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Microbial protein production from lactose-rich effluents through food-grade mixed cultures: Effect of carbon to nitrogen ratio and dilution rate

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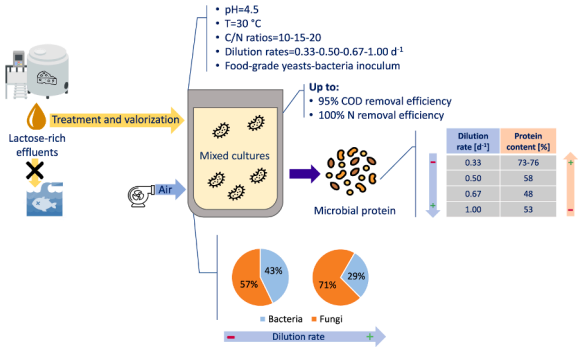
HIGHLIGHTS

- Microbial protein (MP) production from synthetic cheese whey permeate was evaluated.
- The dilution rate (D) mostly influenced the overall process than the C/N ratio.
- Biomass productivities of up to 15.00 g TSS•L^{-1}•d^{-1} were reached at a D of 0.67 d^{-1}.
- The MP biomass protein content (max 76%, min 48%) was negatively correlated with D.
- A mixed culture dominated by food-fermenting yeasts and bacteria was established.

GRAPHICAL ABSTRACT



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ABSTRACT

Overabundant agro-industrial side streams such as lactose-rich effluents from dairy activities offer multiple valorisation opportunities. In the present study, a food-grade mixed culture of bacteria and yeasts was tested under different operational conditions for the treatment and the valorisation of cheese whey permeate (CWP), the residue of whey protein recovery, into microbial protein (MP). Under continuous aerobic fermentation settings, the carbon-to-nitrogen (C/N) ratio showed little to no influence on the system performances and MP quality as compared to dilution rates (D), leading to a final protein content as high as 76%. Under high D values, instead, while biomass productivity increased, N-efficiency and protein content decreased. Unlike the bacterial community, the yeast one proved to be highly stable and less influenced by the increase of D. A preliminary estimate indicated that 2–11% of the future MP-based food production could be satisfied by only valorising lactose-rich dairy residues such as CWP.

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1. Introduction

Global population is projected to reach 9 billion people by 2050 and to further exceed 10 billion by 2100 (Verstraete et al., 2016). The increasing anthropogenic pressure will pose the challenge of a constantly growing food production, particularly by ensuring a high-quality protein supply to the population (Areniello et al., 2023). As a matter of fact, by 2050 about one-third of the global protein consumption is expected to be satisfied through products of animal origin (Godber and Wall, 2014), resulting in a 102 and 78% increase in meat and protein demand (Areniello et al., 2023; Reihani and Khosravi-Darani, 2019), respectively. In light of this, not only the production potential but also the overall resource efficiency of the current food chain requires urgent and major improvements.

Among the possible solutions, the valorisation of overabundant waste and residues through the production of protein-rich microbial biomass from yeasts, microalgae, fungi and bacteria, also known as microbial protein (MP), stands out as one of the most promising solutions (Matassa et al., 2016a; Reihani and Khosravi-Darani, 2019). The main advantages offered by MP are linked to their high protein content (up to 70% by weight), high volumetric biomass productivities (up to 4 $kg{\cdot}m^{-3}{\cdot}h^{-1}$) (Matassa et al., 2016a), rapid doubling times (from 30 min to 6 h), low consumption of primary resources and upcycling of waste and residues (Bajpai, 2017).

The sustainable and circular protein production potential of MP could be leveraged by strategically important agrifood sectors, such as that of the dairy industry. Indeed, the production of 1 kg of cheese generates up to 9 kg of cheese whey (CW), a yellowish liquid that is formed by coagulation of the milk after the curd phase, and consisting mainly of water (~93%), lactose (5%), proteins (1%), lactic acid (<1%), fats and minerals (Dubois Frigon, 2020). The high chemical (COD) (60–80 $g{\cdot}L^{-1}$) and biochemical oxygen demand (BOD_5) (30–50 $g{\cdot}L^{-1}$) of CW, which exceed by several orders of magnitude the typical values of urban sewage (Dubois Frigon, 2020), entail considerable management costs and its direct discharge into water bodies or public sewage systems is no longer allowed.

Within this framework, a mounting research effort is being devoted to CW valorisation. For instance, CW could be directly subjected to biological or physic-chemical treatments aimed at the production of biofuels such as methane (Papirio et al., 2020), biobased chemicals such as acetic acid (Moscariello et al., 2022), lactic acid (Mediboyina et al., 2022), butanol (Kallarakkal et al., 2021) and methanol (Reihani and Khosravi-Darani, 2019), or it could be treated to recover valuable components such as soluble whey proteins. In the latter case, the processing of CW through thermal/*iso*-electric precipitation or through coagulant/flocculant precipitation combined with membrane filtration (*i.e.,* ultrafiltration), allows to recover whey protein along with cheese whey permeate (CWP) (Yadav et al., 2015). CWP is mainly composed of lactose (4–8%), a relevant amount of residual salts (0.5%) and traces of urea, free amino acids and creatine, whose removal to purify the lactose fraction requires the implementation of additional, expensive and energy-demanding steps such as nanofiltration (Pires et al., 2021).

Some microorganisms, including yeasts and lactobacilli, are capable of directly metabolizing lactose-rich dairy residues such as CWP to produce MP (Yadav et al., 2015). Using CWP for MP production could thus enable the co-production, along with the recovered whey protein, of an alternative high protein source also containing vitamins, lipids, carbohydrates, nucleic acid and minerals (Raziq, 2020). The upcycling of CW and CWP into MP has been previously investigated mainly by implementing single yeast species such as those belonging to the genera *Kluyveromyces* (*e.g., K. lactis* and *K. marxianus*), *Candida* (*e.g., Candida crusei*) and *Trichosporon* (*Trichoderma harzianum*) (Dubois Frigon, 2020; Raziq, 2020). Although largely established in conventional industrial settings, monoculture fermentation faces several issues linked to the accumulation of metabolic byproducts such as organic solvents (*e.g.,* ethanol), aldehydes and esters in the fermentation broth, leading to a decrease in biomass yield on lactose and low soluble COD (sCOD) removal efficiencies (Yadav et al., 2015). Thanks to a more diverse set of potential metabolic interactions, the use of mixed culture fermentation processes could contribute to solve these issues (Areniello et al., 2023). In this context, food fermentation offers a broad and yet untapped source of food-grade microorganisms, especially in the domain of fermented dairy products (*e.g.,* kefir), which could facilitate both regulatory and consumer acceptance of the final MP product (Raziq, 2020).

