Morpho-physiological aspects of Scenedesmus acutus PVUW12 cultivated with a dairy industry waste and after starvation

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Complete List of Authors:	Giovanardi, Martina; University of Ferrara, Dept. Life Sciences and Biotechnologies Baldisserotto, Costanza; University of Ferrara, Dept. Life Sciences and Biotechnologies Daglia, Maria; Univeristy of Pavia, Dept. of Drug Sciences, Medicinal Chemistry and Pharmaceutical Technology Section, Ferroni, Lorenzo; University of Ferrara, Dept. Life Sciences and Biotechnologies Sabia, Alessandra; University of Ferrara, Dept. Life Sciences and Biotechnologies Pancaldi, Simonetta; University of Ferrara, Dept. Life Sciences and Biotechnologies
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7 3 M. GIOVANARDI[1§], C. BALDISSEROTTO[1§], M. DAGLIA[2], L. FERRONI[1], A. SABIA[1] & S. PANCALDI[1]
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9
10 4 [1] *Dept. of Life Sciences and Biotechnologies, University of Ferrara, C.so Ercole I d'Este, 32 – 44121*
11
12 5 *Ferrara, Italy*
13
14
15 6 [2] *Dept. of Drug Sciences, Medicinal Chemistry and Pharmaceutical Technology Section, University*
16 7 *of Pavia, Via Tramelli, 12 – 27100 Pavia, Italy*
17
18
19 8
20
21 9 Correspondence: S. Pancaldi, Dept. of Life Sciences and Biotechnologies, University of Ferrara,
22
23 10 Corso Ercole I d'Este, 32 – 44121 Ferrara, Italy. Tel.: +39.0532.293786. Fax.: +39.0532.208561. E-
24
25 11 mail: simonetta.pancaldi@unife.it.
26
27 12 [§] Equal contribution
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39 17 thanked for providing scotta.
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41 18 Other details on Authors addresses
42
43
44 19 MARTINA GIOVANARDI: Tel. +39.0532.293779; e-mail: martina.giovanardi@unife.it
45
46
47 20 COSTANZA BALDISSEROTTO: Tel. +39.0532.293779; e-mail: costanza.baldisserotto@unife.it
48
49 21 MARIA DAGLIA: Tel. +39.0382.987388; e-mail: maria.daglia@unipv.it
50
51
52 22 LORENZO FERRONI: Tel. +39.0532.293779; e-mail: lorenzo.ferroni@unife.it
53
54 23 ALESSANDRA SABIA: Tel. +39.0532.293779; e-mail: alessandra.sabia@unife.it
55
56
57 24 SIMONETTA PANCALDI: Tel. +39.0532.293786; e-mail: simonetta.pancaldi@unife.it
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Abstract

Among green microalgae, *Scenedesmus* sp. is known for its potential in wastewater remediation and lipid production, especially under starvation. Moreover it is often characterised by a mixotrophic metabolism. In this work, we cultivated *S. acutus* PVUW12 in the presence of a liquid fraction of scotta (LFS), a cheese whey by-product, as source of nutrients. Subsequently, cultures were starved to evaluate lipid production. Cells were analysed to obtain information about growth, nutrient consumption during LFS cultivation, morphology and photosynthetic efficiency. We found that the alga boosted its growth when cultured in presence of LFS. Production of stromatic starch grains, polyphosphate granules, cell wall enlargement and reduction of the photosynthetic efficiency were also induced. Massive lipid accumulation was observed only during starvation, which also induced a strong slowdown of growth, loss of polyphosphate grains, and further decrease in photosynthetic efficiency. This study demonstrates that *S. acutus* PVUW12 can be involved in a two-steps cultivation, firstly promoting growth using a by-product from cheese industry and secondly transferring the microalgae on starvation to induce lipid accumulation for bioenergetics purposes.

Keywords: *Scenedesmus*, scotta, mixotrophy, photosynthesis, starvation.

Running title: Cultivation with scotta and subsequent starvation of *Scenedesmus*.

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Introduction

Microalgae are photosynthetic organisms well known for their possible use in many fields, which vary from simple biomass production to the obtainment of valuable products with commercial and ecological implications (Pulz & Gross 2004; Mimouni et al. 2012). At present, the interest on microalgae is mainly oriented to their capability to accumulate lipids suitable for biofuel production, focussing on the strategies to improve the economic feasibility and overcome the competition with fossil fuels (Chisti 2007). In this perspective, nitrate depletion in growth media is the most common nutritional variation used to induce lipid accumulation in several microalgae, though the biomass productivity under starvation is low (Chisti 2007). Conversely, studies show that mixotrophy, the condition in which an organic carbon source is supplied in the culture medium together with light, allows to obtain high biomass yields (Heredia-Arroyo et al. 2010; Abreu et al. 2012; Giovanardi et al. 2013; Baldisserotto et al. 2014). However, the organic carbon supply, usually glucose, acetate, glycerol or fructose (Heredia-Arroyo et al. 2010), is predicted to account for around 80% of the cost of the culture media (Bhatnagar et al. 2011). Then, research is recently focussing on the valorisation of wastes derived from agro-food industries, which can be used as cheap organic carbon sources for the microalgal growth (Heredia-Arroyo et al. 2010; Giovanardi et al. 2013), simultaneously solving problems like freshwater demand, high cost of nutrients and waste remediation (Bhatnagar et al. 2011). Among agro-industrial waste products, cheese whey is considered as the most polluting waste product derived from the dairy industry (Abreu et al. 2012; Prazeres et al. 2012). Cheese whey is a green-yellowish, nutrient-rich effluent composed by lactose, soluble proteins, lipids and mineral salts containing nitrogen and phosphorous. Other components, like lactic and citric acids, are also contained in appreciable quantities (Siso 1996). For its high organic content, cheese whey represents an important environmental problem, and needs proper disposal treatments (Siso 1996; Markou & Georgakakis 2011; Abreu et al. 2012; Prazeres et al. 2012). Ricotta cheese whey, named scotta, shares with other cheese whey both environmental implications and chemical composition, but has a reduced protein content (Sansonetti et al. 2010). Scotta can be considered a good raw material suitable for the fermentation by microorganisms and the production of different bio-products, though its biotechnological potential has not been sufficiently studied yet (Pisponen et al. 2013). Alternatively, Freyssinet & Nigon (1980) proposed the possibility of growing microalgae using cheese whey as organic carbon source. Recently, Abreu and co-workers (2012) evaluated growth parameters, biochemical composition and lipid accumulation of *Chlorella vulgaris* cultivated in the

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presence of a hydrolysate of cheese whey, concluding that the mixotrophic cultivation using dairy effluents is feasible. Beside these data, however, at the best of our knowledge, no information about growth of microalgal species in the presence of scotta is available.

Among green microalgae, *Scenedesmus* sp. has been widely studied as a promising organism to be employed for biotechnological purposes, mainly biofuel production and bioremediation (Doria et al. 2012; Damiani et al. 2013). In a recent work, Mandal and Mallick (2009) have tested the capability of a strain of *Scenedesmus* to accumulate lipids under different growth conditions, mixotrophy and nutrient depletion included. Results confirmed that *Scenedesmus* sp. is a mixotrophic organism, insofar as cell densities increased when glucose was supplemented to the growth media. However, lipid accumulation was not promoted by mixotrophy, but only by nitrogen starvation (Mandal & Mallick 2009). In this work, *Scenedesmus acutus* PVUW12 has been cultivated in the presence of a liquid fraction of scotta (LFS). Preliminary experiments were done using different dilutions of LFS to select the best one in promoting *Scenedesmus* growth. In the perspective of an economically sustainable cultivation and to test the potential of LFS to provide all nutrients required for growing the microalga, LFS was supplied in tap water. Then, a two-stage experiment was set up, firstly cultivating the microalga in the presence of the best LFS dilution to obtain high cell densities, and subsequently transferring the cells in tap water to induce lipid accumulation. Information about growth, nutrient consumption, morphology and photosynthetic efficiency was collected, also with the aim of improving knowledge about the morpho-physiology of this algal strain, in order to ensure a fruitful utilisation of these organisms in biotechnological applications.

Materials and methods

Whey treatment and substrate preparation

The liquid fraction derived from the preparation of ricotta cheese was kindly provided from a dairy factory located in Trentino-Alto Adige Region (Italy). This by-product was maintained at -20°C to preserve its composition. Before experiments, scotta was treated as described in supplementary materials (Figure S1). Trial tests using the LFS "as is" were also performed, but did not lead to positive results (data not shown).

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Algal strain and culture condition

The microalga *Scenedesmus acutus* strain PVUW12 (Chlorophyta, Sphaeropleales) (deposited as *Scenedesmus* cf. *acutus* Meyen CCALA-935 at the Culture Collection of Autotrophic Organisms from Institute of Botany, Academy of Sciences of the Czech Republic, Centre of Phycology, Dukelská), was kindly provided by Prof. Erik Nielsen (Department of Biology and Biotechnology, University of Pavia, Italy) and maintained in axenic Bristol GR^+ medium (Bold 1949) in a growth chamber (24 ± 1 °C, 80 µmol photons $m^{-2}s^{-1}$ PAR, 16:8 h light-dark photoperiod). For preliminary experiments, axenic cultures were prepared in 100 mL Erlenmeyer flasks (50 mL total volume) at a density of about $1x10^6$ cells mL^{-1} in only tap water or tap water added with LFS at the increasing dilutions of 1:2, 1:5, 1:10 and 1:20. Microalgal cultures grown in Bristol GR^+ were used as controls. Tap water was provided from the aqueduct of Ferrara (Italy) and its chemical composition is reported in Tab. S1. During the experiments, the flasks were maintained in the growth chamber described above, without CO_2 supply. To avoid sedimentation, flasks were daily hand-shaken. For analyses, aliquots of cells were weekly collected up to 35 days of growth. For subsequent experiments, samples showing the best growth potential were selected together with their relative control samples, and grown for 14 days in 500 mL Erlenmeyer flasks (250 mL final volume) in the same culture conditions previously described. After that period, cells were harvested by centrifugation (600*g*, 10 min) and resuspended in 250 mL of tap water (starvation). Flasks were then maintained for 28 days in the growth chamber and aliquots of samples were weekly collected for analyses. Experiments were performed at least in triplicate.

Growth evaluation

Growth was estimated by counting the cells with Thoma's haemocytometer under a light microscope (Carl Zeiss, Jena, Germany, model Axiophot). Growth rates (µ) were expressed as number of divisions (div) per day (Giovanardi et al. 2013).

Scotta composition and nutrient consumption determination

For each of the following determinations, culture media samples were obtained by centrifugation.

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Free sugar determination. Lactose was quantified by an UV-method in foodstuff, using an enzymatic assay (Boehringer Mannheim, R-Biopharm, Italia srl, Cerro Al Lambro, Italy). A stabilized standard lactose solution was used as control.

Organic acid determination. Citric and L-lactic acids were determined by two UV-methods in foodstuff, using an enzymatic assay (Boehringer Mannheim, R-Biopharm, Italia srl, Cerro Al Lambro, Italy). L-lactic acid and citric acid standard solutions were used as assay controls.

Protein content determination. Protein content was determined according to Lowry et al. (1951). OD values were determined at 660 nm with an UV/Vis spectrophotometer (Pharmacia Biotech Ultrospec® 2000) (1 nm resolution). A calibration curve with bovine serum albumin was used for the quantification.

Morphological and ultrastructure observations

Microscopic observations were done using the same microscope described above, equipped with conventional or fluorescent attachments, as described in Baldisserotto et al. (2012). For ultrastructural analyses, aliquots of cells were collected at 0 days (inoculum time), after 14 days of growth and after subsequent 28 days of starvation. Cells were harvested by centrifugation (600*g*, 10 min) and then prepared as reported in Baldisserotto et al. (2012). Observation of ultrathin sections was performed with a Hitachi H800 electron microscope (Hitachi, Tokyo, Japan). Images were used to measure the cell diameter and to check the presence of stromatic starch granules and cytoplasmic lipid globules.

