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Yeast bioaugmentation fosters medium-chain carboxylates and polyhydroxyalkanoates production from cheese whey

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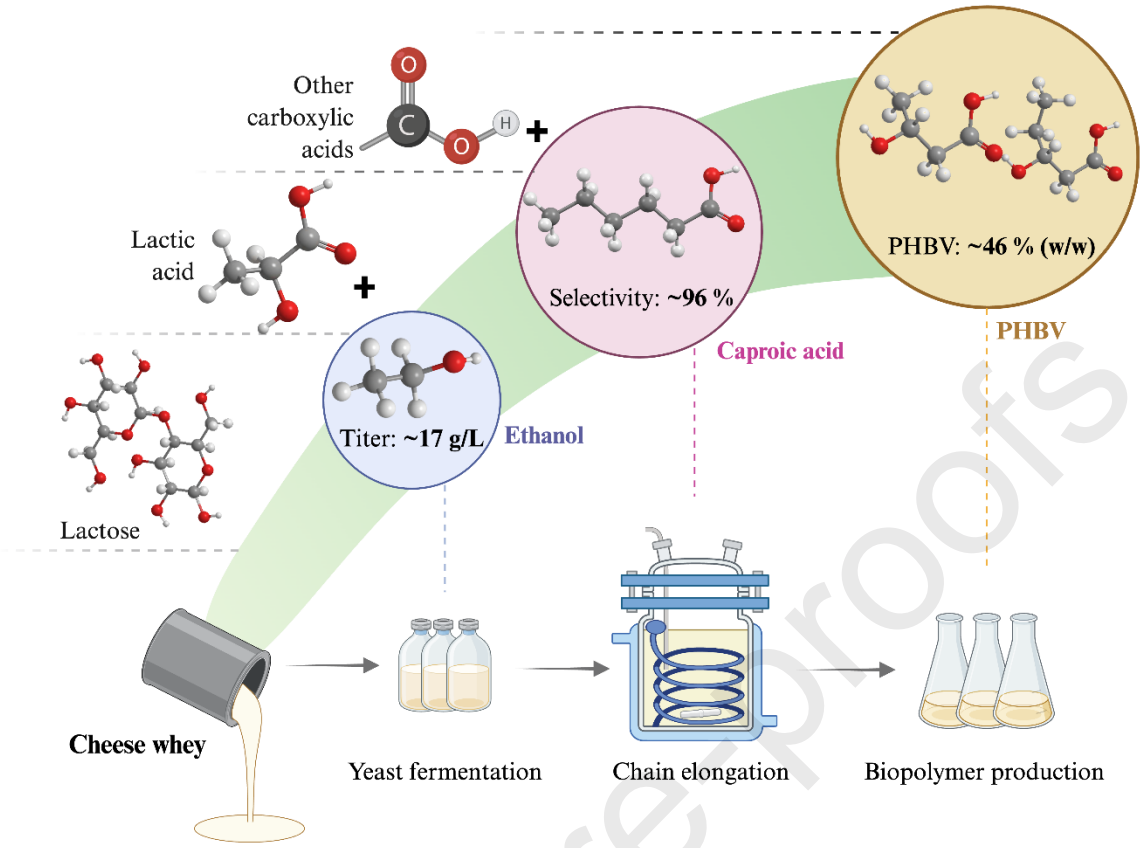

Highlights

- Cheese whey is valorized in a three-step process to produce PHBV bioplastics
- Yeast-assisted fermentation of whey to ethanol favoured chain elongation
- Membrane-assisted fermentation of whey achieved ~96% selectivity for caproic acid
- *Cupriavidus necator* preferentially consumed C4-C6 acids for PHA biosynthesis
- PHV fraction in PHBV was controlled by the odd-chain acid content in the feedstock

Abstract

This study investigated polyhydroxyalkanoates (PHA) production from carboxylic acids obtained by yeast-assisted cheese whey (CW) fermentation. A three-stage hybrid strategy was employed. First, a lactose-fermenting yeast (*Kluyveromyces marxianus*) was inoculated into raw or sterilized CW to produce ethanol and/or lactic acid. Second, these intermediates were converted to carboxylic acids, with an emphasis on caproic acid, using a chain-elongating microbial culture. Batch and fermenter experiments under different operating conditions produced four distinct fermentation products with total carboxylic acid and alcohol concentrations from 4.5 to 14.1 g C/L, and a caproic acid fraction ranging from 17.8 to 72.5%. Finally, synthetic solutions simulating these complex acid mixtures were used as feedstocks for fed-batch PHA synthesis by *Cupriavidus necator*. The process achieved a maximum cell dry weight (CDW) of 6.54 ± 0.17 g/L with a PHA content of 44.21 ± 1.67%. The resulting PHA contained variable proportions of polyhydroxybutyrate (PHB) and polyhydroxyvalerate