In addition to the type and composition of the microbial culture employed, other MP production variables that influence not only biomass yield and sCOD removal, but also biomass quality (*i.e.,* protein, carbohydrates and lipid content) and productivities, are the carbon to nitrogen (C/N) ratio, the dilution (D) and the organic loading (OLR) rates. The latter process variables play thus a major role in the economic feasibility of the overall process. To the best of the authors' knowledge, a systematic investigation of the influence of these parameters on MP production through food-grade mixed cultures from lactose-rich substrates such as CWP is not yet available.

In light of the above, the present study investigated the use of a synthetic lactose-based substrate simulating a real CWP (later briefly named "synthetic CWP") as a substrate for the production of MP through a mixed culture of yeasts and bacteria, conventionally used for the production of lactose-free milk derivatives such as kefir drinks and yogurts. The experimental work involved the production of MP in a continuously stirred tank reactor (CSTR) fed with a synthetic CWP. The influence of different C/N ratios and D values, along with OLRs, were evaluated with respect to key process performance indicators such as biomass yield and productivities, sCOD removal and nitrogen assimilation efficiencies, as well as biomass quality for the evaluation of the final MP product. Furthermore, the effect of different D and OLR values on the structure of the acclimated mixed culture of yeasts and bacteria, which could in turn influence process performances as well as the final MP quality (*e.g.,* protein content), was evaluated by means of microbial community analyses.

2. Materials and methods

2.1. Composition of the synthetic cheese whey permeate

A synthetic solution of lactose and other macro- and micro-nutrients, adapted from Dubois Frigon (2020), was used to simulate an organic substrate for MP production based on CWP (see supplementary information). Different C/N ratios were simulated by varying the concentration of the nitrogen source (NH_4Cl), while keeping the lactose concentration constant to obtain C/N molar ratios equal to 10, 15 and 20. To avoid foaming, a volume of 1 mL of antifoaming agent (Arauner defoamer, Germany) was added to each liter of synthetic CWP solution. Once prepared, the synthetic CWP was pasteurized at 70 °C for 30 min before being fed to the bioreactor. The synthetic CWP solution was characterized by a pH value of about 4.3 and by a sCOD content of 40.75 $g{\cdot}L^{-1}$.

2.2. Source and activation of the microbial inoculum

A lyophilized mixed culture of $>1{\cdot}10^9$ $CFU{\cdot}g^{-1}$ of yeasts and bacteria (*Lactobacillales* and *Saccharomycetales)*, commercially available for the production of kefir drinks (Genesis Laboratories, Bulgaria) was used as source of microorganisms. Prior to its use for the aerobic fermentation process, the inoculum was activated under batch conditions by dosing 2.4 g of the lyophilized culture in a 1 L Schott glass bottle filled with 500 mL of pasteurized synthetic CWP. The bottle was kept at a constant temperature of 30 °C through a thermostatic water bath and stirred at 600 rpm through a magnetic stirrer. The aeration was provided by a stainless steel air sparger connected to a 520S peristaltic pump (Watson Marlow, United Kingdom). The activation period lasted 14 days, during which 50 mL of liquid culture were sampled and exchanged daily with

fresh synthetic CWP.

2.3. Experimental setup of continuous MP production

The experimental set up used for continuous MP production consisted of a 2 L CSTR, controlled by a Biostat B Plus (Sartorius, Germany) and operated between 1.0 (periods V, VI, VII) and 1.5 (periods I, II, III, IV) L of working volume. The CSTR was initially seeded with 0.5 L of the activated microbial inoculum. Dissolved oxygen levels above 2 mg·L^{-1} were guaranteed by regulating aeration (1–4 L·min^{-1}) and mechanical stirring (600–800 rpm) in the system. The process was maintained at a constant temperature of 30 °C, while pH was automatically controlled at 4.5±0.1 by the addition of 1 M of NaOH or HCl solutions. This pH value was chosen as representative of the typical values of acidic whey, as reported in literature (Dubois Frigon, 2020; Muñoz-Páez et al., 2023). The dissolved oxygen and pH were constantly monitored through built-in probes.

2.4. Experimental conditions

The experimental work was divided into seven experimental periods in order to test the effect of varying C/N ratios and D values. The reactor was initially operated with a D of 0.33 d^{-1} (with D equal to the reciprocal of the hydraulic retention time) and a C/N ratio of 15 (days 1–24, period I). Subsequently, the C/N ratio was first decreased to 10 (days 25–53, period II) and then increased to 20 (days 54–80, period III) to evaluate the influence of this parameter at the same D. In the following experimental periods, the C/N ratio was set to 15, while D was gradually increased. Therefore, after a transition period during which the reactor was operated again with a C/N ratio of 15 and with a D of 0.33 d^{-1} (days 81–93, period IV), the influence of increasing dilution rates was studied by testing D values of 0.5 d^{-1} (days 94–121, period V), 0.67 d^{-1} (days 122–130, period VI) and 1.00 d^{-1} (days 131–138, period VII). By keeping a constant influent lactose concentration, the increase of D also resulted in progressively higher OLRs, ranging from 9.06 g sCOD·L^{-1}·d^{-1} for D = 0.33 d^{-1} to 40.75 g sCOD·L^{-1}·d^{-1} for D = 1.00 d^{-1}. A summary of the tested experimental conditions is provided in the supplementary information section (see supplementary information).

During the first experimental period, part of the bioreactor effluent was stored to be used as backup inoculum in case of process failure. The inoculum consisted of 200 mL of effluent concentrated up to 19.1 g TSS•L^{-1} by means of gravity settling and stored at −18 °C until further use.

2.5. Analytical procedures and microbial community analysis

Prior to analyses, all samples were centrifuged at 4000 rpm for 15 min and the liquid fraction was filtered through 0.45 μm polypropylene membranes (VWR, Italy). The sCOD was determined by the closed reflux colorimetric method (APHA, 2007), while the concentration of ammonium nitrogen (N-NH_4^+) was analyzed spectrophotometrically through the indophenol blue method (Aminot et al., 1997). Total (TSS) and volatile suspended solids (VSS) concentrations were analyzed according to Standard Methods (APHA, 2007). The difference between the TSS and VSS values was used to calculate the ash content of the biomass. The macromolecular characterization of the produced MP was performed on the biomass harvested through centrifugation and washed in a 0.9% NaCl saline solution. The carbohydrate content of the MP biomass was determined through the Dubois method (Dubois et al., 1956), while the organic nitrogen content, measured by means of the total Kjeldahl nitrogen (TKN) analysis (APHA, 2007), was used to calculate the protein content by applying a 6.25 conversion factor (Salo-Väänänen and Koivistoinen, 1996). The unaccounted biomass fraction, complementary to the sum of proteins, carbohydrates and ashes, was used to estimate the lipid content of the MP biomass. TSS, VSS, sCOD and N-NH_4^+ analyses were carried out daily, while the macromolecular characterization of the produced MP was performed and averaged on at least half of the samples collected during each experimental period.

