOF, ABSCIEX) was used for the peptide profile analysis. Peptides were loaded onto an analytical column (3 μ C18-CL 120 Ă, 0.075 × 150 mm; Eksigent) equilibrated in 5 % acetonitrile 0.1 % FA (formic acid). Elution was carried out with a linear gradient of 15 to 40 % B in A for 20 min (A: 0.1 % FA; B: ACN, 0.1 % FA) at a flow rate of 300 nL/min. The sample was ionised in a Source Type: Optiflow <1 uL Nano applying 3.0 kV to the spray emitter at 175 °C. Analysis was carried out in a data-dependent mode. Survey MS1 scans were acquired from 350 to1400 *m/z* for 250 ms. The quadrupole resolution was set to 'LOW' for MS2 experiments, which were acquired from 100 to1500 *m/z* for 25 ms in 'high sensitivity' mode. The following switch criteria were used: charge 2+ to 4+; minimum intensity; 100 counts per second (cps). Up to 100 ions were selected for fragmentation after each survey scan. Dynamic exclusion was set to 15 s. The system sensitivity was controlled by analysing 500 ng of K562 trypsin digestion (Sciex); in these conditions, 2206 proteins were identified (FDR <1 %) in 45 min gradient.

ProteinPilot default parameters generated the peak list directly from 6600 plus TripleTOF wiff files. The Paragon algorithm (Shilov, I. V., S. L. Seymour, et al. (2007). Mol Cell Proteomics 6(9): 1638–55) of ProteinPilot v 5.0 was used to search the SwisProt _210126 with the following parameters: trypsin specificity, cys-alkylation, taxonomy non restricted, the search effort set to rapid.

2.6. Metabolomic analysis

Nuclear Magnetic Resonance (NMR) metabolomic analysis was conducted to study secondary metabolites differential production in LAB fermented substrates, comparing untreated and CAPP treated Spirulina samples at 1.1, 1.7, 2.2 and 3.3 W. After 48 h of media fermentation, the metabolomic profile was obtained according to the studies of Tomita et al. [28]. It was carried out by the General Metabolomics Service of the Faculty of Medicine – *Universitat de València* (Molecular Imaging and Metabolomics laboratory). The target aliquots (frozen at −80 °C) in our study were centrifuged at 12.000 rpm for 10 min. Briefly, 140 μL of each supernatant was diluted in 560 μL of potassium phosphate buffer. After centrifugation, the clarified supernatant was transferred to NMR equipment (QXI probe 5.0 mm O.D. × 103.5 mm; Norell, Landisville, NJ). The NMR spectrum was analysed by an Avance-500 spectrometer (Bruker BioSpin, Karlsruhe, Germany) equipped with a CPDUL CryoProbe (Bruker BioSpin) at proton/carbon frequencies of 600 MHz. The SpinAssign – Platform for RIKEN Metabolomics (http://prime.psc.riken.jp/) was used to identify the different metabolites by comparison with standard compounds. The databases used to compare the metabolites obtained were The Human Metabolome Database (http://www.hmdb.ca/) and Biological Magnetic Resonance Data Bank (http://www.bmrb.wisc.edu/). The concentrations of selected metabolites were detected, particularly (i) Short Chain Fatty Acids (SCFAs): acetate,

butyrate and propionate; (ii) fermentable carbohydrates: glucose and fructose; (iii) Organic acids: lactate and succinate; (iv) Amino acid arginine; and (v) Others: 2-aminobutyrate [GABA] andacetoin. The leading (majors and minors) metabolites of untreated and CAPP-treated Spirulina fermented preparations are shown in Figs. 4 and 5.

2.7. Data analysis

Logarithmic comparisons of CFU/mL were performed to determine significant differences between treatments. Statgraphics Centurion XV (Statgraphics Technologies Inc., The Plains, Virginia, USA) was used for the statistical analysis. Statistical differences between the different means (bacterial load, $\log_{10}$ CFU/mL) were determined using ANOVA and multiple range tests. Differences were considered statistically significant at a p-value <0.05.

3. Results and discussion

3.1. SMD-CAPP impact on the prebiotic potential of Spirulina

A positive growth effect was observed for both L. *reuteri* and L. *rhamnosus* cultured in MRSB when inulin and control *Spirulina* (not treated by cold plasma) were added to the media (p-value <0.05) (Fig. 1). The MRSB supplementation with Spirulina (1 mg/mL) accelerated the probiotics growth rate and final concentration by $\approx$ 1–3 $\log_{10}$ cycles. *L. rhamnosus* achieved a final concentration of 7.21 $\log_{10}$ cycles in MRSB and 8.97 $\log_{10}$ cycles in MRSB supplemented with control Spirulina. Meanwhile, *L. reuteri* achieved a bacterial load of 5.41 $\log_{10}$