PAM fluorimetry

The maximum quantum yield of PSII was determined using an ADC OS1-FL fluorometer (ADC Bioscientific Ltd, Hoddesdon, Hertfordshire, UK). Aliquots of samples were collected by centrifugation (10,000*g*, 3 min). Pellets were then deposited onto pieces of wet filter paper (Schleicher & Schuell) (Ferroni et al. 2011) and maintained in the dark for 15 min. Initial fluorescence (F_0) and maximum fluorescence (F_M) values were measured flashing the samples with a saturating light pulse, then used to calculate the maximum quantum yield of PSII (F_V/F_M, where $F_V = F_M - F_0$ is variable fluorescence) (Lichtenthaler et al. 2005).

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Data treatment

Data were processed with Microcal Origin 6.0 software (OriginLab, Northampton, MA, USA). In each case means ± standard deviations for N number of samples are given. Data obtained from control and treated samples were compared using Student's t-test with a significance level of 0.05.

Results

Selection of the best LFS dilution for growth

Cell densities of *S. acutus* PVUW12 grown on different dilutions of LFS were monitored to select the best concentration for further experiments (Figure 1a). Growth kinetics of cells cultivated in Bristol GR^+ and tap water were also included. Cell concentration progressively increased in control GR^+ samples throughout the experiment, reaching values of about 13 x 10^6 cell mL^{-1} at 35 days. During the first 14 days of growth, cells in tap water showed the same cell densities as controls, but soon after entered the stationary phase, which led to a very low cell density at the end of the experiment (about 5 x 10^6 cells mL^{-1}). While the addition of LFS at 1:5 to 1:20 dilutions did not promote growth as compared with tap water (Figure 1a), the 1:2 dilution led to a strongly enhanced growth during the first 14 days of cultivation (μ: 0.31 and 0.15 div d^{-1} for 1:2 LFS samples and both control and tap water samples, respectively; $p < 0.001$), before entering the stationary phase. In these samples, cell densities of about 30 x 10^6 cells mL^{-1} (2.3 and 6 times higher than those observed in controls and in tap water samples, respectively; $p < 0.01$ in both cases) were reached at the end of the experiment. So, the most interesting culture condition, i.e. 1:2 LFS in tap water (hereafter named 1:2 LFS), was selected to perform new experiments in comparison with tap water and Bristol GR^+. New cultures were grown for 14 days, just before cells entered the stationary phase, to analyse nutrient consumption, cell growth, cell morphology and photosynthetic efficiency. After 14 days of cultivation, samples were transferred to tap water, with the exception of cells already growing in water, to test the impact of the previous cultivation on the starvation phase.

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Nutrient consumption in the culture media during growth with LFS

Aliquots of 1:2 LFS medium were harvested during the microalgal cultivation at 0, 5, 7 and 14 days, for monitoring the consumption of the main organic compounds (lactose, proteins, citric and L-lactic acids). The composition of LFS analyzed before experiments was consistent with that of scotta reported by Sansonetti et al. (2010), with the exception of organic acids, which were present at lower concentrations (Table S2). As shown in Figure 1b, lactose slightly decreased its concentration during the experimental time, with an overall consumption of 5.2% (consumption rate 0-14 days: 0.11 $gL^{-1}d^{-1}$). Such decrease was mostly evident between 5 and 7 days of cultivation, when a consumption rate of 0.45 $gL^{-1}d^{-1}$, corresponding to 3.2% of the substrate, was observed. After 7 days of growth, lactose concentration did not change inside the culture medium. Concerning the organic acids content, a gradual decrease in citric and L-lactic acid was measured (consumption rate 0-14 days: 0.004 and 0.005 $gL^{-1}d^{-1}$, respectively). Indeed, at the end of the experiment, concentrations 43 and 41% lower than those present at time 0 were respectively found. Finally, a progressive decrease in protein content was also observed throughout the experiment, and reached, at the 14th day of growth, values about 38% lower than that at time 0 (consumption rate 0-14 days: 0.020 $gL^{-1}d^{-1}$).

Growth kinetics in starvation

During starvation, cells maintained in tap water slightly continued to grow for 7 days, reaching cell densities of about 5 x 10^6 cells mL^{-1}, and subsequently entered the stationary phase. Conversely, cells previously cultivated in GR^+ medium and in 1:2 LFS slightly continued to grow until the 14th day of starvation, reaching cell densities of about 8 x 10^6 cells mL^{-1} and 35 x 10^6 cells mL^{-1} (µ: 0.07 and 0.05 div d^{-1}) respectively (Figure 1c). After that period, even these cells entered the stationary phase.

Cell morphology

Cell morphology of *S. acutus* PVUW12 was examined at time 0, after 14 days of growth in all media, and after 28 days of starvation (Figures 2,3). In Bristol GR^+ cultures, at time 0, either oblong single cells alternated to aggregates were observed (Figure 2a). The characteristic tetrads, with cells more fusiform with respect to the single cells, were also found (Figure 2b). Generally, cell dimensions were 5-6 µm of length and 2 µm of width. A single chloroplast, occupying most of the

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cell volume and containing a big pyrenoid, was always visible (Figures 2a-c). TEM observation showed that the round pyrenoid was surrounded by a starch shell (Figure 2d). Cell morphology did not change throughout the growth period (not shown).

Cells grown in tap water for 14 days appeared similar to controls in GR^+, although roundish cells were observed beside the characteristic oblong cells (Figure 2e). Moreover, TEM analyses did not show differences in morphology, except for the detachment of the protoplast from the cell wall that was sometimes observed (Figure 2f).

When cells were grown for 14 days in 1:2 LFS, a great variability in cell morphology and dimensions was observed. Indeed, cells were often roundish, even though oblong cells were also detected, and dimensions were 3-6 µm (Figure 2g-i). Sporocysts were observed too, confirming that cells were in active division (Figure 2g). Pyrenoid was not detected inside the plastid (Figures 2h,i). Conversely, starch granules appeared interspersed among the thylakoids (Figures 2h,i). Moreover, small lipid globules and polyphosphate grains were well detectable in the cytoplasm and the cell wall appeared thicker compared with controls (Figures 2h,i).

When cells grown in GR^+ were starved for 28 days, both fusiform and oblong, single or grouped cells were observed (Figure 3a). Interestingly, the cell width was higher (2.5-4.5 µm) relative to that found at 14 days of growth in GR^+ medium (2 µm). TEM observations showed that the chloroplast, containing a clearly visible pyrenoid, still occupied most of the cell volume (Figure 3b). However, several starch granules frequently occurred inside the plastid, and several small lipid droplets accumulated in the cytoplasm. Finally, the cell wall appeared thicker than that observed in cells grown for 14 days in GR^+ (190 *versus* 80 nm; $p<0.001$). Two layers were distinguished: a clearer inner layer and a more electrondense external layer (Figure 3c). Even cells maintained in tap water showed relevant differences as compared with the previous sampling time (compare Figures 2e,f and Figures 3d-f). Tetrads were frequent and the cytoplasm appeared granulated (Figure 3d). TEM analyses confirmed that these granulations were either stromatic starch granules or cytoplasmic lipid globules (Figures 3e,f). Lipid droplets usually converged in a single larger globule (Figure 3f). Even in this case, thickening and bistratification of the cell wall were clearly evident (Figures 3e,f). When cells grown in 1:2 LFS were starved for 28 days, they maintained a more roundish shape than the other samples (Figures 3g-j). Moreover, these samples acquired several distinctive characteristics, such as the disappearance of the polyphosphate granulations, observed after 14 days of growth, and the presence around cells of polysaccharidic materials,

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never detected in the other samples (Figures 3i-j). Accumulation of lipid globules and starch granules, and bilayering of cell wall were also observed, as in the other samples (Figure 3).

Maximum PSII quantum yield determination

During the growth period, F_V/F_M values ranged between 0.71 and 0.76 in cells cultivated in GR^+. Conversely, in tap water-cultivated cells the values decreased from about 0.71 at time 0 to about 0.67 at 14 days of growth, whereas in 1:2 LFS samples the decrease was even more pronounced with F_V/F_M values of about 0.61. When Bristol GR^+ cells were starved, F_V/F_M strongly decreased from 0.75 to 0.61 at the end of the experiment. The maximum drop already occurred during the first 7 days of starvation, when values of 0.65 were measured (-13% with respect to time 0). Interestingly, cells grown in tap water, with/without the addition of LFS, maintained a parallel trend. Indeed, in both samples a slight increase in F_V/F_M was observed after 7 days of starvation (+6% in cells grown in tap water and +8% in cells previously grown with LFS), followed by a gradual decrease during the rest of experiment. However, starved cells previously grown in 1:2 LFS maintained F_V/F_M values always lower with respect to cells grown in tap water and previously grown in GR^+, reaching at the end of the experiment values about 12% lower than in the latter two samples ($p<0.01$ with respect to both samples).

Discussion

The use of cheese industry effluents as substrates for microalgal growth is not new in the literature, but still requires further investigations. Some Authors referred that growth was enhanced only when whey was supplied after hydrolysis, due to the incapability of the used microalgae to assimilate lactose (Freyssinet & Nigon 1980; Abreu et al. 2012). However, it is also reported that some microalgae, including *Scenedesmus* (Danforth 1962), can metabolize disaccharides such as lactose (Zhang et al. 2011). This capability might be exploited for the growth of *S. acutus* PVUW12 with scotta, considering that several strains of *Scenedesmus* can grow mixotrophically in the presence of different organic carbon sources (Mandal & Mallick 2009; El-Sheekh et al. 2013). In this work, the addition of LFS to tap water strongly enhanced the cell density with respect not only to tap water, but also to other standard growth media (Doria et al.

2012). However, growth was: 1. promoted only when the LFS was supplied at the lowest dilution (1:2), suggesting that at higher dilutions (1:5 to 1:20) the cultivation medium could be affected by some limiting nutrients; 2. suppressed by undiluted LFS (not shown), conceptually due to some components contained at inhibiting concentrations in the pure substrate (Prazeres et al. 2012).

The composition of scotta used for experiments was in line with literature (Sansonetti et al. 2010). As expected, lactose was the main component. Here, the capability of *S. acutus* PVUW12 to degrade and use this disaccharide has been confirmed. Indeed, although only 5.2% of the lactose was consumed, the consumption rate was the highest of all the components considered. Beside lactose, citric and L-lactic acids were also used as organic carbon sources. Especially for citric acid, this is not surprising, because this compound is commonly added to many growth media, where it increases solubility and availability of trace metal ions (Provasoli & Pintner 1953). More interesting was the capability to assimilate part of the lactic acid, which in several microalgae is instead reported at most to slightly promote growth (Tanoi et al. 2011). A total protein consumption of about 38% was also found. Microalgae, in fact, can exploit a great variety of organic nitrogen-containing molecules dissolved in natural waters as direct or indirect nitrogen sources (Berman & Chava 1999). Moreover, it has been demonstrated that different organic nitrogen sources are even preferred to the inorganic sources by heterotrophic *Chlorella protothecoides* (Xiong et al. 2008). Similarly, the mixotrophic *S. acutus* PVUW12 proves to be able to assimilate proteins available in the LFS as nitrogen sources. As shown in Figure 1a, mixotrophic *S. acutus* PVUW12 entered the stationary phase between 14 and 21 days of growth, apparently still having organic nutrients available (Figure 1b). The combination of two different causes can explain this growth slowdown: 1. some of the minor components of LFS have become limiting; 2. during cultivation, some growth-inhibiting catabolites have been released (Moheimani & Borowitzka 2006).