2.6. Microbial community analysis

The composition and the evolution of the mixed microbial community was evaluated by means of 16S and ITS2 DNA amplicon sequencing for bacteria and yeasts, respectively. The analyses were performed by the BMR Genomics laboratory (Padova, Italy) on samples collected at the end of period IV and VII. The abundance of yeasts and bacteria was calculated by comparing the total number of their respective operational taxonomic units (OTUs) to the sum of the OTUs detected from both yeast and bacterial community analyses.

2.7. Calculations

The biomass yield coefficient (Y), which represents the amount of biomass produced per unit of substrate consumed (g TSS•g $sCOD^{-1}$), was calculated based on Eq. (1):

$$Y = \left[\frac{V_{IN} \bullet \left(\frac{TSS_i + TSS_{i-1}}{2}\right) + (TSS_i - TSS_{i-1}) \bullet V_{TOT}}{V_{IN} \bullet sCOD^{IN} - \left(sCOD_i^{OUT} - sCOD_{i-1}^{OUT}\right) \bullet V_{TOT}}\right] \left[\frac{g_{TSS}}{g_{sCOD}}\right] \quad (1)$$

where V_{IN} indicates the volume of the influent in the reference time, while V_{TOT} indicates the working volume of the reactor. The subscripts *i* and *i-1* indicate the values of each parameter at the chosen sampling time and at the previous one, respectively.

The N-biomass yield (Y_N), which represents the amount of biomass produced per unit of nitrogen consumed (g TSS•g N^{-1}), was calculated based on Eq. (2):

$$Y_N = \left[\frac{V_{IN} \bullet \left(\frac{TSS_i + TSS_{i-1}}{2}\right) + (TSS_i - TSS_{i-1}) \bullet V_{TOT}}{V_{IN} \bullet N^{IN} - \left(N_i^{OUT} - N_{i-1}^{OUT}\right) \bullet V_{TOT}}\right] \left[\frac{g_{TSS}}{g_N}\right] \quad (2)$$

The biomass productivity (P), which represents the amount of biomass produced in the unit of time per unit of working volume (g TSS•L^{-1}•d^{-1}), was calculated based on Eq. (3):

$$P = \frac{\left[V_{OUT} \bullet \left(\frac{TSS_i + TSS_{i-1}}{2}\right) + (TSS_i - TSS_{i-1}) \bullet V_{TOT}\right]}{V_{TOT}} \left[\frac{g_{TSS}}{L \bullet d}\right] \quad (3)$$

where V_{OUT} indicates the volume of the effluent in the reference time.

The protein productivity (PP), which indicates the amount of protein produced in the unit of time per unit of working volume (g protein•L^{-1}•d^{-1}), was calculated based on Eq. (4):

$$PP = P \bullet \%\,protein \left[\frac{g_{protein}}{L \bullet d}\right] \quad (4)$$

Finally, the sCOD removal and N-assimilation efficiencies were calculated according to Eqs. (5) and (6), respectively:

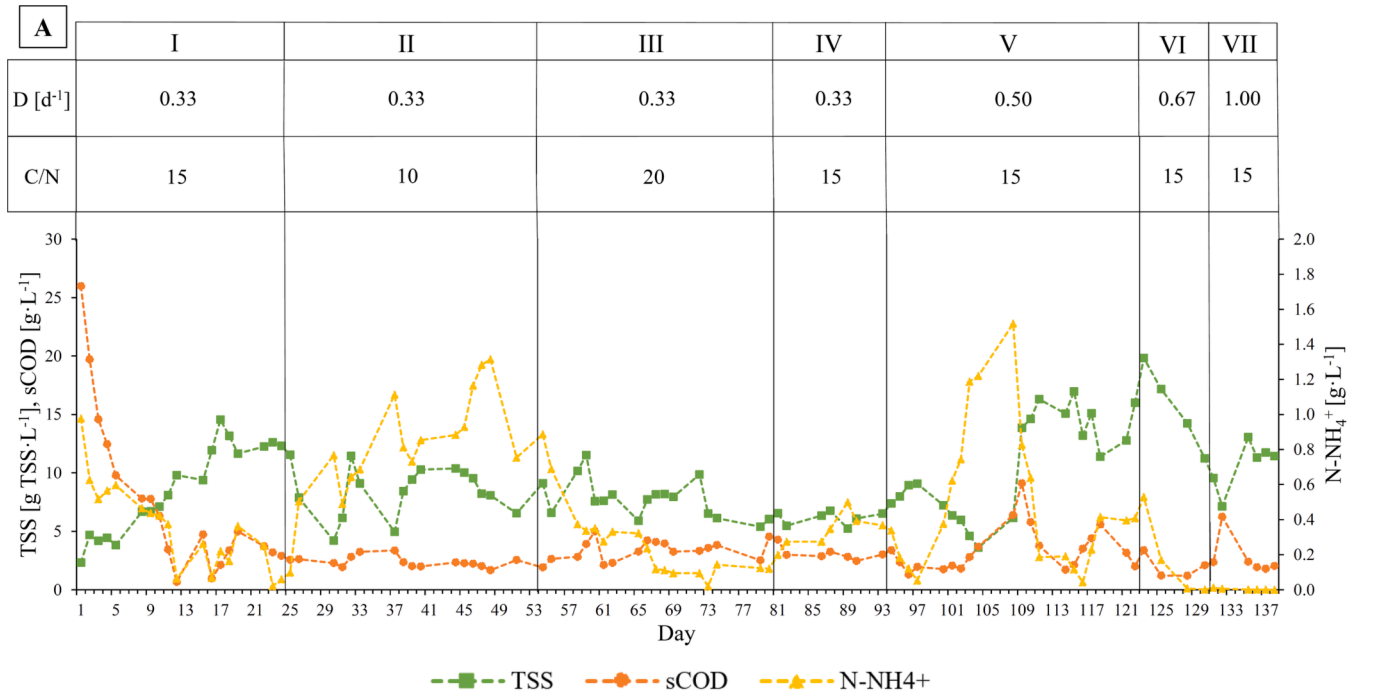
$$sCOD\,removal\,efficiency = \frac{sCOD^{IN} - sCOD^{OUT}}{sCOD^{IN}} \bullet 100 \quad (5)$$

$$N - assimilation\,efficiency = \frac{N - NH_4^{+IN} - N - NH_4^{+OUT}}{N - NH_4^{+IN}} \bullet 100 \quad (6)$$

3. Results and discussion

3.1. Effect of carbon to nitrogen ratio and dilution rate on the aerobic fermentation performances

The time-dependent trends of TSS, sCOD and N-NH_4^+ concentrations observed during the experimentation are shown in panel A of Fig. 1,