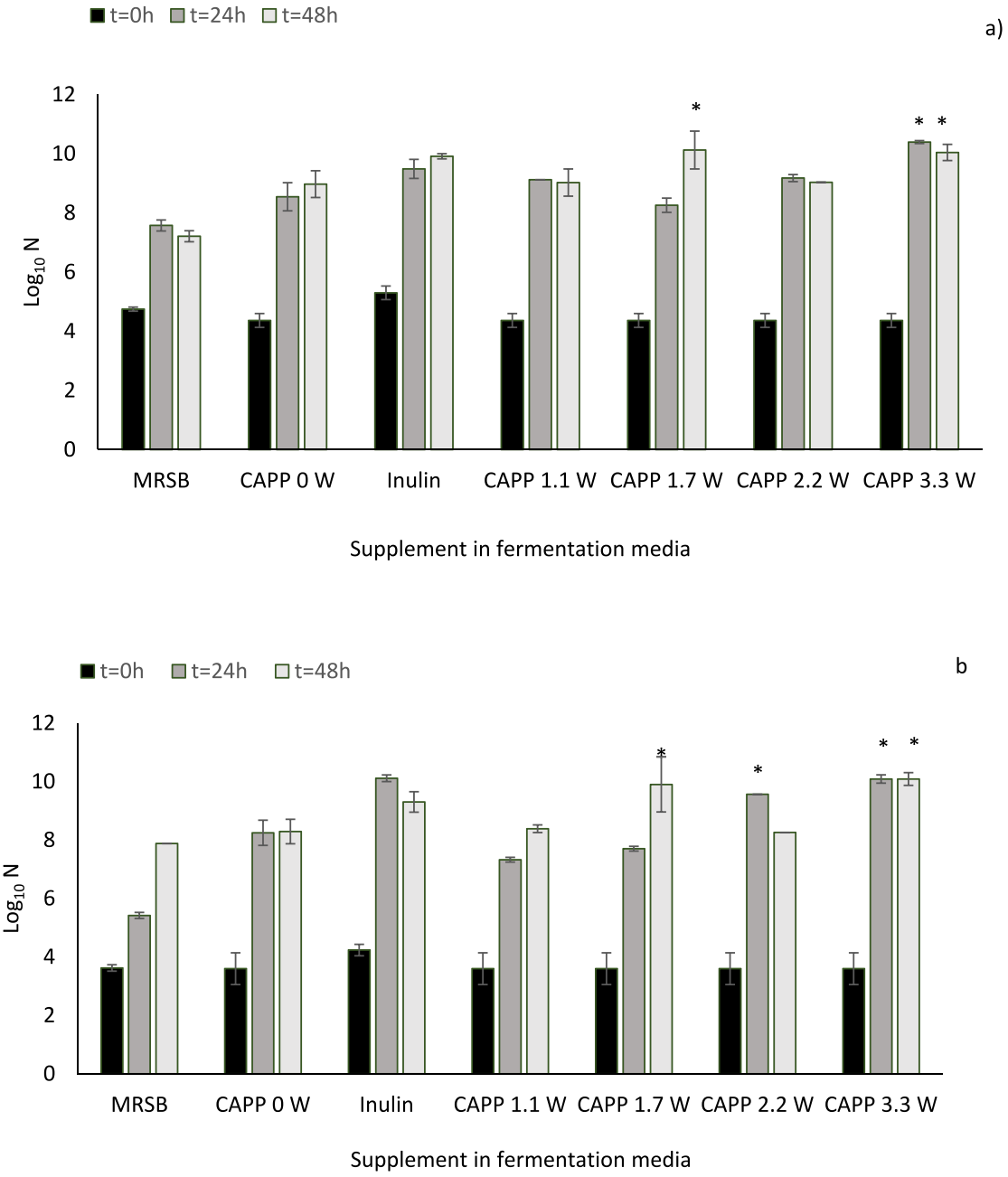

Fig. 1. Microbial growth (Final Log_{10} N (CFU/mL)) of *Lacticaseibacillus rhamnosus* (a) and *Limosilactobacillus reuteri* (b) during 48 h of incubation in Man Rogose Sharpe broth (MRSB) media (control), MRSB supplemented with inulin (as reference prebiotic), MRSB supplemented with untreated Spirulina (CAPP 0 W), and MRSB supplemented with CAPP-treated Spirulina at different discharge power (5 min). Significant differences between bacterial growth on MRSB supplemented with untreated Spirulina and treated Spirulina are marked as (*).

cycles in MRSB and 8.24 $\log_{10}$ cycles in MRSB supplemented with control Spirulina (after 24 h incubation). The application of SMD-CAPP increase the stimulating growth capacity of Spirulina, being final load of L. *reuteri* close to 9–10 $\log_{10}$ cycles in MRSB (after 48 h incubation) supplemented with Spirulina treated at 2.2 and 3.3 W discharge power. In the same way, *L. rhamnosus* final load achieved close to 9–10.5 $\log_{10}$ cycles after 24–48 h fermentation in MRSB media supplemented with 2.2 and 3.3 W SMD-CAPP treated Spirulina. Consequently, he substrate in which the probiotic growth rate was maximised corresponds to MRSB supplemented with SMD-CAPP 3.3 W treated Spirulina (10.5 ± 0.05 $\log_{10}$ cycles), being this growth even higher than the obtained one on MRSB supplemented with the inulin as the reference prebiotic substance (Fig. 1a and b).