Scenedesmus sp. shows alternative morphologies depending on different environmental conditions (Lürling 2003). Here, the addition of LFS to tap water induced many morphological modifications. Thickened cell wall, enlarged cell volumes, accumulation of stromatic starch and of cytoplasmic polyphosphate granules probably played a role in osmoregulation, considering that scotta contains many solutes (Table S2; Ferroni et al. 2007; Seufferheld & Curzi 2010; Baldisserotto et al. 2012). However, starch and polyphosphates might also be accumulated as a consequence of the organic nutrient uptake from the medium, as observed in other mixotrophic microalgae (Markou & Georgakakis 2011; Baldisserotto et al. 2014). This is also supported by the

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disappearance of the pyrenoid (containing RuBisCO enzyme), which testifies that the starch synthesis relies more on the exogenous carbon source than on photosynthesis (Solymosi 2012).

Effects of LFS on the photosynthetic efficiency were also investigated. The PSII maximum quantum yield is a useful parameter for estimating the physiological state of plants (Koller et al. 2014) and microorganisms (White et al. 2011). As expected, when *S. acutus* PVUW12 was grown in only tap water, values lower than in controls were recorded. Indeed, it is well known that nitrogen limitation has detrimental effects on PSII efficiency (Berges et al. 1996; White et al. 2011). However, in this *Scenedesmus* strain grown in only tap water for 28 days, F_V/F_M remained in the normal range for green microalgae (around 0.7; White et al. 2011), suggesting a great capability to resist nutrient stress conditions (Figure 4). Conversely, the addition of LFS led to a strong and rapid decrease in F_V/F_M. This effect can be attributed to mixotrophy, where a decreased F_V/F_M ratio is linked to the downregulation of photosynthesis (Liu et al. 2009). On the other hand, addition of organic carbon sources often makes mixotrophic microalgae able to not be strictly dependent on light and photosynthesis. For this reason, the growth rates of *S. acutus* PVUW12 were enhanced probably benefiting from the heterotrophic component of the mixotrophic metabolism.

S. acutus PVUW12 can be considered a suitable candidate for biofuel production, since under starvation it accumulates a good quality oil (Damiani et al. 2013). As this work was aimed to find an economically sustainable cultivation protocol to obtain lipid-rich algal biomass for bioenergetics purposes, 14 days-grown cells were transferred in tap water and special attention was focussed on lipid accumulation. During the first two weeks of starvation, cells previously grown in Bristol GR^+ and in 1:2 LFS still slightly continued to grow, benefiting from the carbonates dissolved in fresh tap water (El-Sheekh et al. 2013; Giovanardi et al. 2013). Additionally, in LFS starved samples, the polyphosphate granules, accumulated during the mixotrophic cultivation, might also be degraded for the cell growth (Markou & Georgakakis 2011). A decrease was observed in F_V/F_M throughout starvation in all samples, due to the incapability of cells to maintain a normal photosynthetic efficiency (Berges et al. 1996; White et al. 2011). The most interesting information on starved-samples was given by morphological observations. Cells previously grown in Bristol GR^+ showed enlarged cell volumes, whereas thickening of the cell wall, lipid and starch accumulation were observed independently of the previous cultivation. These characteristics have often been observed in several Chlorophyta (van Donk et al. 1997; Baldisserotto et al. 2014), *Scenedesmus* included (Lürling 2003), grown under nutrient-limiting conditions. Increased cell volume and

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alteration of the cell wall have been attributed to a strategy for limiting cell growth under stress conditions (van Donk et al. 1997; Lürling 2003; Baldisserotto et al. 2014), whereas starch and lipids were regarded as a consequence of the excess reducing power produced during the photoassimilation of the organic carbon under N-depletion (White et al. 2011; Baldisserotto et al. 2014). The extracellular polysaccharides production has also been associated with other N-depleted Chlorophyta, for the same reason described above (Yang et al. 2010). However, in our case the presence of polysaccharides around cells was observed only in samples previously cultivated in 1:2 LFS, thus they might as well represent the remnants of the mother cell wall in daughter cells at a late phase of autosporulation (Somogyi et al. 2011), associated with the previous high mixotrophic growth rate.

This work provides new information on the capability of *S. acutus* PVUW12 to be cultivated in a two-stage process for obtaining high quantities of cells enriched in valuable molecules for different biotechnological applications. During the first stage, the use of a by-product obtained from the dairy industry could provide all the nutrients required for the growth of the microalga, simultaneously providing added-value to an environmentally problematic product. The second stage (starvation) could be subsequently applied to enhance the lipid globules production. Further investigation on lipid profile and quantification will be planned for more direct bioenergetics perspectives.

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Supplementary materials contain information about scotta treatment, LFS and tap water composition.

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Figure captions

Figure 1. (a) Growth kinetics of *S. acutus* PVUW12 in Bristol GR^+ (filled circles), tap water (filled squares), tap water added with LFS at increasing dilution of 1:2 (empty squares), 1:5 (empty circles), 1:10 (empty triangles) and 1:20 (empty diamonds). (b) Organic nutrient concentration in 1:2 LFS medium during the first 14 days of growth of *S. acutus* PVUW12. Black: proteins; light grey: lactic acid; white: citric acid; stripes: lactose. (c) Growth kinetics of *S. acutus* PVUW12 in Bristol GR^+ (filled circles), tap water (empty circles) and 1:2 LFS (empty squares) for 14 days and subsequent 28-days-long starvation. Y scales in (a) and (c) are logarithmic. Dashed line indicates the beginning of starvation. Data are means ± s.d. ($N \geq 3$).

Figure 2. (a-d) Cell morphology of *S. acutus* PVUW12 grown in Bristol GR^+ at the inoculum time. (a-c) light microscopy images. (d) TEM micrograph. (e) Cell morphology of *S. acutus* PVUW12 grown in tap water after 14 days. (f) Corresponding TEM micrograph. (g) Cell morphology of *S. acutus* PVUW12 grown in 1:2 LFS after 14 days. (h-i) Corresponding TEM micrographs. White arrows: pyrenoid; black arrows: chloroplast; d: detachment of the cytoplasm from the cell wall; black arrowhead: sporocyst; white arrowheads: cytoplasmic lipid globules; double arrowheads: cell wall; asterisks: poliphosphate granules; s: starch granules.

Figure 3. Cell morphology of starved *S. acutus* PVUW12 after 28 days of cultivation on tap water. (a) light microscopy and (b, c) TEM micrographs of cells previously grown in Bristol GR^+. (d) light microscopy and (e, f) TEM micrographs of cells mantained in tap water. (g) light microscopy and (h, j) TEM micrographs of cells previously grown in 1:2 LFS. White arrows: pyrenoid embedded in the plastid; black arrowheads: cytoplasmic lipid globules; s: starch granules; il: internal layer; el: external layer of the cell wall; double arrowheads: polysaccharide materials;

Figure 4. Timecourse of F_V/F_M of *S. acutus* PVUW12 grown in Bristol GR^+ (filled circles), tap water (empty circles) and 1:2 LFS (empty squares) for 14 days and under starvation on tap water. Dashed line indicates the beginning of starvation. Data are averages ± s.d. ($N \geq 3$).

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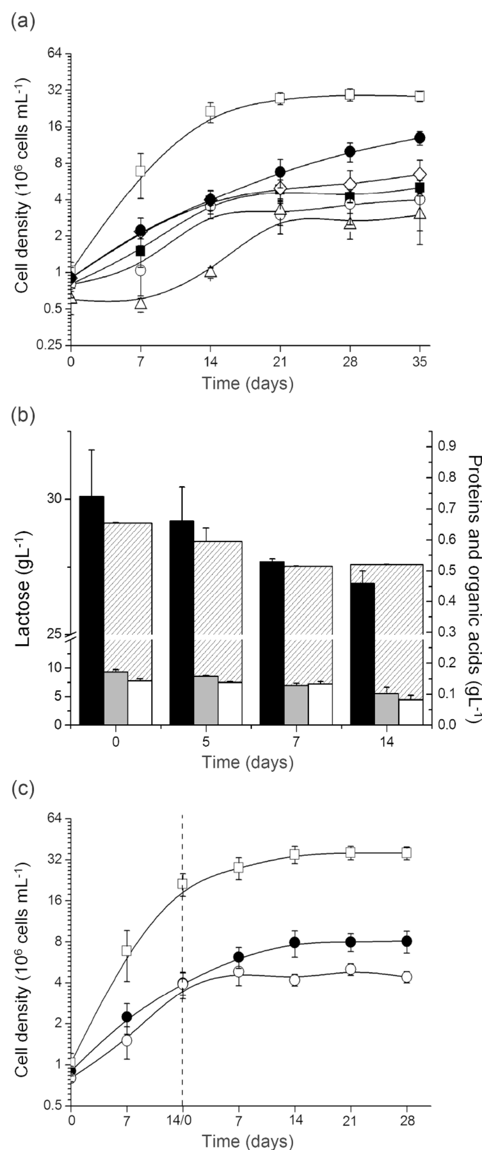

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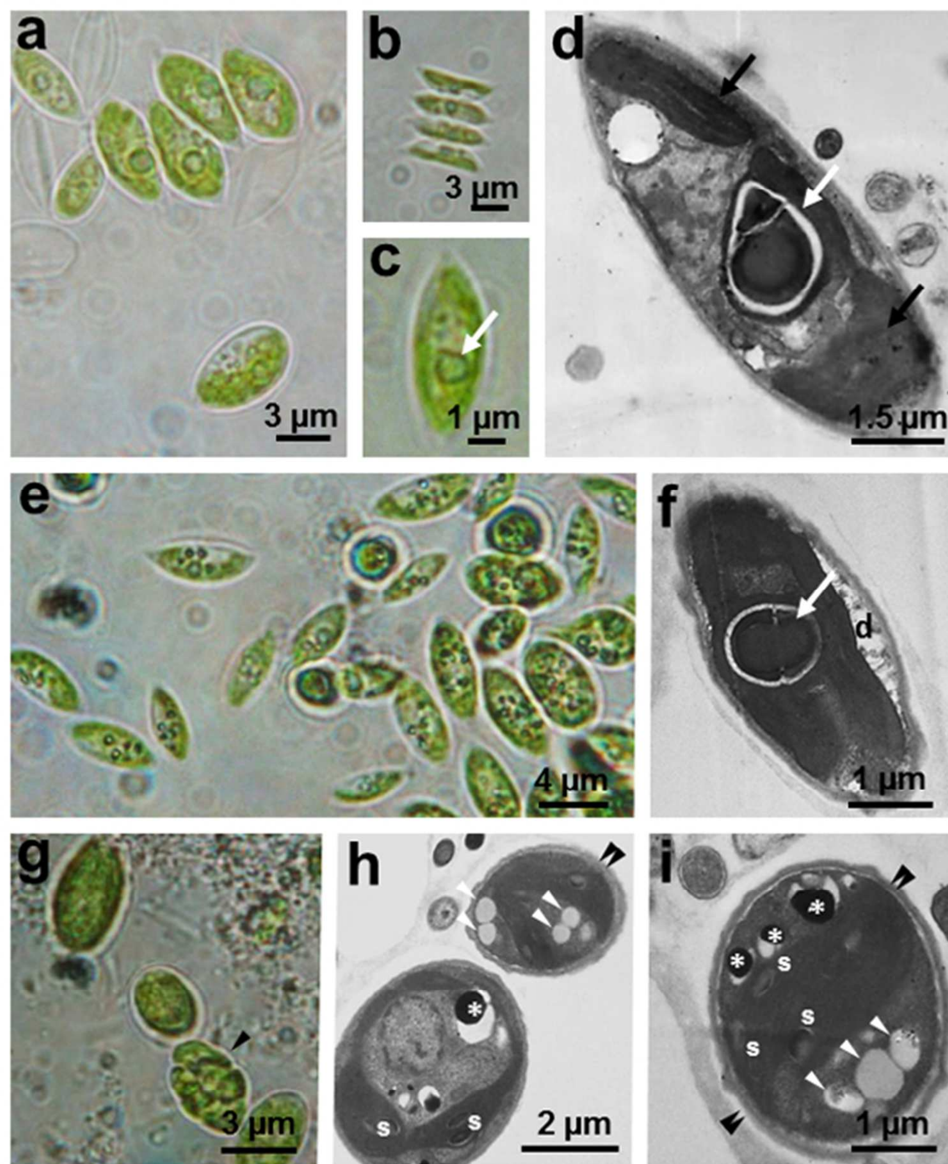