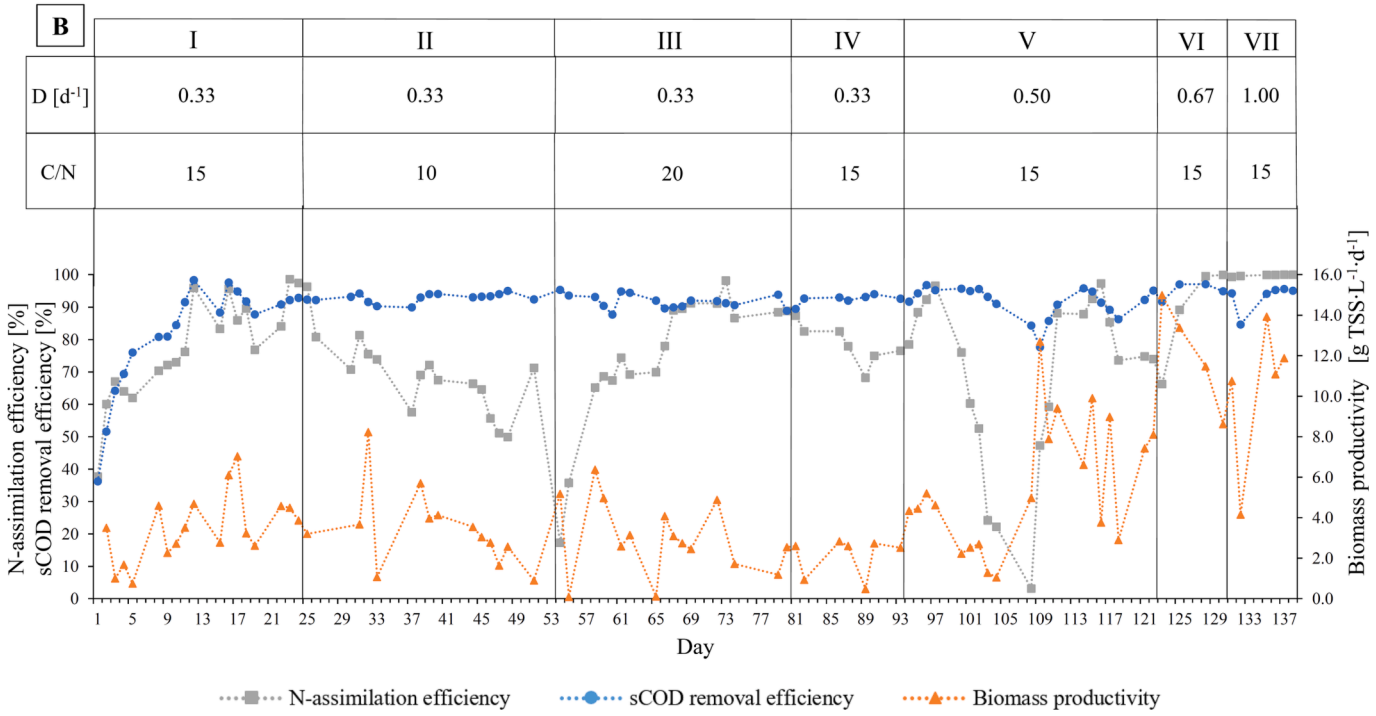


Fig. 1. Performances of the aerobic fermentation process during the different experimental periods in terms of biomass, effluent sCOD and N-NH_4^+ concentrations (Panel A), as well as sCOD removal and N-assimilation efficiencies and biomass productivity (Panel B).

while the values of sCOD removal efficiency, N-assimilation efficiency and biomass productivity are reported in panel B of Fig. 1. Since the first days of reactor operation, the yeast-bacterial culture developed rapidly, increasing its concentration from 2.3 g TSS•L^{-1} on day 1 to 14.6 g TSS•L^{-1} on day 17, and reaching a maximum value of 19.8 g TSS•L^{-1} on day 123. Only during periods V and VII, significant drops of biomass concentrations were observed (Fig. 1, panel A). Therefore, on days 104 and 132 a reinoculation was necessary to facilitate the recovery of the process.

In general, the average biomass concentration was not affected by the C/N ratio, but increased clearly with increasing D, moving from 9.0, 8.5, 8.1 and 6.1 g TSS•L^{-1} with a D of 0.33 d^{-1} in period I, II, III and IV, respectively, up to 14.4, 15.7 and 10.6 g TSS•L^{-1} with D values of 0.50, 0.67 and 1.00 d^{-1} in period V, VI and VII, respectively. The latter can be

explained by the fact that, together with the increasing dilution rates, also the organic loading rates increased during periods V, VI, VII, promoting higher biomass concentrations. The average and maximum biomass concentrations obtained in the present study are generally comparable or higher than those obtained in previous studies using pure cultures. Moeini et al. (2004) studied the production of MP from CW using pure cultures of *Kluyveromyces lactis* and *Kluyveromyces marxianus* and obtained biomass concentrations of 11.8 and 11.5 g TSS•L^{-1}, respectively. Similar values were observed by Carlotti et al. (1991), who reached a maximum biomass concentration value of 14.0 g TSS•L^{-1} by culturing *Candida kefyr* on a medium containing 42.0 g•L^{-1} of lactose, thus similar to the synthetic CWP used in the present study. Hence, the values of biomass concentration here obtained represent a first indicator of the good performances of the employed mixed yeast-bacterial culture grown on CWP, as also observed by Yadav et al. (2014a) who noted a 19% biomass yield increase by 19% when using mixed yeast cultures over mono-culture.

In terms of sCOD removal efficiency, the process showed stable and high performances regardless of the applied D and C/N ratio, with average values constantly above 84%. As shown in Table 1, a high average sCOD removal efficiency, ranging between 84.3±9.5 (period I) and 95.1±1.9% (period VI), was observed across the whole experimentation. These values are slightly higher than those reported in the literature for similar processes applied to real CW treatment. Yadav et al. (2014c) achieved a maximum of 80% sCOD removal by operating a CW fermentation process for 36 h with *Kluyveromyces marxianus*. Matassa et al. (2022), using a similar yeast-bacterial mixed culture on real CW, reached sCOD removal efficiencies ranging from 77 to 89%. The values obtained in this study are also in line with the sCOD removal efficiencies of 85 to 93% obtained by Dubois Frigon (2020), while treating a synthetic CW characterized by different sCOD concentrations (32–65 g·L^{-1}). Clearly, the simpler composition of the synthetic CWP with respect to that of real CW substrates led to an overall more complete degradation of the organic substrate, thus resulting into slightly higher sCOD removal efficiencies. In addition, it is reported that mixed cultures improve sCOD removal from CW due to the capability of yeasts to hydrolyze lactose and make it more bioavailable for the subsequent fermentation by other microorganisms (Areniello et al., 2023; Xia et al., 2021). As an example, Yadav (2014a) obtained a 8.8% higher sCOD removal efficiency moving from mono to mixed cultures.