L. *rhamnosus* and L. *reuteri* growth curves and kinetic parameter values were obtained according to Gompertz equation [29] in MRSB growth media supplemented with control Spirulina (untreated) and cold plasma treated Spirulina (Fig. 2). Growth curves homogeneously reached saturation indicating a plateau after 20 h incubation. Kinetic parameters (maximum specific growth rate, μ_{max} ($(\log_{10}$ (CFU/mL))/h); and lag duration, ƛ (h)) are revealing a more efficient growth of

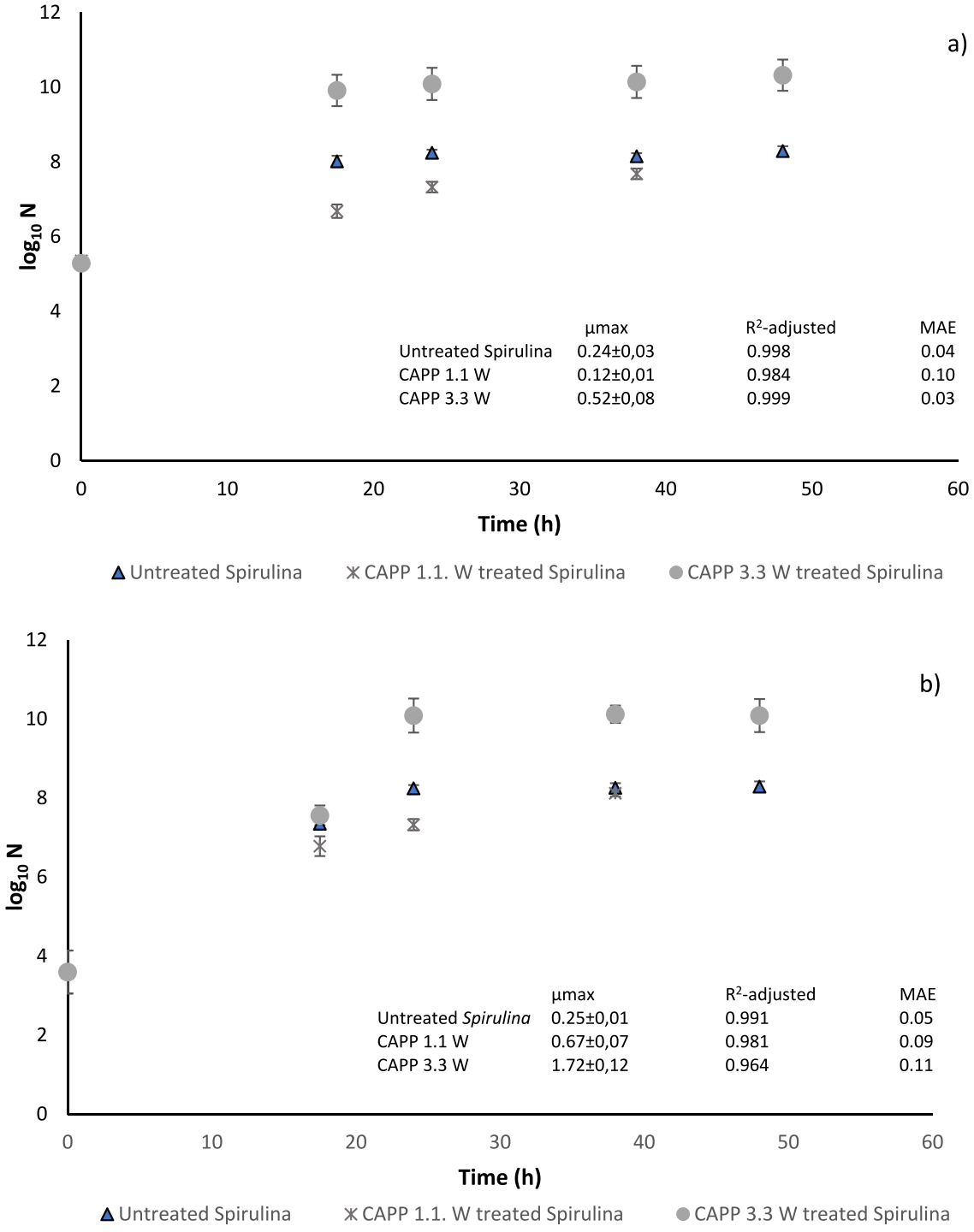

Fig. 2. Growth curves (and kinetic values from Gompertz equation) obtained for *Lacticaseibacillus rhamnosus* (panel a) and *Limosilactobacillus reuteri* (panel b) in reference MRSB media supplemented with Spirulina, treated and untreated by cold plasma technology (CAPP), at representative 1.1 W and maximum 3.3 W effective discharge power (5 min).

probiotics during 48 h incubation under optimal conditions when media is supplemented with CAPP treated Spirulina.

According to our results, the impact of SMD-CAPP treatments on Spirulina powder not only preserve the prebiotic potential of this matrix, but even increase L. *rhamnosus* and *L. reuteri* growth from 1 to 2 $\log_{10}$ cycles, in comparison to the growth observed in MRSB supplemented with control Spirulina.

Previous studies by Niccolai et al. [30] revealed that concentrations around 100 mg/mL of *A. platensis* exerted a prebiotic effect on *Lactobacillus plantarum* (initial concentration 5.3 ± 0.05 $\log_{10}$ CFU/mL), stimulating bacterial growth to a final concentration of 10.6 ± 0.2 $\log_{10}$ CFU/mL, after 48 h of fermentation. A similar final microbial load (9 $\log_{10}$ CFU/g) was reached by the studies of Martelli et al. [5], in which the growth of L. *casei* was studied in the presence of Spirulina previously subjected to ultraviolet (UV) radiation.