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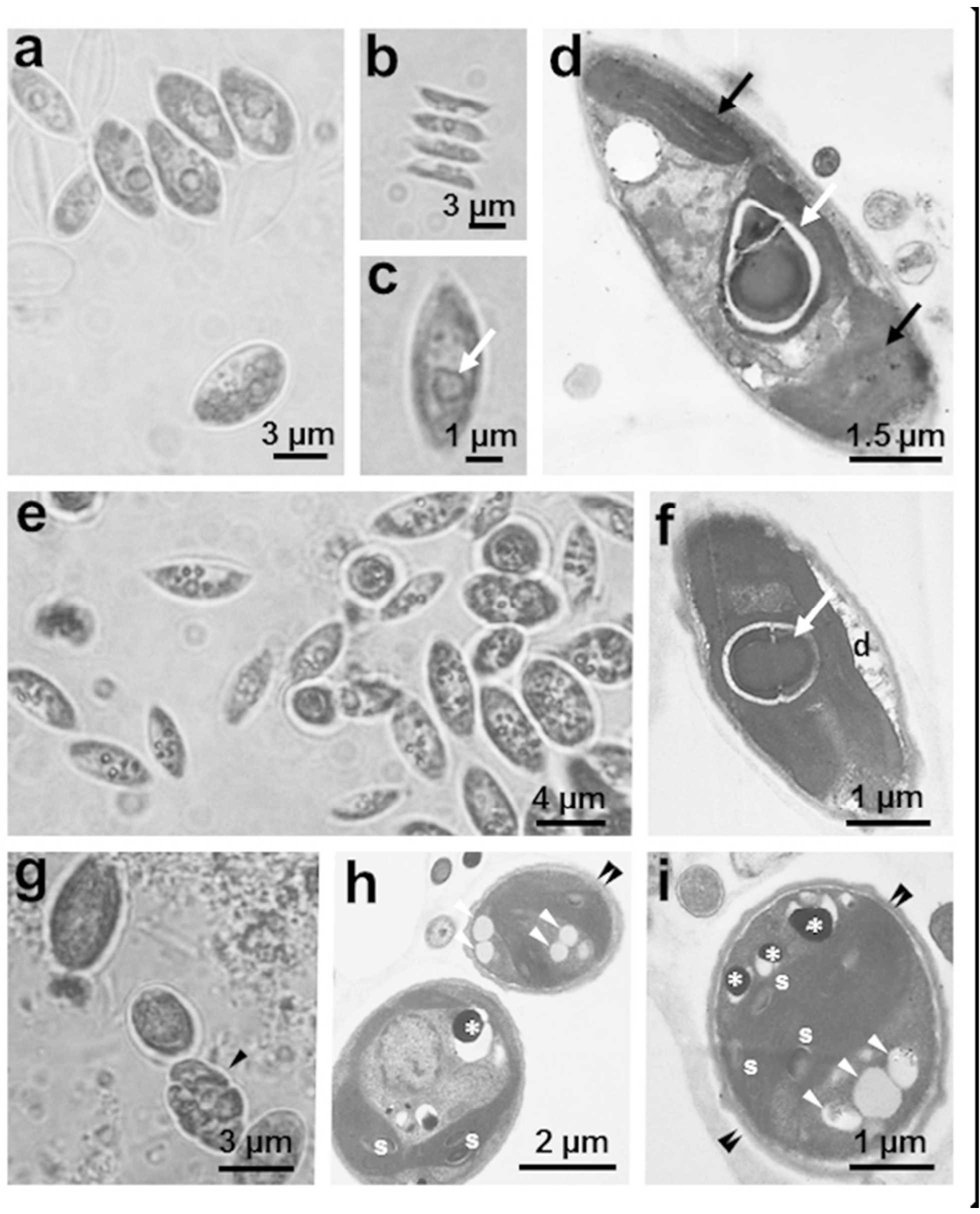

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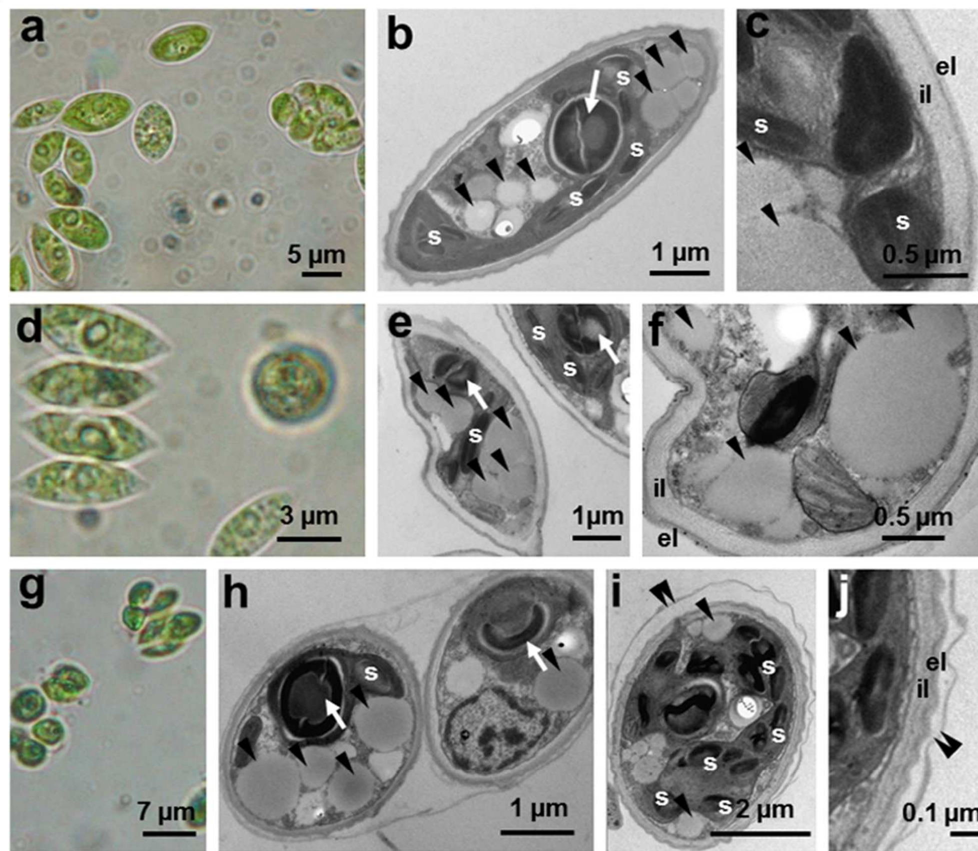

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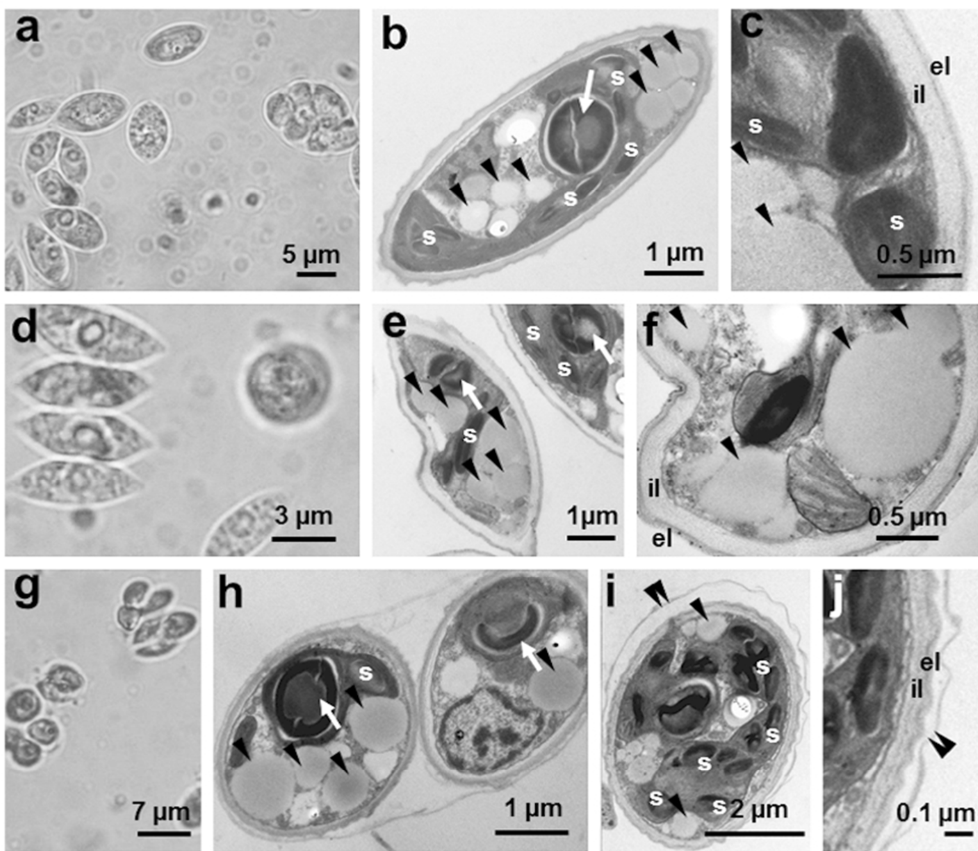

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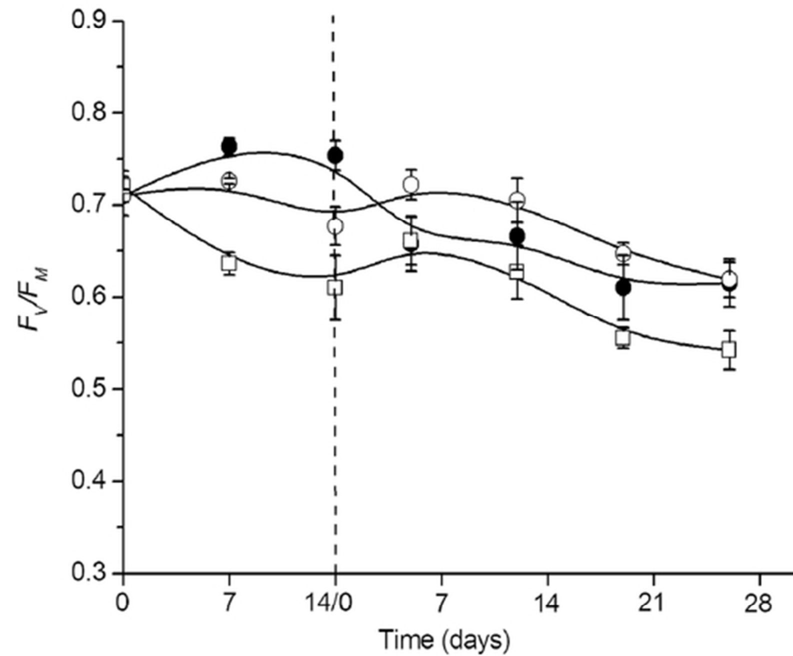

Figure 4. Timecourse of FV/FM of S. acutus PVUW12 grown in Bristol GR+ (filled circles), tap water (empty circles) and 1:2 LFS (empty squares) for 14 days and under starvation on tap water. Dashed line indicates the beginning of starvation. Data are averages ± s.d. (N≥ 3).
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Supplemental

Whey treatment for the obtained of the liquid fraction of scotta (LFS)

The LFS used in the experiments was obtained from untreated scotta (Figure S1a) after several steps. First of all, pH was adjusted to 6.8 with KOH (original pH was 6.1 ± 0.1), then the liquid fraction was centrifuged (600*g*, 10 min) to allow a first clarification. Then, the upper lipid phase was removed, whereas the lower liquid phase was further filtered and subsequently autoclaved for sterilisation. This latter step allowed the formation of curd (Figure S1b), which was completely separated in axenic conditions from the liquid phase after cooling by centrifugation (600*g*, 10 min). Finally, the liquid phase was transferred into sterile bottles (Figure S1c). The obtained LFS was used for the experiments. Aliquots of 20 ml of LFS were freeze-dried in order to determine the dry weight, which was 7.8% (*w*:*v*). Chemical composition of the obtained LFS was also determined as described in "Material and methods" section. The analyses confirmed that the composition is similar to that of unsterilized scotta reported by Sansonetti et al. (2010).