The average N-assimilation efficiency was constantly above 69% and strongly dependent on D rather than on C/N ratio, reaching almost 100% during period VII. In the presence of a dilution rate of 0.33 d^{-1} (periods I, II, III, IV), the N-assimilation efficiency ranged from a minimum of 73.8±10.6% (C/N = 15) to a maximum of 78.6±5.8% (C/N = 20). When a constant C/N of 15 was applied and D increased from 0.33 to 1.00 d^{-1}, the N-assimilation efficiency, except for singularities due to physiological adaptations of the culture on days 54, 108 and 123, increased progressively by reaching values of 78.5±11.5, 85.8±13.6 and 99.7±0.3% in periods V, VI and VII, respectively. These values are in line with those reported from Dubois Frigon (2020), who achieved N-assimilation efficiencies of 82.6 and 96.6% under D values equal to 1.20 and 0.67 d^{-1}, respectively.

The maximum average biomass productivity achieved with a dilution rate of 0.33 d^{-1} was equal to 3.50±1.62 g TSS•L^{-1}•d^{-1} (period I) (Table 1). This value is quite similar to that of 4.08 g TSS•L^{-1}•d^{-1} obtained by Yadav et al. (2014b) using real CW and a dilution rate of 1 d^{-1}. When higher dilution rates were applied, also the average biomass productivity increased substantially up to a maximum value of 15.00 g TSS•L^{-1}•d^{-1} (day 123) (Fig. 1, panel B) and a maximum averaged value of 11.31 g TSS•L^{-1}•d^{-1} reached in period VI (Table 1). The latter can be explained by the increasing biomass concentrations obtained during periods V, VI, VII promoted by the increased OLR. As already discussed above, such high productivities were also matched by an almost complete N-assimilation efficiency during period VII, highlighting how the process reached optimal conditions for biomass production and N-substrate utilization.

Regarding the N-biomass yield (Table 1), this parameter ranged between 3.7±1.1 (period I) and 7.1±3.8 g TSS•g N^{-1} (period III) when D was equal to 0.33 d^{-1} and C/N to 15 and 20, respectively, reaching up to 9.6±4.5 g TSS•g N^{-1} (period VI) when D was equal to 0.67 d^{-1} and C/N to 15. The low Y_N values observed during the initial experimental periods (I, II and IV) of this study indicate an overall high nitrogen consumption, which was apparently needed to sustain high protein contents when the dilution rate was low (Section 3.2). On the other hand, when D increased, less nitrogen was required to produce the same amount of biomass, as demonstrated by the higher Y_N values constantly observed in periods V, VI and VII, during which the final protein content was generally lower (Section 3.2). When these values are compared with those observed under different experimental settings by other studies, the production of MP from CWP through a mixed culture seems to require higher nitrogen. Van Peteghem et al. (2022), for instance, observed a minimum Y_N of 10.0±0.29 g TSS•g N^{-1} for the bacterium *Corynebacterium glutamicum* and a maximum Y_N of 32.8±0.84 g TSS•g N^{-1} for the yeast *Cyberlindnera saturnus* when cultivating different species of yeasts and bacteria on ethanol under a C/N ratio of 20. Nevertheless, the same authors also observed lower protein contents in the produced microbial biomass, as it will be further discussed in Section 3.2.

Concerning the biomass yield (Table 1), which ranged from a minimum of 0.17±0.06 g TSS•g $sCOD^{-1}$ (period IV) to a maximum of 0.42 ±0.12 g TSS•g $sCOD^{-1}$ (period VI), this parameter showed values comparable with the results obtained in previous studies. Yadav et al. (2014b) achieved a biomass yield of 0.26 g TSS•g $sCOD^{-1}$ by co-cultivating *Kluyveromyces marxianus* and *Candida krusei* on a CW characterized by a sCOD concentration of approximately 30.0 g•L^{-1}. Lukondeh et al. (2005) reached a yield of 0.38 g TSS•g $sCOD^{-1}$ growing *Kluyveromyces marxianus* in a synthetic medium characterized by a lactose content of 40 g•L^{-1}, thus similar to the synthetic CWP used in this study.

Finally, as shown in Table 1, it can be observed that slightly different results were obtained in period I and period IV, although they were operated under the same conditions. In period IV, the sCOD removal efficiency was higher than in period I (92.4 vs. 84.3%) while Y_N

Table 1
Summary of the performances observed during the different experimental periods in terms of sCOD removal efficiency, N-assimilation efficiency, N-biomass yield (Y_N), biomass yield (Y) and biomass productivity (P). The values are reported as the average±standard deviation for each experimental period and were obtained by applying to each data point the equations reported in Section 2.6.

Period	D [d^{-1}]	C/N [mol/mol]	sCOD removal efficiency [%]	N-assimilation efficiency [%]	Y_N $\left[\frac{g_{TSS}}{g_N}\right]$	Y $\left[\frac{g_{TSS}}{g_{COD}}\right]$	P $\left[\frac{g_{TSS}}{L \bullet d}\right]$
I	0.33	15	84.3±9.5	78.4±12.5	3.7±1.1	0.24±0.12	3.50±1.62
II	0.33	10	92.9±1.3	69.0±11.6	5.7±2.2	0.25±0.14	3.42±1.87
III	0.33	20	91.9±2.1	73.8±10.6	7.1±3.8	0.22±0.14	3.04±1.83
IV	0.33	15	92.4±1.3	78.6±5.8	4.2±1.7	0.17±0.06	2.10±0.90
V	0.50	15	89.3±5.2	78.5±11.5	6.8±3.2	0.38±0.16	7.46±2.85
VI	0.67	15	95.1±1.9	85.8±13.6	9.6±4.5	0.42±0.12	11.31±2.66
VII	1.00	15	92.1±4.3	99.7±0.3	7.5±1.5	0.29±0.03	10.36±3.29

increased from 3.7 (period I) to 4.2 g TSS•g N^{-1} (period IV). The latter differences were probably due to a longer exposure of the microorganisms to CWP in the system that allowed a faster and more complete microbial activity.

3.2. Effect of carbon to nitrogen ratio and dilution rate on microbial protein composition

As shown in Fig. 2, protein was the main macromolecular component of the produced biomass, constituting at least half (>48%) of its dry weight. The remaining part of the biomass was made up of carbohydrates and, in smaller quantities, lipids and ashes.

The highest protein contents were observed when the aerobic fermentation process was operated with a D of 0.33 d^{-1} regardless of the C/N ratio. Under these conditions, the produced MP biomass was constantly characterized by an average protein content above 70%. The highest average protein content of 76.3% was observed with a C/N ratio of 15 (period I). Similar values of 73.8 (period II) and 72.9% (period III) were obtained in the presence of a C/N equal to 10 or 20, respectively. The protein content here observed is generally higher than the average values found in other studies. Raziq et al. (2020) report the protein content of *Trichoderma harzianum*, *Kluyveromyces marxianus*, *Candida crusei* and *Kefir species* grown using CW as substrate to be 34, 43, 48 and 54%, respectively. Paraskevopoulou et al. (2003) produced a MP biomass characterized by protein levels of 54%, while performing aerobic fermentation of CW under a D value equal to 2.66 d^{-1} by means of a kefir inoculum, thus with a source of microorganisms similar to that used in the present study.