An approach estimating the relative growth ratio of probiotic bacteria growth to studied substrates was determined. For this approach, the prebiotic activity scores of inulin and untreated Spirulina are compared with the scores obtained for CAPP-treated Spirulina at 1.1, 1.7, 2.2 and 3.3 W (Table 1). No significant differences can be detected between the prebiotic activity scores for inulin and untreated/treated Spirulina, which corroborates Spirulina's prebiotic capacity as being similar to the prebiotic capacity of inulin. The growth of L. *rhamnosus* was slightly more favored in untreated Spirulina (1.81) than in inulin (1.52) supplemented media. Regarding CAPP treatments, Mehta et al. [22] showed that cold plasma (cold atmospheric plasma applied at 260 V, 10 min) could affect glucose diffusion and in vitro fermentation properties of dietary fibers from rice and corn bran (in a positive or negative manner depending on the applied plasma treatment conditions). These results align with the results in the present study regarding the possible decomposition of complex polysaccharides from Spirulina by SMD-CAPP treatment.

To date, no previous study has evaluated Spirulina's prebiotic potential to stimulate the growth of L. *reuteri* and L. *rhamnosus*. In the present study, the addition of Spirulina to the medium at a concentration of 1 mg/mL leads to a final concentration values above 7.0 $\log_{10}$ cycles for the studied probiotics, which is promising for the future development of nutraceutical products fermented with both bacteria, since 7.0 $\log_{10}$ CFU/mL is considered the minimum recommended dose to be considered an effective prebiotic [31].

3.2. Protein modification by CAPP treatments and bacterial fermentation

SDS-PAGE analysis of the Spirulina protein content (before and after fermentation of treated and untreated CAPP matrices) is shown in Fig. 3. In the present study, the fingerprint after enzymatic protein fragmentation was the same for substrates fermented with the two LAB species. A band between 15 and 20 kDa can be observed for all samples, which corresponds to the unfragmented alpha (α) and beta (β) subunits of C-phycocyanin (C-PC) [32,33]. Regarding the contribution of CAPP processing on protein fragmentation and hydrolysis during fermentation, the study reveals different protein hydrolyzation pattern depending on CAPP Spirulina pretreatment, with significant differences in fermentation results obtained just for most intensive treatment conditions (3.3 W). A main line is detected at 50 kDa, which is slightly attenuated in the case of CAPP-treated Spirulina at 1.1 W and disappears in the case of CAPP-treated Spirulina at 3.3 W. The effect is suggested to be related to CAPP-triggered, improved hydrolysis of peptide bonds during fermentation [34]. The bands detected at 150 kDa correspond with previous bands detected by Verni et al. [35]. Studies by Verni et al. [35] revealed a different proteolysis pattern, depending on the applied Spirulina pretreatments (lyophilized and low temperature air-dried Spirulina biomass), being detected at 150 kDa, in between 15 and 20 kDa, and also between 37 and 50 kDa.

Table 1
Estimation of the prebiotic score (as relative growth ratio) of each ingredient under consideration, inulin (prebiotic reference), untreated Spirulina, and CAPP-treated Spirulina, on *Lacticaseibacillus rhamnosus* and *Limosilactobacillus reuteri* growth stimulating capacity.

	Inulin	Untreated Spirulina	CAPP-treated Spirulina			
			1.1 W	1.7 W	2.2 W	3.3 W
L. rhamnosus	1.52[a]	1.81[b]	1.76[b]	1.88[b]	1.70[b]	1.76[b]
L. reuteri	3.27[a]	3.27[a]	3.34[a]	3.31[a]	3.29[a]	3.24[a]

[a–b]Different superscript letters are referred to significant differences between columns in the same row.

The nutritional value of Spirulina is well-recognized due to its high protein content, which can be hydrolysed into bioactive peptides (BPs) with potential in vitro and ex vivo bioactivity. The peptide profile in the hydrolysed fermentation media was also characterised by LC-MS/MS. In our study, 21 individual peptide sequences were attributed to C-PC or allophycocyanin (APC), eight corresponding to the APC alpha subunit, six to the APC beta subunit, three to the C-PC alpha subunit, and four to the C-PC beta subunit. Table 2 shows the differential peptide profile (APC and C-PC) produced by bacteria after each substrate fermentation, depending on the Spirulina processing conditions applied (CAPP treated 3.3 W–5 min, or untreated). Specific peptides were no longer detected when Spirulina CAPP-treated matrix was used to supplement the MRSB media. Sequences SLGTPIEAVAEGVR, EVTAGLVGADAGK, MQDAITSVINSSDVQGK, ADSLISGAAQAVYNK, DIGYYLR, MKTPLTEAVSIADSQGR were detected on fermented MRSB supplemented with untreated Spirulina and MRSB supplemented with CAPP-treated Spirulina at 1.1, 1.7, and 2.2. W, but not with 3.3 W CAPP-treated Spirulina (5 min of treatment time). The Peptide Ranker software can estimate the potential bioactivity associated with a specific peptide sequence. For example, the disappearance of the DIGYYLR sequence of the α C-PC, with an estimated bioactive potential of 70 %, is an important indicator that CAPP processing has induced a deviation from the initial state. The AA coverage of the analysis was also reduced for CAPP-treated samples and indicated a reduced nutritional value, at least for the samples treated at 3.3 W of plasma power (Table 2).

Among the potential biological activities that have been attributed to Spirulina fermentation following peptides are included: ACE inhibitor, an alpha-glucosidase inhibitor, antioxidative peptides potential, dipeptidyl peptidase III inhibitor, dipeptidyl peptidase IV inhibitor, neuropeptide activity, immunomodulatory effect, antithrombotic effect, and anti-inflammatory potential, among others [36,46]. Further research is necessary to understand in depth to what extent these non-LAB fermentation produced peptides in CAPP-treated Spirulina contribute to the fermented matrix's bioactive potential.