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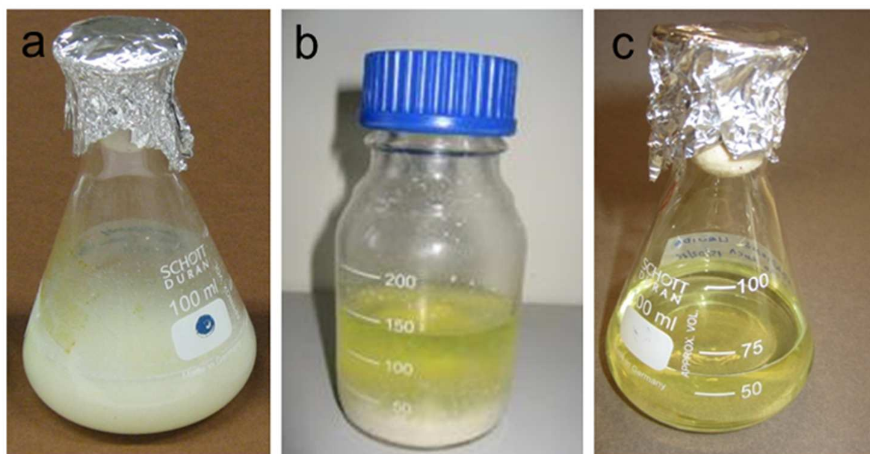

Figure S1. Different treatment steps of the scotta obtained from the Ricotta cheese production. (a) untreated scotta. (b) liquid fraction of scotta after sterilisation. (c) liquid phase axenically harvested by centrifugation and subsequently used for the experiments.

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Table S1. Chemical composition of tap water used for experiments. Values are available in HERA website (www.gruppohera.it) and are referred to a monthly average registered at the time when experiments were executed.

Parameters	Units	Average values
pH	pH	7.7
Hardness	°F	20
Cl^-	mgL^{-1}	24
F^-	mgL^{-1}	0.1
NH_4^+	mgL^{-1}	< 0.02
NO_3^-	mgL^{-1}	10.0
NO_2^-	mgL^{-1}	< 0.02
CO_3^{2-} and HCO_3^-	mgL^{-1}	246
Na^+	mgL^{-1}	15

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3 Table S2. Average organic compound composition of LFS. Data are averages ± s.d. ($N \geq 3$).

Parameter	g L^{-1}
Lactose	58.23 ± 0.05
Citric Acid	0.29 ± 0.01
L-Lactic Acid	0.34 ± 0.01
Total protein	1.48 ± 0.3

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1 **Morpho-physiological aspects of *Scenedesmus acutus* PVUW12 ~~CCALA 935~~ cultivated with a 2 dairy industry waste and after starvation**

3 M. GIOVANARDI[1§], C. BALDISSEROTTO[1§], M. DAGLIA[2], L. FERRONI[1], A. SABIA[1] & S. PANCALDI[1]

4 [1] *Dept. of Life Sciences and Biotechnologies, University of Ferrara, C.so Ercole I d'Este, 32 – 44121 5 Ferrara, Italy*

6 [2] *Dept. of Drug Sciences, Medicinal Chemistry and Pharmaceutical Technology Section, University 7 of Pavia, Via Tramelli, 12 – 27100 Pavia, Italy*

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9 Correspondence: S. Pancaldi, Dept. of Life Sciences and Biotechnologies, University of Ferrara, 10 Corso Ercole I d'Este, 32 – 44121 Ferrara, Italy. Tel.: +39.0532.293786. Fax.: +39.0532.208561. E-11 mail: simonetta.pancaldi@unife.it.

12 [§] Equal contribution

13

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18 Other details on Authors addresses

19 MARTINA GIOVANARDI: Tel. +39.0532.293779; e-mail: martina.giovanardi@unife.it

20 COSTANZA BALDISSEROTTO: Tel. +39.0532.293779; e-mail: costanza.baldisserotto@unife.it

21 MARIA DAGLIA: Tel. +39.0382.987388; e-mail: maria.daglia@unipv.it

22 LORENZO FERRONI: Tel. +39.0532.293779; e-mail: lorenzo.ferroni@unife.it

23 ALESSANDRA SABIA: Tel. +39.0532.293779; e-mail: alessandra.sabia@unife.it

24 SIMONETTA PANCALDI: Tel. +39.0532.293786; e-mail: simonetta.pancaldi@unife.it

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Abstract

~~Microalgae are photosynthetic microorganisms with a wide metabolic plasticity and largely studied for their potential in many fields.~~ Among green microalgae, *Scenedesmus* sp. is known for its potential in wastewater remediation and lipid production, especially under starvation. Moreover~~, *Scenedesmus* sp.~~ it is often characterised by a mixotrophic metabolism. ~~Cheese industry is highly expanding and originates large amounts of organic polluting wastes and by-products, as cheese whey, including scotta (from ricotta cheese production). So Therefore, w~~ In this work, we ~~tested the ability of~~ cultivated *S. acutus* PVUW12 ~~CCALA 935~~ ~~to grow~~ in the presence of a liquid fraction of scotta (~~SLF~~ LFS), a cheese whey by-product, ~~diluted in tap water,~~ as source of nutrients. ~~Moreover~~ Subsequently, cultures were starved to evaluate lipid production. Cells were analysed to obtain information about growth, nutrient consumption during ~~SLF~~ LFS cultivation, morphology and photosynthetic efficiency. We found that the alga boosted its growth when cultured in presence of ~~SLF~~ LFS. ~~Moreover, cCultivation with SLF LFS induced sSome morphological modifications, as p~~Production ~~or enhancement~~ of stromatic starch grains, polyphosphate granules, cell wall enlargement~~,~~ and reduction of the photosynthetic efficiency~~,~~ were also induced. Massive ~~L~~lipid accumulation was observed only during starvation, which also induced a strong slowdown of growth, loss of polyphosphate grains, ~~but not of starch grains,~~ ~~production of some polysaccharide parietal material~~ and further decrease in photosynthetic efficiency. This study demonstrates that *S. acutus* PVUW12 can be involved in a two-steps cultivation, firstly promoting growth using a by-product from cheese industry and secondly transferring the microalgae on starvation to induce lipid accumulation for bioenergetics purposes.

Keywords: *Scenedesmus*, scotta, mixotrophy, photosynthesis, starvation.

Running title: Cultivation with scotta and subsequent starvation of *Scenedesmus*.

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Introduction

Microalgae are photosynthetic organisms ~~that have gained considerable importance during the last decades~~ well known for their possible use in many fields, which vary from simple biomass production ~~up~~ to the obtainment of valuable products with commercial and ecological implications (Pulz & Gross 2004; Mimouni et al. 2012). At present, the interest on microalgae is mainly oriented to their capability to accumulate lipids suitable for biofuel production, ~~especially biodiesel,~~ focussing on the strategies to improve the economic feasibility and overcome the competition with fossil fuels (Chisti 2007~~; Wijffels & Barbosa 2010~~). ~~One of the most important advantages of using microalgae is their ability to efficiently modify their metabolism in response to changes in environmental conditions (Gushina & Harwood 2006; Markou & Georgakakis 2011). Therefore, the chemical composition of the culture medium can be manipulated to obtain variable ratios of lipids, carbohydrates and proteins, depending on the target of the cultivation (Mandal & Mallick 2009). For example,~~ In this perspective, nitrate depletion in growth media is the most common nutritional variation used to induce lipid accumulation in several microalga~~el~~ ~~species~~, ~~even if~~ though the biomass productivity under starvation is low (Chisti 2007; ~~Li et al. 2008~~). ~~On the other hand~~ Conversely, studies show that mixotrophy, ~~i.e.~~ the condition in which an organic carbon source is supplied in the culture medium together with light, allows to obtain high biomass yields (~~Andrade & Costa 2007;~~ Heredia-Arroyo et al. 2010; ~~Chen et al. 2011;~~ Abreu et al. 2012; Giovanardi et al. 2013, ~~2014;~~ Baldisserotto et al. 2014). However, the organic carbon supply, usually glucose, acetate, glycerol or fructose (Heredia-Arroyo et al. 2010; ~~Chen et al. 2011~~), is predicted to account for around 80% of the cost of the culture media (Bhatnagar et al. 2011~~;~~ ~~Abreu et al. 2012~~). ~~For this reason~~ Then, research is recently focussing on the valorisation of wastes ~~products or organic effluents~~ derived from agro-food industries, which can be used as cheap organic carbon sources for the microalgal growth (Heredia-Arroyo et al. 2010; Giovanardi et al. 2013~~; Baldisserotto et al. 2014~~)~~.~~, ~~In this way, microalgae might be cultivated in wastes rich in organic and inorganic nutrients,~~ simultaneously solving problems~~,~~ like freshwater demand, high cost of nutrients~~,~~ and waste remediation (Bhatnagar et al. 2011). Among agro-industrial waste products~~, a large amount originates from the food processing industry (Markou & Georgakakis 2011). In this context~~, cheese whey ~~can be~~ is considered as the most polluting waste product derived from the dairy industry (~~Dragone et al. 2009;~~ Abreu et al. 2012; Prazeres et al. 2012). Cheese whey is a green-yellowish, nutrient-rich effluent composed by lactose, soluble proteins ~~(mainly β-lactoglobulin, and α-lactalbumin)~~, lipids and mineral salts containing nitrogen and

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phosphorous. Other components, ~~such as~~ like lactic and citric acids, are also contained in appreciable quantities (Siso 1996). For its high organic content, cheese whey ~~is considered~~ represents an important environmental problem, and needs proper disposal treatments ~~systems for its disposal~~ (Siso 1996; ~~Dragone et al. 2009;~~ Markou & Georgakakis 2011; Abreu et al. 2012; Prazeres et al. 2012). Ricotta cheese whey, named scotta, shares with ~~the~~ other cheese whey both environmental implications and chemical composition, ~~except for~~ but has a reduced protein content (Sansonetti et al. 2010). Scotta can be considered a good raw material suitable for the fermentation by microorganisms and the production of different bio-products, though ~~it~~ its biotechnological potential has not been sufficiently studied yet (~~Panesar et al. 2007;~~ ~~Dragone et al. 2009;~~ ~~Saraceno et al. 2010;~~ Pisponen et al. 2013). Alternatively, Freyssinet & Nigon (1980) proposed the possibility of growing microalgae using cheese whey as organic carbon source. Recently, Abreu and co-workers (2012) evaluated growth parameters, biochemical composition and lipid accumulation of *Chlorella vulgaris* cultivated in the presence of a hydrolysate of cheese whey, concluding that the mixotrophic cultivation using dairy effluents is feasible. Beside these data, however, at the best of our knowledge, no information about growth of microalgal species in the presence of scotta is available.