(PHV), and high PHV fractions (21.91–27.56% w/w) were specifically linked to the presence of propionate and valerate in the feedstock. Yields of 0.79 ± 0.06 g CDW/g C consumed, and 0.36 ± 0.03 g PHA/g C consumed were achieved. This work investigates a novel proof-of-concept pathway for the valorization of CW into a tailored bioplastic, establishing a clear link between upstream fermentation control and the final material properties of the *poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (*PHBV) copolymer using simulated fermentates.

Keywords

Caproic acid; Chain elongation; Cheese whey; *Cupriavidus necator*; *Kluyveromyces marxianus*; Polyhydroxyalkanoates; Waste valorization.

1. Introduction

For over a century, the chemical industry has relied on fossil feedstocks to produce the fuels, chemicals, and materials essential for modern society. This makes it one of the most energy and carbon-intensive industrial sectors, contributing to around 13% of direct industry-based greenhouse gas (GHG) emissions (Yao et al., 2024). However, the depletion of fossil resources, along with increasing CO_2 emission pricing, is driving the chemical industry to seek sustainable alternative feedstocks (Lange, 2021). Organic waste represents a valuable alternative to fossil fuels, as it is abundant, and produced worldwide. Such organic waste, and particularly its carbohydrate-rich fractions, has significant and largely unexploited potential to produce biochemicals, biofuels, and bioplastics (Kannah et al., 2021).

Cheese whey (CW), a side-product of the dairy industry, possesses specific chemical and biological properties that make it a highly suitable feedstock for producing platform chemicals *via* fermentation (Asunis et al., 2020). CW is the liquid by-product generated after casein and fat are removed from milk by isoelectric or rennet coagulation. On average, 8-10 L of CW per kg of cheese are produced, resulting in a yearly global production of nearly 200 million m^3 (Buchanan et al., 2023). CW composition significantly varies depending on the type of cheese and the method used for casein extraction. On average, it contains 45-50 g/L of lactose and is also rich in nitrogen and in minerals. The microbial fermentation of this nutrient-rich substrate can yield a range of valuable metabolites, including lactic acid (LA), ethanol (EtOH) and various other carboxylic acids (Asunis et al., 2019). Among the potential downstream products, medium-chain carboxylic acids (MCCAs) possess significant biotechnological potential. Their market value is up to five times higher than that of short-chain carboxylic acids (SCCAs) like acetic acid (AA) and butyric acid (BA). Furthermore, due to their low solubility in water, MCCAs are easier to extract from the fermentation broth (Dessì et al., 2024). MCCAs are biologically produced from an electron donor (e.g., EtOH or LA) and an electron acceptor (SCCA) through the reverse *β*-oxidation pathway, in a process known as chain elongation. Microorganisms capable of performing chain elongation include several *Clostridium*, *Megasphaera* and *Caproiciproducens* species (Wang & Yin, 2022). In CW fermentation, chain elongation can be initiated in the presence of SCCAs and LA and/or EtOH, resulting in caproic acid (CA) production (Ordoñez-Frías et al., 2024). While EtOH is the preferred electron donor for CA production in terms of carbon conversion (Wang & Yin, 2022),

spontaneous CW fermentation predominantly results in LA production (Mazorra-Manzano et al., 2022). Therefore, CW inoculation with yeasts capable of fermenting lactose to EtOH, such as *Kluyveromyces marxianus*, emerges as a viable strategy to improve CA production (Tesfaw, 2023).