3.3. Metabolomic profile of fermented samples

LAB can hydrolyse plant cells and cyanobacteria cell walls to convert complex organic components into smaller molecules with anti-inflammatory, antioxidant, and immunomodulatory properties [37,38]. Thus, the use of LAB can improve certain foods' nutraceutical profiles [49].

According to NMR results, the predominant molecules in all the matrices before fermentation ($t = 0$ h) were acetate and glucose (Fig. 4). Other metabolites present at lower but significant concentrations were lactate (Fig. 4), butyrate, propionate, succinate, fructose, arginine, acetoin and GABA (Fig. 5). At 0 h, CAPP treatments increased the presence of specific molecules in the medium significantly such as, glucose, fructose and arginine whose concentrations were substantially higher in the media, especially for 1.7 W and 3.3 W CAPP-treated Spirulina, in comparison to the control (Figs. 4 and 5). For instance, the concentration of glucose in the 3.3 W CAPP-treated is approximately twice the concentration of the untreated sample (31.9 versus 16.4 ppm, Fig. 4). This increase may be due to an increase in bioavailable intracellular microalgae compounds for probiotics caused by CAPP induced

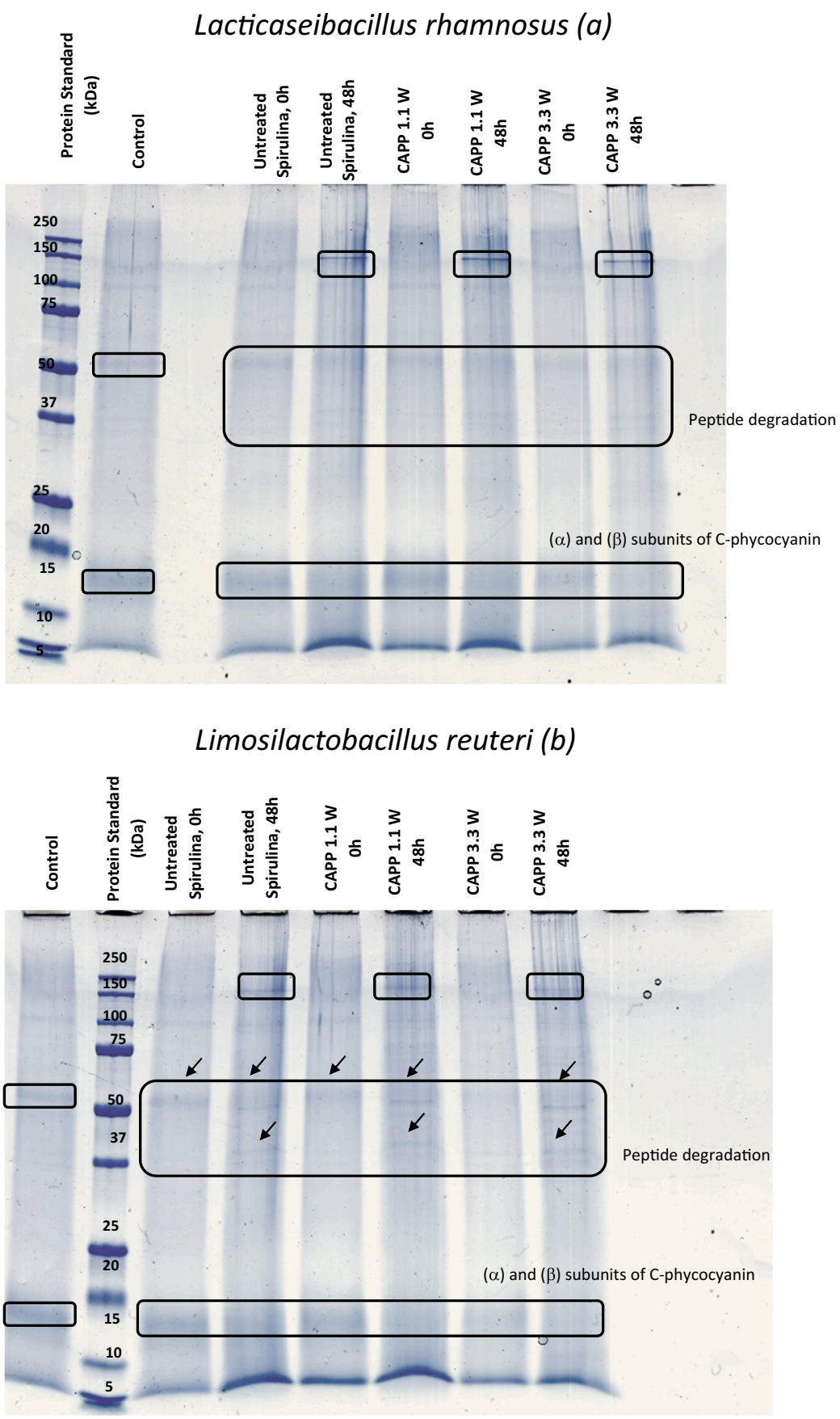

Fig. 3. 1D-SDS-PAGE electrophoresis for protein fragmentation detection after lactic acid bacteria (LAB), *Lacticaseibacillus rhamnosus (a)* and *Limosilactobacillus reuteri (b)*, fermentation of different Spirulina supplemented matrices, treated and untreated by CAPP.