Among green microalgae, *Scenedesmus* sp. has been widely studied as a promising organism to be employed for biotechnological purposes, mainly biofuel production and bioremediation (~~Chisti 2007; Mandal & Mallick 2009;~~ Doria et al. 2012; Damiani et al. 2013~~, Ördög et al. 2013~~). In a recent work, Mandal and Mallick (2009) have tested the capability of a strain of *Scenedesmus* to accumulate lipids under different growth conditions, mixotrophy and nutrient depletion included. Results confirmed that *Scenedesmus* sp. is a mixotrophic organism, insofar as ~~the~~ cell densities ~~boosted~~ increased when glucose was supplemented to the growth media. However, lipid accumulation was not promoted by mixotrophy, but only by nitrogen starvation (Mandal & Mallick 2009). In this work, *Scenedesmus acutus* PVUW12 ~~CCALA 935~~ ~~(Chlorophyta)~~ has been cultivated in the presence of ~~the~~ a liquid fraction of scotta (~~SLF~~ LFS). Preliminary experiments were done using different dilutions of ~~SLF~~ LFS to select the best one in promoting *Scenedesmus* growth. In the perspective of an economically sustainable cultivation and to test the potential of ~~SLF~~ LFS to provide all nutrients required for growing the microalga, ~~SLF~~ LFS was supplied in tap water. Then, a two-stage experiment was set up, firstly cultivating the microalga in the presence of the best ~~SLF~~ LFS dilution to obtain high cell densities, and subsequently transferring the cells in ~~simple~~ tap water to induce lipid accumulation. ~~Cells were periodically analysed in order to obtain~~

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2
3 1 Iinformation about growth, nutrient consumption, morphology and photosynthetic efficiency was
4
5 2 collected, also with the aim of improving knowledge about the morpho-physiology of this algal
6
7 3 strain., Gaining deeper knowledge Knowledge deepening on microalgae is, in fact, a necessary
8
9 4 step in order to ensure a fruitful utilisation of these organisms in biotechnological applications
10
11 5 (Wijffels & Barbosa 2010).
12
13 6
14 7 **Materials and methods**
15
16 8 *Whey treatment and substrate preparation*
17
18
19 9 In this work, Tthe liquid fraction derived from the preparation of ricotta cheese was kindly
20
21 10 provided from a dairy factory located in Trentino-Alto Adige Region (Italy). This waste by-product
22
23 11 was maintained at -20°C in Falcon tubes to preserve the chemical its composition until use. Before
24
25 12 experiments, scotta was treated in different steps as described in supplementary materials (Figure
26
27 13 S1). First of all, pH was adjusted to 6.8 with KOH (original pH was 6.1 ± 0.1), then the liquid fraction
28
29 14 was centrifuged (600*g*, 10 min) to allow a first clarification. Then, the upper lipid phase was
30
31 15 removed, whereas the lower liquid phase was further filtered and subsequently autoclaved for
32
33 16 sterilisation. This latter step allowed the formation of curd, which was completely separated in
34
35 17 axenic conditions from the liquid phase after cooling by centrifugation (600*g*, 10 min). Finally, the
36
37 18 liquid phase was transferred into sterile bottles. The obtained SLF LFS was used for the
38
39 19 experiments described below. Trial tests using the SLF LFS "as is" were also performed, but did not
40
41 20 lead to positive results (data not shown).
42
43 21
44
45 22 *Algal strain and culture condition*
46
47 23 The microalga *Scenedesmus acutus* strain PVUW12 cf. *acutus* Meyen CCALA-935 (Chlorophyta,
48
49 24 Sphaeropleales) (syn. of *S. acutus* strain PVUW12 deposited as *Scenedesmus* cf. *acutus* Meyen
50
51 25 CCALA-935 at the Culture Collection of Autotrophic Organisms from Institute of Botany, Academy
52
53 26 of Sciences of the Czech Republic, Centre of Phycology, Dukelská), an isolate from an urban
54
55 27 wastewater treatment plant in Pavia (Italy), was kindly provided by Prof. Erik Nielsen (Department
56
57 28 of Biology and Biotechnology, University of Pavia, Italy) and maintained in axenic Bristol GR^+
58
59 29 medium (Bold 1949; web.biosci.utexas.edu) in a growth chamber (24 ± 1 °C temperature, 80 µmol
60
30 photons $m^{-2}s^{-1}$ PAR, 16:8 h light-dark photoperiod). For preliminary experiments, axenic cultures

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were prepared in 100 mL Erlenmeyer flasks (50 mL total volume) at a density of about 1x10^6 cell mL^{-1} in only tap water or tap water added with SLF LFS at the increasing dilutions of 1:2, 1:5, 1:10 and 1:20. Microalgal cultures grown in Bristol GR^+ were used as controls. Tap water was provided from the aqueduct of Ferrara (Italy) and its chemical composition is reported in Tab. S1 by HERA (Holding Energia Risorse Ambiente), the local agency for water, energy and environmental management (www.gruppohera.it). During the experiments, the flasks were maintained in the same growth chamber described above, without CO_2 supply and stirring. To avoid sedimentation, flasks were daily hand-shaken. For analyses, aliquots of cells were weekly collected up to 35 days of growth. For subsequent experiments, samples showing the best growth potential were selected together with their relative control samples, and grown for 14 days in 500 mL Erlenmeyer flasks (250 mL final volume) in the same culture conditions as previously described. After that period, cells were harvested by centrifugation (600*g*, 10 min) and resuspended in 250 mL of tap water (starvation). Flasks were then maintained for 28 days in the growth chamber and aliquots of samples were weekly collected for analyses. All the eExperiments were performed at least in triplicate.

Growth evaluation

Growth was estimated by counting the cells with a Thoma's haemocytometer under a light microscope (Carl Zeiss, Jena, Germany, model Axiophot). Growth rates (μ) were calculated according to Issa et al. (1995) and were expressed as number of divisions (div) per day (Giovanardi et al. 2013).

Scotta composition and nutrient consumption determination

For each of the following determinations, culture media samples, free of algal cells, were obtained by centrifugation throughout the experiment.

Free sugar determination. Lactose content was determined quantified by an UV-method for the determination of this sugar in foodstuff, using an enzymatic assay (Boehringer Mannheim, R-Biopharm, Italia srl, Cerro Al Lambro, Italy). A stabilized standard lactose solution was used as control for the enzymatic determination of lactose in whey.

Organic acid determination. Citric and L-lactic acids were determined by two UV-methods ~~for the determination of these organic acids~~ in foodstuff, using an enzymatic assay (Boehringer Mannheim, R-Biopharm, Italia srl, Cerro Al Lambro, Italy). ~~Also in these cases~~ L-lactic acid and citric acid standard solutions were used as assay controls.

Protein content determination. Protein content was determined according to ~~by~~ Lowry et al. assay (~~Lowry et al.~~ 1951). ~~After reaction,~~ OD values were determined at 660 nm with an UV/Vis spectrophotometer (Pharmacia Biotech Ultrospec® 2000) (1 nm resolution). A calibration curve ~~obtained with increasing concentrations of~~ with bovine serum albumin was used for the quantification ~~of proteins~~.

Morphological and ultrastructure observations

Microscopic observations were done using the same microscope described above, equipped with conventional or fluorescent attachments, as described in Baldisserotto et al. (2012). ~~The light source for chlorophyll fluorescence observation was a HBO 100W pressure mercury vapour lamp (filters: BP436/10, LP470). Pictures of cells were taken with a Canon Powershot S40 digital camera (4 megapixels), mounted on the ocular lens through a Leica DC150 system (Leica camera AG, Solms, Germany).~~ For ultrastructural analyses, aliquots of cells were collected at 0 days (inoculum time), after 14 days of growth ~~in the presence of LFS~~ and after subsequent 28 days of starvation. Cells were harvested by centrifugation (600*g*, 10 min) and then prepared as reported in Baldisserotto et al. (2012)~~Giovanardi et al. (2013). Samples were finally embedded in Araldite-Epon 812 resin (Fluka, Buchs, Switzerland) and stained for transmission electron microscopy (TEM), following standard protocols (Baldisserotto et al., 2012; Giovanardi et al., 2013).~~ Observation of ultrathin sections was performed with a Hitachi H800 electron microscope (Hitachi, Tokyo, Japan). Images were ~~employed~~ used to measure the cell diameter and to check the presence of stromatic starch granules and cytoplasmic lipid globules.

PAM fluorimetry

The maximum quantum yield of PSII was determined using an ADC OS1-FL fluorometer (ADC Bioscientific Ltd, Hoddesdon, Hertfordshire, UK). Aliquots of samples were collected by

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centrifugation (10,000*g*, 3 min). Pellets were then deposited onto pieces of wet filter paper (Schleicher & Schuell) (Ferroni et al.~~,~~ 2011) and maintained in the dark for 15 min. Initial fluorescence (F_0) and maximum fluorescence (F_M) values were measured flashing the samples with a saturating light pulse, then ~~. These values were~~ used to calculate the maximum quantum yield of PSII~~,~~ ~~i.e. the~~ (F_V/F_M ~~ratio~~, where $F_V = F_M - F_0$ is variable fluorescence) (Lichtenthaler et al. 2005).

Data treatment

Data were processed with Microcal Origin 6.0 software (OriginLab, Northampton, MA, USA). In each case means ± standard deviations for *N* number of samples are given. ~~Different d~~ Data obtained from control and treated samples were compared using Student's *t*-test with a significance level of 0.05.

Results

Selection of the best ~~SLF~~ LFS dilution for growth

Cell densities of *S. acutus* PVUW12 ~~CCALA 935~~ grown on different dilutions of LFS ~~with~~ ~~SLF~~ ~~at~~ ~~increasing dilutions~~ were monitored ~~for 35 days~~ to select the best ~~one~~ concentration for further experiments (Figure 1a). Growth kinetics of cells cultivated in Bristol GR^+ and tap water were also included. ~~The c~~Cell concentration progressively increased in control GR^+ samples throughout the experiment, reaching values of about 13 x 10^6 cell mL^{-1} at 35 days. During the first 14 days of growth, cells in tap water showed the same cell densities ~~of~~ as controls, but soon after entered the stationary phase, which led to a very low cell density at the end of the experiment (about 5 x 10^6 cells mL^{-1}). While the addition of ~~SLF~~ LFS at 1:5 to 1:20 dilutions did not promote growth ~~with respect to~~ as compared with tap water (Figure 1a), the 1:2 dilution led to a strongly enhanced growth during the first 14 days of cultivation (μ: 0.31 and 0.15 div d^{-1} for 1:2 ~~SLF~~ LFS samples and both control and tap water samples, respectively; $p < 0.001$), before entering the stationary phase. In these samples, cell densities of about 30 x 10^6 cells mL^{-1} (2.3 and 6 times higher than those observed in controls and in tap water samples, respectively; $p < 0.01$ in both cases) were reached at the end of the experiment. So, the most interesting culture condition, i.e. 1:2 ~~SLF~~ LFS in tap

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water (hereafter simply named 1:2 SLF LFS), was selected to perform new experiments in comparison with tap water and Bristol GR^+. New cultures were grown for 14 days, just before cells entered the stationary phase, to analyse nutrient consumption, cell growth, cell morphology and photosynthetic efficiency. After 14 days of cultivation, samples were then transferred in to tap water, with the exception of cells already growing in water, to test the impact of the previous cultivation on the starvation phase.