When the dilution rate was gradually increased across the experimental periods V, VI and VII, the protein content of the MP biomass decreased from 72.9 to 52.7%, leading to the increment of carbohydrates and lipids up of 23.5 and 17.7%, respectively. A similar negative correlation between protein content and growth rate, which under a chemostat reactor configuration is assimilated to D, was also observed by Van Peteghem et al. (2022). Using ethanol as carbon source under a C/N ratio of 5 and growing, separately, different species of yeasts and bacteria, the authors observed a maximum protein content of 63% with *Corynebacterium glutamicum* having a growth rate of 1.2 d^{-1}, while the minimum protein content, equal to 6.9%, was obtained for the yeast *Cyberlindnera saturnus* having a higher growth rate of 4.2 d^{-1}. In this study, the differences in macromolecular composition, as it will be further discussed in Section 3.3, can be possibly explained by the shift in microbial community composition promoted by the increase of D, especially in terms of relative abundances of yeasts and bacteria.

Regarding the other biomass components, the carbohydrate content was rather stable and tended to increase with increasing C/N ratio and dilution rate, averaging 16.5 (period II), 15.4 (period I) and 21.6% (period III) when D was equal to 0.33 d^{-1} and C/N to 10, 15 and 20, respectively, and reaching up to 23.5% with D equal to 0.67 d^{-1} (period VI). The carbohydrate content obtained in this study using a yeast-bacterial culture is lower than the 33.6% reported by Yadav et al. (2014b), who used *Kluyveromyces marxianus* and *Candida krusei* for the aerobic fermentation of CW. Finally, the estimated lipid content was very low when D = 0.33 d^{-1}, as demonstrated by the values of 0.9 (period I), 1.6 (period II) and 2.5% (period III) observed in the presence of C/N ratios of 15, 10 and 20, respectively. These lipid values are lower than the typical ones of 8–10% reported in literature for bacteria (Ravindra, 2000). However, lipid content tended to increase with higher D, reaching values of 13.2 (period V), 17.7 (period VI) and 14.0% (period VII) when D was equal to 0.50, 0.67 and 1.00 d^{-1}, respectively. Similarly to the protein content variation, such a high increase in lipids could be related to changes in the microbial community composition (Section 3.3).

The average protein productivities for each period, as calculated according to Eq. (4), are reported in Fig. 3 along with the average biomass productivities and biomass protein content. When D was equal to 0.33 d^{-1} during periods I, II, III, IV, despite the high protein content, the overall protein productivity values were rather low and stable at 2.67, 2.52, 2.22 and 1.30 g protein•L^{-1}•d^{-1}, respectively. This was mainly due to the poor biomass productivities occurred during these experimental periods. When D was increased in periods V, VI, VII, PP values increased as well, reaching 4.35, 5.39, 5.47 g protein•L^{-1}•d^{-1}, respectively. In the latter cases, therefore, although the microbial biomass was characterized by a lower protein content, the higher biomass productivities resulted in increased protein productivities.

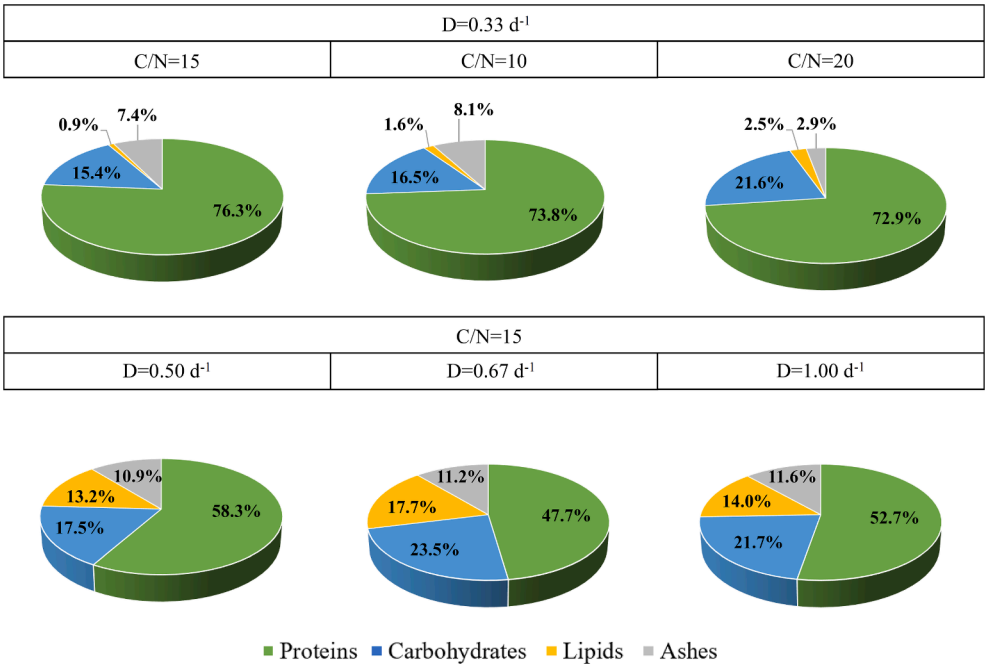


Fig. 2. Overall composition of the microbial protein biomass in terms of proteins, carbohydrates, lipids and ashes under the different operating conditions tested. The values were obtained by averaging the results of the analyses performed on at least half of the samples collected within each experimental period.