Table 2
HPLC-Chip-ESI-MS/MS differential peptides produced by *Lacticaseibacillus rhamnosus* and *Limosilactobacillus reuteri* fermentation of untreated Spirulina ingredient in Man Rogose Sharpe broth (MRSB), in relation to fermented hydrolysate obtained from cold plasma treated Spirulina in MRSB (from allophycocyanin (APC) and C-phycocyanin (C-CP) proteins).

Fermentation substrate	Protein name	Peptide sequence	% AA coverage
MRSB+not treated Spirulina powder (1 mg/mL)	Allophycocyanin alpha chain OS = Arthrospira platensis	SIVNADAEAR SLGTPIEAVAEGVR[a] YLSPGELDR SFVTSGER DLDYYLR IAETMTGAR EAGNQLFQK	42.9
	Allophycocyanin beta chain OS = Arthrospira platensis	AATTISANAANIVK EVTAGLVGADAGK[a] MQDAITSVINSSDVQGK[a] YAACIR AATTISANAANIVK AYFATGELR DLDYYLR	40,9
	C-phycocyanin alpha subunit OS = Arthrospira platensis	ADSLISGAAQAVYNK[a] DIGYYLR[a] MKTPLTEAVSIADSQGR[a] DIGYYLR	24
	C-phycocyanin beta subunit OS = Arthrospira platensis	ITSNASTIVSNAAR VVSQADTR DMEIILR MFDAFTK	24,4
MRSB + CAPP treated Spirulina powder (1 mg/mL)	Allophycocyanin alpha chain OS = Arthrospira platensis	IAETMTGAR SIVNADAEAR DLDYYLR EAGNQLFQK YLSPGELDR SFVTSGER	21
	Allophycocyanin beta chain OS = Arthrospira platensis	AATTISANAANIVK AYFATGELR YAACIR DLDYYLR	18,6
	C-phycocyanin alpha subunit OS = Arthrospira platensis	DIGYYLR	4
	C-phycocyanin beta subunit OS = Arthrospira platensis	DMEIILR ITSNASTIVSNAAR VVSQADTR MFDAFTK	17

[a] Differential identified peptides in a fermented substrate, including not treated Spirulina.

disintegration of the cell membrane and CAPP induced depolymerisation of the structural polysaccharide compounds [22]. Yan et al. [39] observed an increase in amylose content in the media due to the decomposition of starch granules after DBD plasma treatment of banana starch, which could have favored nutrient bioavailability during microbial growth. This phenomenon was also reported by Herceg et al. [40], where an increase of polyphenols and antioxidants in pomegranate juice was observed after cold plasma treatment due to the treatment induced cell membrane breakdown and induced depolymerisation of polymeric polyphenols.

de Marco Castro et al. [34] conducted a study that indicated a Spirulina cell wall biodegradation by LAB fermentation. Niccolai et al. [30] proved that the fermentation of Spirulina with *L. plantarum* increased digestibility by 4.4 %, antioxidant activity by 79 %, and total phenolic content by 320 %. In addition, Bao et al. [41] demonstrated that fermentation with L. *plantarum* and *B. subtilis* removed off-flavours, favored protein hydrolysis, and yielded an improved ratio of essential-to-total amino-acids, compared to the unfermented Spirulina.

After 48-h fermentation, L. *rhamnosus* and *L. reuteri* showed a different predominant metabolic pattern. A significant decrease in sugar content (glucose and fructose) and increase in lactate was observed after 48 h incubation. Glucose reduced from 15.5 ± 1.25 ppm in untreated and 31.8 ± 0.8 ppm in CAPP-treated (3.3 W) samples to 0.17 ppm and 2.2 ppm, respectively (t = 48 h) (Fig. 4). The fructose concentration decreases from 2.10 ppm to 0.70 ppm in the untreated Spirulina matrix by fermentation with L. *rhamnosus* (Fig. 5a) and from 3.91 ppm to 0.88 ppm (1.7 W CAPP-treated Spirulina matrix). In the case of fermentation with L. *reuteri*, fructose concentration drops from 2.09 ppm to 0.98 ppm (untreated Spirulina-supplemented media) and from 3.88 ppm to 1.21 ppm (3.3 W CAPP-treated Spirulina supplemented media) (Fig. 5b). Besides the bacterial count, the reduced substrate concentration indicates, the performance and end point of the fermentation.

Fermentations carried out with L. *rhamnosus* resulted in lower final lactate concentration (3.29 ppm in untreated Spirulina media and 4.40 ppm in 1.7 W CAPP-treated media), compared to fermentations with L. *reuteri* (8.91 ppm in untreated Spirulina and 9.67 ppm in 1.7 W CAPP-treated Spirulina media) (Fig. 4a and b). This dynamic is consistent with the change in pH from approximately 6 (t = 0 h) to 4 (after 48 h fermentation) in all treatments. The decrease in pH is also due to the increase of acetate, propionate, butyrate and succinate concentration 48 h after starting the incubation. SCFAs indicate the heterofermentative metabolic pathway associated with L. *reuteri* and L. *rhamnosus*. In this pathway, glucose and fructose are catabolised into lactate, SCFAs (acetate, propionate and butyrate), succinate, phenylacetate, ethanol, methane, carbon dioxide and hydrogen, which are all important for maintaining the redox balance during the fermentation process [42]. Mehta et al. [22] demonstrated that cold plasma treatment of dietary fiber from rice and corn bran enhances the release of SCFAs (lactate, propionate and acetate) during fermentation. These SCFAs have important functions for the host's physiology. Colonocytes take up butyrate as an energy source, while propionate and acetate are metabolised by the liver and muscles, respectively [43].