Nutrient consumption in the culture media during growth with SLF LFS

Aliquots of 1:2 SLF LFS medium were harvested during the microalgal cultivation at 0, 5, 7 and 14 days, for monitoring the consumption of the main organic compounds (lactose, proteins, citric and L-lactic acids). The composition of SLF LFS analyzed before experiments was consistent with that of scotta reported by Sansonetti et al. (2010), with the exception of the concentration of organic acids, which were present at lower concentrations (Table S2). As shown in Figure 1b, lactose, the main component of SLF LFS, slightly decreased its concentration during the experimental time, with an overall consumption of 5.2% (consumption rate 0-14 days: 0.11 g L^{-1} d^{-1}). In particular, sSuch decrease was mostly evident between the 5th and the 7th days of cultivation, when a consumption rate of 0.45 $gL^{-1}d^{-1}$, corresponding to the 3.2% of the substrate, was observed. After the 7th days of growth, lactose concentration did not change inside the culture medium. As concernsConcerning the organic acids content, a gradual decrease in citric and L-lactic acid was measured (consumption rate 0-14 days: 0.004 and 0.005 $gL^{-1}d^{-1}$ for citric and lactic acid, respectively). Indeed, at the end of the experiment, concentrations 43 and 41% lower than those present at time 0 were respectively found. Finally, a progressive decrease in protein content was also observed throughout the experiment, and reached, at the 14th day of growth, values about 38% lower than that at time 0 (consumption rate 0-14 days: 0.020 $gL^{-1}d^{-1}$).

Growth kinetics in starvation

During starvation, cells maintained in tap water slightly continued to grow for 7 days, reaching cell densities of about 5 x 10^6 cells mL^{-1}, and subsequently entered the stationary phase. Conversely, After being transferred in tap water, cells previously cultivated in GR^+ medium and in 1:2 LFS still slightly continued to grow during the first until the 14th day of starvation, reaching cell densities of

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about 8 x 10^6 cells mL^{-1} ~~(μ: 0.07 div d^{-1})~~ and 35 x 10^6 cells mL^{-1} (μ: 0.07 and 0.05 div d^{-1}) respectively (Figure 1c). After that period, even these cells entered the stationary phase. ~~The same trend was observed for cells previously grown in presence of 1:2 SLF LFS (Figure 1c). In this case, after 14 days of starvation, cultures reached densities of about 35 x 10^6 cells mL^{-1}, comparable to those observed at the end of the preliminary experiments (Figure 1a). Thereafter, also these cells entered the stationary phase.~~

Cell morphology

Cell morphology of *S. acutus* PVUW12 ~~CCALA 935~~ was examined at time 0, after 14 days of growth in all media, and after 28 days of starvation (Figures 2,3). ~~Pictures of cells grown in Bristol GR^+ at time 0 (inoculum) are reported in Figure 2.~~ In Bristol GR^+ cultures, at time 0, ~~different algal forms were found inside the cultures. Indeed,~~ either oblong single cells alternated to aggregates were observed (Figure 2a). The characteristic tetrads, with cells more fusiform with respect to the single cells, were also found ~~inside the cell population~~ (Figure 2b). Generally, cell dimensions were ~~between~~ 5-6 µm of length and 2 µm of width. A single chloroplast, occupying most of the cell volume and containing a big pyrenoid, was always visible (Figures 2a-c). TEM observation showed that the round pyrenoid was surrounded by a starch shell (Figure 2d). Cell morphology did not change throughout the growth period (not shown).

Cells grown in tap water for 14 days appeared similar to controls in GR^+, although roundish cells were observed beside the characteristic oblong cells (Figure 2e). Moreover, TEM analyses did not show differences in morphology, except for the ~~a sometimes-observed~~ detachment of the protoplast from the cell wall that was sometimes observed (Figure 2f).

When cells were grown for 14 days in 1:2 ~~SLF~~ LFS, a great variability in cell morphology and dimensions was observed. Indeed, cells were often roundish, even though oblong cells were also detected, and dimensions were ~~between and~~ 3-6 µm (Figure 2g-i). Sporocysts were observed too, confirming that cells were in active division (Figure 2g). Pyrenoid was not detected inside the plastid (Figures 2h,i). Conversely, starch granules appeared ~~in granulations~~ interspersed among ~~between~~ the thylakoids (Figures 2h,i). Moreover, small lipid globules and polyphosphate grains were well detectable in the cytoplasm and the cell wall appeared thicker compared with ~~respect to~~ controls (Figures 2h,i).

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When cells grown in GR^+ were starved ~~transferred in for starvation to tap water~~ for 28 days, both fusiform and oblong, single or grouped cells~~,~~ were observed (Figure 3a). Interestingly, the cell width was higher (2.5-4.5 µm) ~~with respect~~ relative to that found at ~~the~~ 14th days of growth in GR^+ medium (2 µm). TEM observations showed that the chloroplast, containing a clearly visible pyrenoid, still occupied most of the cell volume (Figure 3b). However, several starch granules frequently occurred inside the plastid, and several small lipid droplets ~~were~~ accumulated in the cytoplasm. Finally, the cell wall appeared thicker than that observed in cells grown for 14 days in GR^+ (190 *versus* 80 nm; p<0.001). Two layers were distinguished: a clearer inner layer and a more electrondense external layer (Figure 3c). Even cells ~~grown~~ maintained in tap water showed relevant differences ~~with respect to previous time of growth~~ as compared with the previous sampling time (~~14 days;~~ compare Figures 2e,f and Figures 3d-f). Tetrads were frequent ~~among the cell population,~~ and the cytoplasm appeared granulated (Figure 3d). TEM analyses confirmed that ~~such~~ these granulations were either stromatic starch ~~stromatic~~ granules or cytoplasmic lipid ~~cytoplasmic~~ globules (Figures 3e,f). Lipid droplets usually ~~tended to~~ converged in a single larger globule (Figure 3f). Even in this case, thickening and bistratification of the cell wall ~~was~~ were clearly evident (Figures 3e,f). When cells grown in 1:2 ~~SLF~~ LFS were starved for 28 days, they maintained a more roundish shape than the other samples (Figures 3g-j). Moreover, these samples acquired several distinctive characteristics, such as the disappearance of the polyphosphate granulations, observed after 14 days of growth, and~~, more interestingly,~~ the presence around cells ~~extrusion~~ of polysaccharidic materials, never detected in the other samples (Figures 3i-j). Accumulation of lipid globules and starch granules, and bilayering of cell wall were also observed, as in the other samples (Figure 3).

Maximum PSII quantum yield determination

~~The maximum efficiency of PSII photochemistry of cells grown in the different culture media and during starvation is shown in Figure 4.~~ During the growth period, F_V/F_M values ranged between 0.71 and 0.76 in cells cultivated in GR^+. Conversely, ~~values were lower in samples grown in tap water (with/without SLF LFS). In particular,~~ in tap water-cultivated cells the values decreased from about 0.71 at time 0 to about 0.67 at ~~the~~ 14th days of growth, whereas in 1:2 ~~SLF~~ LFS samples the decrease was even more pronounced with F_V/F_M values of about 0.61. When Bristol GR^+ cells were starved, F_V/F_M ~~ratio~~ strongly decreased from 0.75 to 0.61 at the end of the experiment. The

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maximum drop already occurred during the first 7 days of starvation, when values of 0.65 were measured (-13% with respect to time 0). Interestingly, cells grown in tap water, with/~~or~~without the addition of ~~SLF~~ LFS, maintained a parallel trend. Indeed, in both samples a slight increase in ~~the~~ F_V/F_M ~~ratio~~ was observed after 7 days of starvation (+6% in cells grown in tap water and +8% in cells previously grown with ~~SLF~~ LFS), followed by a gradual decrease during the rest of experiment. However, starved cells previously grown in 1:2 ~~SLF~~ LFS maintained F_V/F_M values always lower with respect to cells grown in tap water and previously grown in GR^+, reaching at the end of the experiment values about 12% lower than in the latter two samples (p<0.01 with respect to both samples).

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Discussion

The use of cheese ~~making~~ industry effluents as substrates for ~~the~~ microalgal growth is not new in the literature, but ~~it is~~ still requires further investigations ~~scantly deepened~~ (Freyssinet & Nigon 1980; Abreu et al. 2012). ~~In those works, only the microalgal growth in the presence of cheese whey and the concomitant removal of its pollutants have been considered (Freyssinet & Nigon, 1980; Abreu et al. 2012). Moreover, no information is available about scotta. In all cases, the microalgal~~ Some Authors referred that growth was enhanced only when whey was supplied after hydrolysis, due to the incapability of the used microalgae ~~strains~~ to assimilate lactose ~~(Freyssinet & Nigon 1980; Abreu et al. 2012)~~. However, it is also reported that some microalgae, including *Scenedesmus* ~~sp.~~ (Danforth 1962), can metabolize disaccharides such as lactose (~~Chen et al. 2011;~~ Zhang et al. 2011). ~~So, t~~This capability might be exploited for the growth of ~~*Scenedesmus*~~ *S. acutus* PVUW12 with scotta~~.~~, considering that ~~Since~~ several strains of *Scenedesmus* can grow mixotrophically in the presence of different organic carbon sources (~~Ogawa & Aiba 1981;~~ Mandal & Mallick 2009; El-Sheekh et al. 2013)~~, in this work the capability of *S. acutus* PVUW12 CCALA-935 to grow in the presence of a ricotta cheese effluent (SLF LFS) was stated is reported~~. In this work ~~fact~~, the addition of ~~SLF~~ LFS to tap water ~~led to a strong enhancement~~ strongly enhanced ~~of~~ the cell density with respect not only to tap water, but also to other standard growth media (Doria et al. 2012). However, growth was: 1. promoted only when the ~~SLF~~ LFS was supplied at the lowest dilution (1:2), suggesting that at higher dilutions (1:5 to 1:20) the cultivation medium could be affected by ~~one or more several~~ some limiting nutrients; 2. suppressed by ~~not~~ undiluted ~~SLF~~ LFS

(not shown), conceptually due to some components contained at inhibiting concentrations in the pure substrate (Prazeres et al. 2012).

The composition of scotta used for experiments was in line with literature (Sansonetti et al. 2010). As expected, lactose was the main component. Here, the capability of *S. acutus* PVUW12 ~~CCALA-935~~ to degrade and use this disaccharide has been confirmed. Indeed, although ~~despite the fact that~~ only ~~the~~ 5.2% of the lactose was consumed, the consumption rate was the highest ~~among~~ of all the ~~other~~ components considered. Beside lactose, ~~scotta contains appreciable amounts of~~ citric and L-lactic acids~~, which~~ were also used as organic carbon sources. Especially for citric acid, this is not surprising, because this compound is commonly added to many growth media, where it increases solubility and availability of trace metal ions ~~and their availability~~ (Provasoli & Pintner 1953). More interesting was the capability to assimilate part of the lactic acid, which in several microalgae is instead reported at most to ~~at most~~ slightly promote growth (~~Perez-Garcia et al. 2011;~~ Tanoi et al. 2011). A ~~In this study, a~~ total protein consumption of about 38% was also found. Microalgae, in fact, can exploit a great variety of organic nitrogen-containing molecules dissolved in natural waters as direct or indirect nitrogen sources (Berman & Chava 1999). Moreover, it has been demonstrated that different organic nitrogen sources are even preferred to the inorganic sources by heterotrophic *Chlorella protothecoides* (Xiong et al. 2008). Similarly, the mixotrophic *S. acutus* PVUW12 ~~used in this work~~ proves to be able to assimilate proteins available in the ~~SLF~~ LFS as nitrogen sources. As shown in Figure 1a, mixotrophic *S. acutus* PVUW12 ~~CCALA-935~~ entered the stationary phase between ~~the~~ 14th and ~~the~~ 21st days of growth, apparently still having organic nutrients available (Figure 1b). The combination of two different causes can explain this growth slowdown: 1. some of the minor components of ~~the~~ ~~SLF~~ LFS have become limiting; 2. during cultivation, some growth-inhibiting catabolites have been released (Moheimani & Borowitzka 2006).