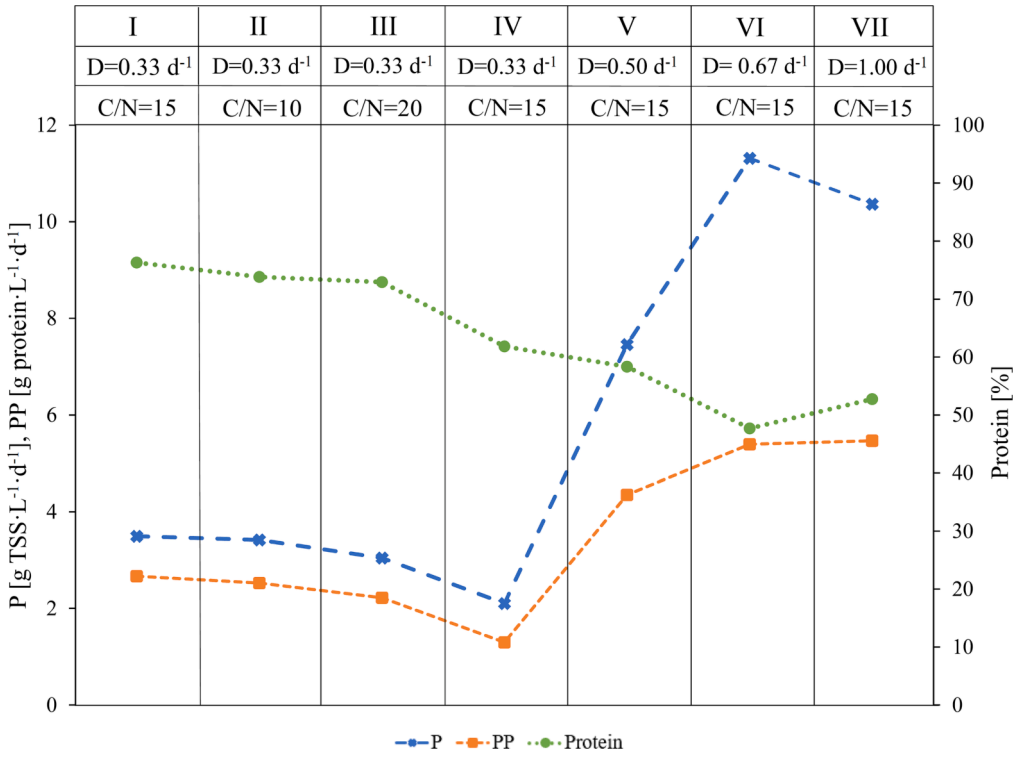


Fig. 3. Average protein productivity (PP) along the seven experimental periods compared to the average biomass productivity (P) and protein content (Protein) of the biomass.

3.3. Effect of dilution rate on microbial community composition and MP quality

Among the tested experimental variables, the dilution rate is known to exert the highest selective pressure on the composition and the stability of mixed microbial cultures, especially under chemostat configurations (Matassa et al., 2016b). Therefore, microbiological analyses were carried out on samples collected at the end of the experimental periods characterized by the same D of 0.33 d^{-1} (end of period IV) (Fig. 4A) and at the end of period VII, when D was 1.00 d^{-1} (Fig. 4B). At the end of period IV, after >90 days of continuous reactor operation at a dilution rate of 0.33 d^{-1}, the microbial community composition appeared rather balanced between yeasts (57%) and bacteria (43%). In terms of relative abundance, the main yeast genera were *Cutaneotrichosporon* (76%), two species belonging to the order of *Saccharomycetales*, namely *Issatchenkia* (9%) and *Lodderomyces* (6%), and *Trichoderma* (6%), while bacteria were mainly composed by the genera *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (65%), *Bifidobacterium* (14%) and *Lactobacillus* (3%).

Conversely, after having gradually increased D up to 1.00 d^{-1} at the end of the experimentation (period VII), yeasts constituted 71% of the microbial population, while the absolute abundance of bacteria decreased to 29%. Moreover, while the yeast species showed a relatively high stability, with 85 and 81% of the yeast community composed by *Cutaneotrichosporon* and *Issatchenkia* in periods IV and VII, the bacterial community showed a greater variability at the end of period VII, with a decreased relative abundance of the species belonging to the genus *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (30%) and the presence of *Enterobacteriaceae* (29%), TM7a (16%), *Microbacterium* (7%), *Luteibacter* (5%) and *Raoultella* (3%).

As already anticipated in Section 3.2, these results could allow to better explain the variation in macromolecular composition of the produced MP. As a matter of fact, being characterized by a lower protein content than bacteria (Bajpai, 2017), the greater abundance of yeasts (71%) at the end of the experimentation could be linked to the lower protein content of the MP biomass produced in periods V, VI and VII with respect to that of periods I, II and III. Moreover, species of the genus *Cutaneotrichosporon*, which largely dominated the yeast population throughout the experimentation, have been identified as fast-growing yeasts capable of producing large amounts of lipids from various substrates including whey and whey-related products, as well as typical components of kefir grains used for milk fermentation(Arastehfar et al., 2021; Bracharz et al., 2017). Additional studies conducted by Moon et al. (1978) also reported that *Cutaneotrichosporon* grows well on cheese whey permeate and tends to accumulate more lipids (20–45%) than proteins (13–15%). Therefore, probably thanks to the high abundance of *Cutaneotrichosporon* and the onset of nitrogen-limiting conditions in periods VI and VII, the MP biomass produced under higher D values (V, VI and VII) was characterized by a higher content of lipids, ranging between 13.2 and 17.7% (see Section 3.2).

Among the most abundant bacterial genera, representatives of the *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* genus have been mostly characterized and studied as nitrogen-fixing bacteria (Farda et al., 2022; Pang et al., 2022), having a protein content higher than 55% (Ritala et al., 2017). The presence of these species has also been often reported in traditional fermented vegetables such as Laotan Suancai (Xiong et al., 2023) and radish paocai (Mi et al., 2022).

In light of the above, the process studied allowed for the acclimatization and stable development of microorganisms that typically constitute the microflora of fermented food and beverage products such as vegetables and kefir. Indeed, in addition to the above mentioned *Cutaneotrichosporon,* previous studies on the microbial diversity of kefir grains reported also the presence of *Saccharomyces*, *Issatchenkia* and *Trichosporon* spp., as well as that of *Enterobacter* and *Lactobacillus* spp., which were also detected in the mixed microbial culture used in this study (Dertli and Çon, 2017).