In the same way, certain amino acids (arginine) showed increasing values, with increasing CAPP treatment intensity applied to Spirulina powder. The arginine content in the control sample (t = 0 h) was close to 1.06 ± 0.22 ppm, and the reported values for1.1, 1.7, 2.2, and 3.3 W CAPP-treated samples were respectively(t = 0 h): 1.76, 2.18, 2.39 and2.7 ppm.

GABA's presence highlights the decarboxylation of glutamic acid, which constitutes 8.39 % of Spirulina's amino acids [4,44]. This mechanism was reported by Jung et al. [44] in a metabolomic study on the functional characterisation of bacterial fermentation of traditional Korean soybean paste, highlighting the potential of fermentation by LAB in obtaining GABA-rich foods. A direct relationship was found between the amount of *Lactobacillus* spp. and GABA concentration, which could promote the production of high-value-added fermented products [5].

Considering the expansion of fields related to microbiome-targeted interventions that are emerging to date, and the evolving landscape for implementation across regulatory, policy, prescriber, and consumer spheres, a new era of significant changes is predicted to occur in next decade [45]. Although the application of CAPP in food processing remains expensive, it could be acceptable for high-added value products targeted to personalized nutrition.

4. Conclusions

The present study provides evidence of Spirulina's prebiotic properties, even after high intensity CAPP treatments, on the evaluated growth of L. *rhamnosus* and L. *reuteri*. The maximum prebiotic value was observed for Spirulina processed by cold plasma at 3.3. W – 5 min when added to the MRSB media stimulating the L. *rhamnosus* growth up to 10.4 ± 0.05 $\log_{10}$ CFU/mL. Moreover, the metabolomic analysis included in the present study revealed a possible improvement in the bioavailability of carbohydrates and amino acids resulting from the disintegration and depolymerization of the cell wall and cell membrane caused by the energy conveyed by the cold plasma. Likewise, metabolomic analysis of these results revealed metabolite dynamics in the fermentation process, which can be used to improve the organoleptic

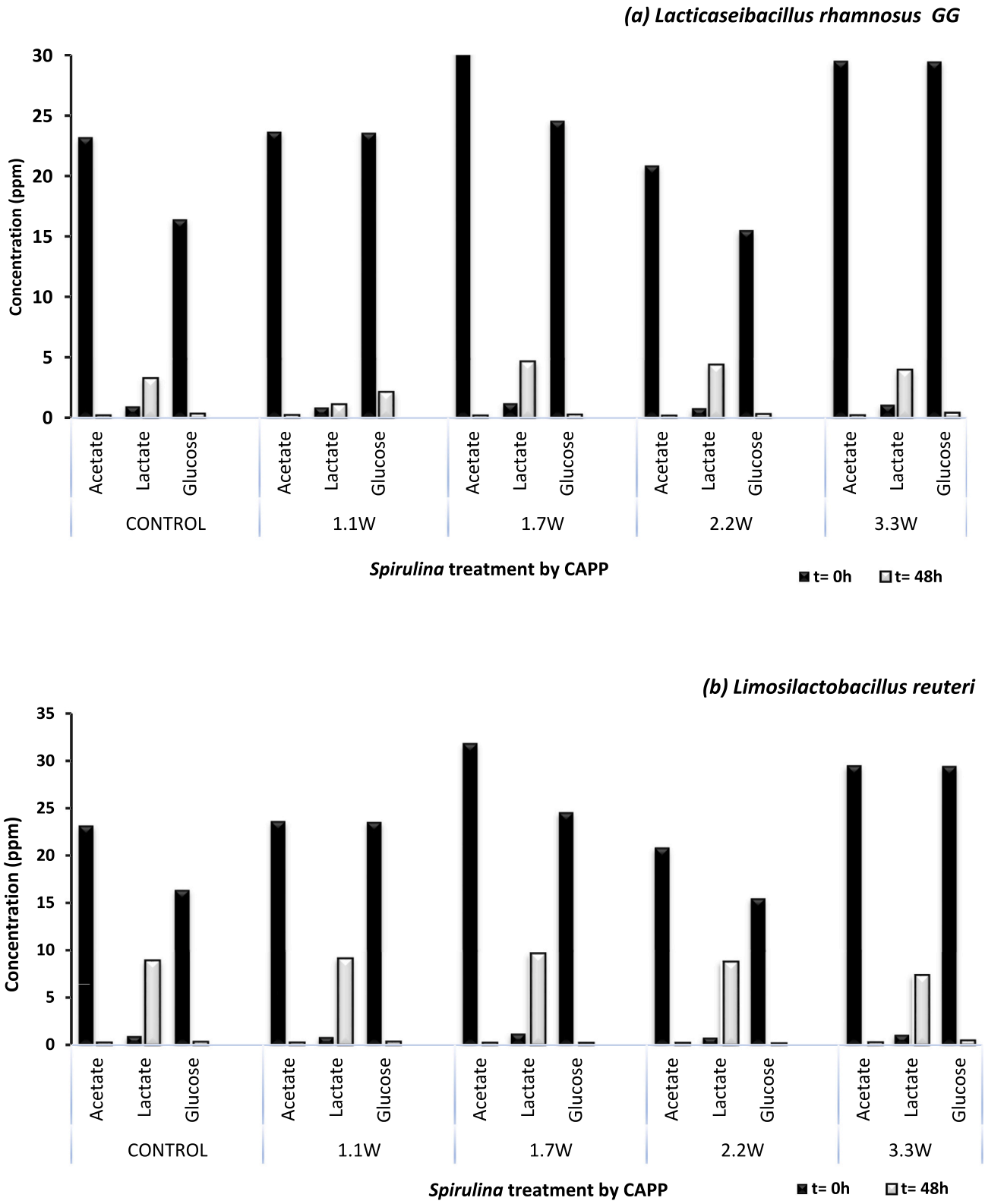

Fig. 4. Main substrates and products profile in the fermentation media supplemented with untreated and CAPP-treated Spirulina for L. *rhamnosus* (a) and L. *reuteri* (b).