~~SLF LFS affected cell morphology of *S. acutus* PVUW12 CCALA-935~~. *Scenedesmus* sp. ~~has a great phenotypic plasticity and~~ shows alternative morphologies depending on different environmental conditions~~, such as nutrient availability, pH and temperature~~ (Lürling 2003). Here, the addition of ~~SLF~~ LFS to tap water induced many morphological modifications. ~~In particular, T~~thickened cell wall, enlarged cell volumes, accumulation of stromatic starch and of cytoplasmic polyphosphate granules probably played a role in osmoregulation, considering that scotta contains ~~is rich in~~ many solutes (Table S2; ~~Sansonetti et al. 2010~~ Ferroni et al. 2007; Seufferheld & Curzi 2010;

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Baldisserotto et al. 2012). ~~All those cell modifications, in fact, are common in freshwater microalgae acclimated to brackish media~~ ~~(Ferroni et al. 2007; Seufferheld & Curzi 2010; Baldisserotto et al. 2012~~). However, starch and polyphosphates might also be accumulated as a consequence of the organic nutrient uptake from the medium, as ~~already~~ observed in other mixotrophic microalgae (~~Akazawa & Okamoto 1980;~~ ~~Li et al. 2011;~~ Markou & Georgakakis 2011; ~~Giovanardi et al. 2013;~~ Baldisserotto et al. 2014). This is also supported by the disappearance of the pyrenoid (containing RuBisCO enzyme), which testifies that the starch synthesis relies more on the exogenous carbon source than on photosynthesis (Solymosi 2012).

Effects of ~~SLF~~ LFS on the photosynthetic efficiency were also investigated. ~~In particular, t~~The PSII maximum quantum yield is a useful parameter for estimating the physiological state of plants (Koller et al. 2014) and microorganisms (White et al. 2011) ~~in different conditions, such as nutrient limitation (Young & Beardall, 2003;; Baldisserotto et al. 2014), changes of salinity (Eggert et al. 2007; Baldisserotto et al. 2012), sub-optimal temperature (Eggert et al. 2003), high-irradiance (Masojídek et al. 1999) and even addition of organic carbon to growth media (Liu et al. 2009; Baldisserotto et al. 2014; Giovanardi et al. 2014).~~ As expected, when *S. acutus* PVUW12 ~~CCALA-935~~ was grown in only tap water, values lower than in controls were recorded. ~~In fact~~ Indeed, it is well known that nitrogen limitation has detrimental effects on PSII efficiency (Berges et al. 1996; ~~Young & Beardall 2003;~~ White et al. 2011; ~~Baldisserotto et al. 2014~~). However, ~~interestingly,~~ in this *Scenedesmus* strain grown in only tap water for 28 days, F_V/F_M ~~ratios~~ remained in the normal range for green microalgae (around 0.7; White et al. 2011 ~~Kromkamp & Peene 1999~~), suggesting a great capability to resist nutrient stress conditions (Figure 4). Conversely, the addition of ~~SLF~~ LFS led to a strong and rapid decrease in ~~the~~ F_V/F_M ~~ratio~~. This effect can be attributed to mixotrophy, where a decreased F_V/F_M ratio is linked to the downregulation of photosynthesis (~~Oesterheld et al. 2007;~~ Liu et al. 2009). On the other hand, addition of organic carbon sources often makes mixotrophic microalgae able to not be strictly dependent on light and photosynthesis~~, because autotrophy and heterotrophy occur independently (Marquez et al. 1993; Andrade & Costa 2007)~~. For this reason, the growth rates of *S. acutus* PVUW12 ~~CCALA-935~~ were enhanced probably benefiting from the heterotrophic component of the mixotrophic metabolism.

~~As this work was aimed to find an economically sustainable cultivation protocol to obtain algal biomass enriched in lipids useful for bioenergetics purposes, we tested~~ *S. acutus* PVUW12 ~~CCALA-935 that~~ can be considered a suitable candidate for biofuel production, since ~~(Damiani et al. 2013).~~

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~~In fact, when this alga is cultivated~~ under starvation~~,~~ it accumulates a good quality oil ~~to be used as a biodiesel feedstock~~ (Damiani et al. 2013). As this work was aimed to find an economically sustainable cultivation protocol to obtain lipid-rich algal biomass ~~useful~~ for bioenergetics purposes,~~In order to evaluate if a previous mixotrophic cultivation with SLF LFS induced interesting morpho-physiological alterations,~~ 14 days-grown cells were transferred in tap water and special attention was focussed on lipid accumulation. During the first two weeks of starvation, cells~~,~~ previously grown in Bristol GR$^+$ and in 1:2 ~~SLF~~ LFS~~,~~ still slightly continued to grow, benefiting from the carbonates dissolved in fresh tap water~~. Indeed, several microalgae, *Scenedesmus* included, can assimilate carbonates~~ (~~Raven 1968;~~ El-Sheekh et al. 2013; Giovanardi et al. 2013). Additionally, in ~~SLF~~ LFS starved samples, ~~the degradation of~~ the polyphosphate granules, accumulated during the mixotrophic cultivation, might also be ~~used~~ degraded for the cell growth (Markou & Georgakakis 2011). ~~About F_V/F_M ratio,~~ ~~a~~ A decrease was observed in ~~the~~ F_V/F_M ~~ratio~~ throughout starvation in all samples, due to the incapability of cells to maintain a normal photosynthetic efficiency (Berges et al. 1996; ~~Young & Beardall 2003;~~ White et al. 2011; ~~Baldisserotto et al. 2014~~).

The most interesting information on starved-samples was given by morphological observations. Cells previously grown in Bristol GR$^+$ showed enlarged cell volumes, whereas thickening of the cell wall, lipid and starch accumulation were observed independently of the previous cultivation. ~~starved cells of *S. acutus* PVUW12 CCALA-935 showed increased cell volumes,.~~ These characteristics have often been observed in several Chlorophyta (van Donk ~~& Lürling~~ et al. 1997; ~~Dragone et al. 2011;~~ Baldisserotto et al. 2014), *Scenedesmus* included (Lürling 2003), grown under nutrient-limiting conditions. Increased cell volume and alteration of the cell wall have been attributed to a strategy for limiting cell growth under stress conditions (van Donk ~~& Lürling~~ et al. 1997; Lürling 2003; Baldisserotto et al. 2014), whereas starch and lipids were regarded ~~linked to~~ as a consequence of the excess ~~of~~ reducing power produced during the photoassimilation of the organic carbon under ~~nitrogen~~ N-depletion (~~Dragone et al. 2011;~~ ~~Li et al. 2011;~~ White et al. 2011; Baldisserotto et al. 2014). The extracellular polysaccharides production has also been associated with other N-depleted Chlorophyta, for the same reason described above (Yang et al., 2010). However, in our case the presence of polysaccharides around cells was observed only in samples previously cultivated in 1:2 LFS, thus they might as well represent the remnants of the mother cell wall in daughter cells at a late phase of autosporulation (Somogyi et al., 2011), associated with the previous high mixotrophic growth rate.

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This work provides new ~~interesting~~ information on the capability of *S. acutus* PVUW12 ~~CCALA 935~~ to be cultivated in a two-stage process for obtaining high quantities of cells enriched in valuable molecules ~~to be exploited~~ for different biotechnological applications. ~~Moreover,~~ During the first stage, the use of a by-product obtained from the dairy industry could provide all the nutrients required for the growth of the microalga, ~~without using expensive growth media and~~ simultaneously providing added-value to ~~valorising~~ an environmentally problematic product. The second stage (starvation) could be subsequently applied to enhance the lipid globules production. Further investigation on lipid profile and quantification will be planned for more direct bioenergetics perspectives.

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3 1 Supplementary materials contain information about scotta treatment, LFS and tap water
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1 **Figure captions**

2 Figure 1. (a) Growth kinetics of *S. acutus* PVUW12 ~~CCALA-935~~ in Bristol GR$^+$ (filled circles), tap
3 water (filled squares), tap water added with ~~SLF~~ LFS at increasing dilution of 1:2 (empty squares),
4 1:5 (empty circles), 1:10 (empty triangles) and 1:20 (empty ~~rhombs~~ diamonds). ~~Values are means ±~~
5 ~~s.d. ($N \geq 3$).~~ (b) Organic nutrient concentration in 1:2 ~~SLF~~ LFS medium during the first 14 days of
6 growth of *S. acutus* PVUW12 ~~CCALA-935~~. Black: proteins; light grey: lactic acid; white: citric acid;
7 stripes: lactose. ~~Data are means ± s.d. ($N \geq 3$).~~ (c) Growth kinetics of *S. acutus* PVUW12 ~~CCALA-935~~
8 in Bristol GR$^+$ (filled circles), tap water (empty circles) and 1:2 ~~SLF~~ LFS (empty squares) for 14 days
9 and subsequent 28-days-long starvation. Dashed line indicates the beginning of starvation. Y scales
10 in (a) and (c) are logarithmic. Data are means ± s.d. ($N \geq 3$).

11 Figure 2. (a-d) Cell morphology of *S. acutus* PVUW12 ~~CCALA-935~~ grown in Bristol GR$^+$ at the
12 inoculum time. (a-c) light microscopy images ~~showing (a) single cells and aggregates, (b) a~~
13 ~~characteristic tetrade and (c) an oblong cell with a big chloroplast and the pyrenoid embedded in~~
14 ~~the plastid (arrow);~~. (d) TEM micrograph. ~~showing the chloroplast (black arrow), the pyrenoid with~~
15 ~~a starch-shell inside the plastid (white arrow).~~ (e) Cell morphology of *S. acutus* PVUW12 ~~CCALA-~~
16 ~~935~~ grown in tap water after 14 days. ~~and~~ (f) Corresponding TEM micrograph. (g) Cell morphology
17 of *S. acutus* PVUW12 grown in ~~tap water with~~ 1:2 ~~SLF~~ LFS after 14 days. ~~and~~ (h-i) Corresponding
18 TEM micrographs~~ a) and c) light microscopy images; b, d-e)~~. White arrows: pyrenoid ~~embedded in~~
19 ~~the plastid~~; black arrows: chloroplast ~~cell wall~~; d: detachment of the cytoplasm from the cell wall;
20 black arrowhead: sporocyst; white arrowheads: cytoplasmic lipid globules; double arrowheads:
21 cell wall; asterisks: poliphosphate granules; s: starch granules.

22 Figure ~~6~~ 3. Cell morphology of starved *S. acutus* PVUW12 ~~CCALA-935~~ after 28 days of cultivation
23 on tap water. (a) light microscopy and (b, c) TEM micrographs of cells previously grown in Bristol
24 GR$^+$. (d) light microscopy and (e, f) TEM micrographs of cells mantained in tap water. (g) light
25 microscopy and (h, j) TEM micrographs of cells previously grown in 1:2 ~~SLF~~ LFS. White arrows:
26 pyrenoid embedded in the plastid; black arrowheads: cytoplasmic lipid globules; s: starch
27 granules; il: internal layer; el: external layer of the cell wall; ~~Black arrows: thickenings of the cell~~
28 ~~wall;; white arrowhead~~ double arrowheads: polysaccharide materials.

29 Figure ~~7~~ 4. Timecourse of F_V/F_M ~~ratio~~ of *S. acutus* PVUW12 ~~CCALA-935~~ grown in Bristol GR$^+$ (filled
30 circles), tap water (empty circles) and 1:2 ~~SLF~~ LFS (empty squares) for 14 days and under

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~~subsequent~~ starvation on tap water. Dashed line indicates the beginning of starvation. Data are averages ± s.d. ($N \geq 3$).

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