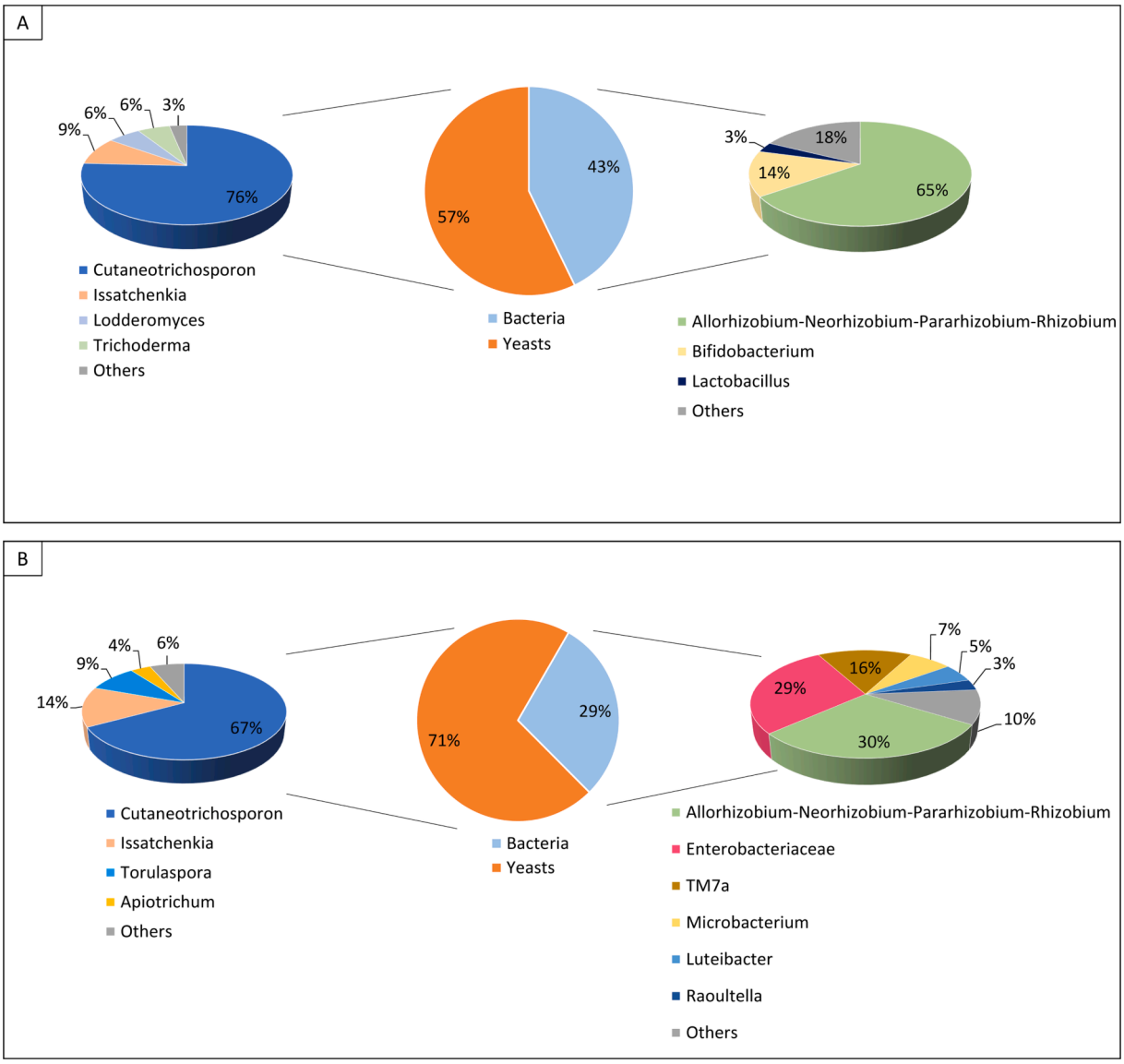


Fig. 4. Microbial community composition at the end of period IV (panel A) and period VII (panel B). In each panel, the central chart shows the absolute abundance between bacteria and yeasts, while the side charts report the relative abundance within yeast and bacterial communities. Operational taxonomic units (OTUs) with relative abundances below 3% were classified as "others".

3.4. Preliminary estimation of the production potential of MP from lactose-rich residues

Over the past decades, Europe, North and South America and Oceania accounted for most of the global cheese production, averaging 19 million tons of cheese per year (Fox et al., 2017). Cheese production results in the generation of about 180 Mtons of CW per year (Xia et al., 2021). Similarly, at the European Union level, the production of 5.5 Mtons of cheese per year (Valta et al., 2017) leads to the annual production of almost 50 Mtons of CW.

By combining the data on CW production with the experimental results obtained in this study, a preliminary estimate of the production potential of MP at European and global level from lactose-rich residues was performed (Table 2). For the analytical calculations, a CW density value equal to 1.03 $kg{\cdot}L^{-1}$ (Fox et al., 2017) was considered, while the sCOD value of 40.75 $g{\cdot}L^{-1}$ used in this study, which is associated with the lactose fraction of CW, was used as a reference, thereby mimicking the use of CWP-like substrates. Since D turned out to be the main parameter affecting the overall process performances, the estimate was performed by considering two scenarios based on different D values. To

Table 2
Estimation of the potential production of microbial protein (MP) at European and global levels from cheese whey based on both literature and experimental data.

	European potential		Global potential	
CW [Mtons]	50		180	
CW [GL (*)]	49		175	
sCOD [$g{\cdot}L^{-1}$]	40.75		40.75	
	D = 0.33 d^{-1}	D >0.33 d^{-1}	D = 0.33 d^{-1}	D >0.33 d^{-1}
sCOD removal efficiency [%]	90.4	92.2	90.4	92.2
Y [g TSS·g $sCOD^{-1}$]	0.22	0.36	0.22	0.36
Protein content [%]	74.3	52.9	74.3	52.9
MP biomass [Mtons]	0.39	0.66	1.42	2.38
Protein [Mtons]	0.29	0.35	1.05	1.26

(*) GL = gigaliter.

this end, two sets of process parameters were obtained by averaging the sCOD removal efficiency, Y and protein content values over the periods with a low D (periods I-IV) and a higher D (periods V-VII).

As shown in Table 2, when a low D is considered, the average process performances would lead to the production of 0.39 Mtons of MP per year, corresponding to 0.29 Mtons of protein per year, at the European level. Conversely, a higher amount of 0.66 Mtons of MP, corresponding to 0.35 Mtons of protein, could be reached annually at European level with higher D values thanks to the higher average process performances. Following the same criteria, between 1.42 and 2.38 Mtons of MP per year, corresponding to 1.05 and 1.26 Mtons of protein per year, respectively, could be produced globally from lactose-rich effluents such as CWP. Considering that the future annual production levels of MP-based food are expected to reach 22 Mtons by 2035 (Areniello et al., 2023), the preliminary estimates presented in this study indicate that between 2 (0.39 Mtons of MP) and 11% (2.38 Mtons of MP) of the global MP-based food market could be covered only by valorising the lactose-rich effluents generated by the dairy industry, thereby increasing its overall sustainability and circularity.

4. Conclusions

A stable and well-performing mixed culture dominated by food-fermenting microorganisms was established during synthetic CWP treatment and valorisation into MP. Process performances were strongly influenced by D rather than the C/N ratio. Under a D higher than 0.33 d^{-1}, a maximum average productivity of up to 11.31 g $TSS \bullet L^{-1} \bullet d^{-1}$, together with sCOD removal efficiencies of up to 95% and an almost complete N-assimilation were achieved. With a D of 0.33 d^{-1}, the highest protein content of 73–76% was observed. Conversely, by increasing D, the abundance of yeasts also increased, thereby reducing the protein (48%) and increasing the lipid content (18%).

CRediT authorship contribution statement

Antonella Scotto di Uccio: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Silvio Matassa:** Project administration, Conceptualization, Supervision, Writing – review & editing. **Alessandra Cesaro:** Conceptualization, Supervision, Writing – review & editing. **Giovanni Esposito:** Supervision, Writing – review & editing. **Stefano Papirio:** Conceptualization, Supervision, 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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2023.129717.

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