characteristics of different products with probiotic and prebiotic potential (e.g., acetoin production). This research opens the possibility of exploring and detailing the optimal CAPP -Spirulina treatment maximizing microbial inactivation [16] and minimizing intensity required to sterilise Spirulina powder in order to promote nutrient bioavailability and probiotic additional growth in novel formulated matrices that will be fermented by studied LAB.

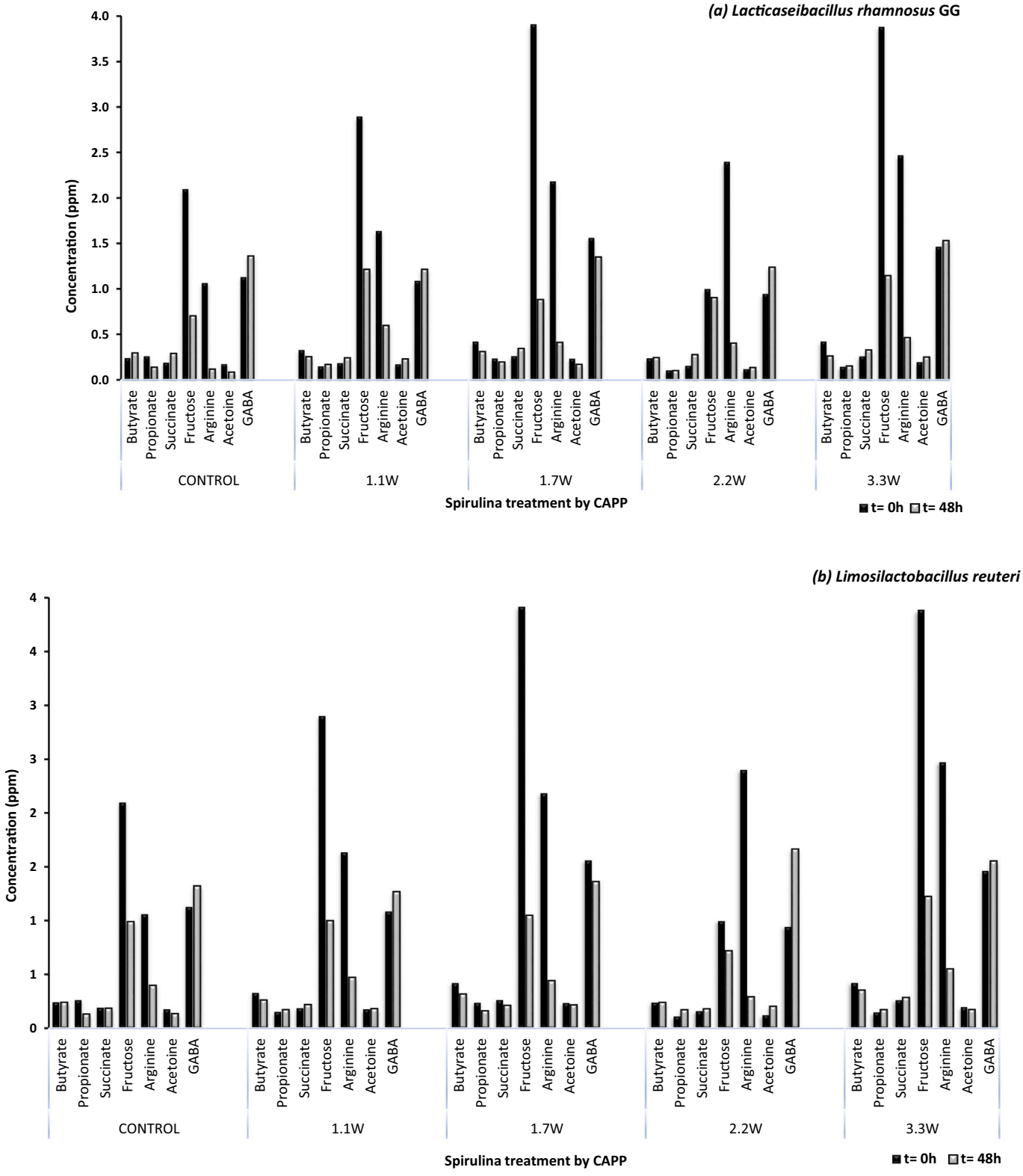

Fig. 5. Profile of low molecular weight metabolites in fermentation media, including not treated and CAPP-treated Spirulina, fermented by L. *rhamnosus* (a) and *L. reuteri* (b).

CRediT authorship contribution statement

María Consuelo Pina-Pérez: Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Neus Ricós-Muñoz:** Investigation. **Ella Karina López-Suárez:** Investigation. **Consuelo Esteve:** Formal analysis, Investigation, Methodology, Validation. **Sergi Maicas:** Formal analysis, Validation. **Michael Beyrer:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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