Both SCCAs and MCCAs are valuable platform molecules for the chemical industry. One of the most promising valorization routes is their further conversion to polyhydroxyalkanoates (PHAs), a family of biodegradable, biocompatible, and renewable thermoplastics (Thamarai et al., 2024; Zeng et al., 2025). *The composition of the carboxylic acid feedstock is critical, as it directly dictates the properties of the PHA polymer. While even-chain acids like butyrate and caproate are precursors to the polyhydroxybutyrate (PHB) monomer, odd-chain acids such as propionate and valerate are essential for producing the polyhydroxyvalerate (PHV) monomer (Zhang et al., 2023). The incorporation of PHV into the polymer chain to form a poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) copolymer is highly desirable, as it reduces crystallinity and improves the material's flexibility (Athanasoulia et al., 2026). Studies on PHA production from CW have primarily focused on mixed microbial cultures (MMCs) (Asunis et al., 2022; Asunis et al., 2025; Otero-Logilde et al., 2025). Although MMCs offer the advantage of non-sterilized operation, they have significant drawbacks, including multi-stage configurations leading to longer production times than pure cultures, and whose variable community structures prevent precise control over the final PHA composition (Asunis et al., 2022; Asunis et al., 2025). In contrast, pure cultures could offer predictable conversion of a defined acid feedstock into a PHA with a specific composition (Hernandez-Herreros et al., 2025). Of the known PHA producers, Cupriavidus necator H16 stands out as a prominent industrial model microbial strain due to its exceptional metabolic versatility, diverse feedstock utilization, and high PHA accumulation capacity (up to 90 % w/w) (Morlino et al., 2023; Nygaard et al., 2025). However, the use of pure cultures on complex SCCA and MCCA mixtures, such as those derived from CW fermentation, remains unexplored.*

To address these gaps, this study develops and evaluates an integrated three-stage hybrid strategy that combines the robustness of mixed-culture fermentation with the precision of pure culture biosynthesis. This approach is designed to explore the conversion of CW into a feedstock for tailored PHBV bioplastics. Specifically, this work aimed to: (i) employ yeast bioaugmentation with *K. marxianus* to create an EtOH-rich feedstock suitable for generating a diverse acid profile; (ii) utilize a mixed-culture chain elongation step to convert this feedstock into distinct acidogenic effluents containing tunable concentrations of odd- and even-chain carboxylic acids; and (iii) evaluate the capacity of *C. necator* H16 to predictably synthesize PHBV copolymers with controlled PHV fractions from these complex, CW-derived acid mixtures, thereby investigating the direct link between upstream process control and final material properties.

2. Materials and methods

2.1 Cheese whey characterization

Raw CW from cow's milk Mozzarella production was obtained from a local dairy in the Naples province, Italy, and used immediately as the fermentation feedstock. The initial lactose concentration was 46.13 ± 1.75 g/L; a complete compositional analysis is provided in the Supplementary Information (**Table S1**).

2.2 Yeast inoculum optimization for ethanol production

To determine the optimal yeast inoculum for EtOH production, batch fermentations were performed in triplicate 120-mL serum bottles with a working volume of 90 mL of CW. The bottles were inoculated with *K. marxianus* NS62, previously isolated from water buffalo Mozzarella cheese (Aponte et al., 2010). A pre-culture was prepared by growing the yeast in YPL broth containing (in g/L): lactose (20), peptone (20), yeast extract (10), and chloramphenicol (0.1). Once the culture reached the exponential phase, the cells were harvested by centrifugation (6000 rpm, 10 min), resuspended in an equal volume of CW, and inoculated at four concentrations of 0% (uninoculated control), 3%, 5%, and 10% (v/v). The serum bottles were incubated at 30 °C under static conditions for 15 days. Samples were periodically collected (every 1-2 days) for monitoring pH, and metabolite concentrations.

2.3 Evaluation of a two-step fermentation for caproic acid production

A two-step batch fermentation was designed to evaluate CA production from CW (**Fig. S1**). The experimental setup included triplicate 120 mL serum bottles (90 mL working volume), prepared under three different conditions: (i) raw CW (uninoculated control), (ii) CW inoculated with 10% (v/v) yeast (Ferm1), and (iii) CW buffered with 100 mM phosphate buffer and inoculated with 10% (v/v) yeast (Ferm2). The initial pH was 5.95 ± 0.05 in the buffered (Ferm2) condition versus 4.95 ± 0.01 in the unbuffered (control and Ferm1) conditions. Fermentations were carried out at 30 °C in a temperature-controlled orbital shaker (SKI 8, Argo Lab, Italy). In the yeast-inoculated trials (Ferm1 and Ferm2), this first step was concluded on day 8 upon the conversion of most of the lactose (79 and 99% for Ferm1 and Ferm2, respectively) to EtOH and LA. In the control bottles, initial lactose consumption was slower, as the process was inhibited by rapid acidification, resulting in only 47% lactose consumption. To overcome this limitation and stimulate fermentation, the pH was adjusted to 5.0 on day 10, leading to complete lactose consumption by day 15.

For the chain elongation step, the pH was initially adjusted to 7.0 in all serum bottles using 3 M NaOH at the point of inoculation. The bottles were sparged with N_2 to create anaerobic conditions and inoculated with a mixed culture of chain elongators (5% *v/v*). The inoculum was obtained from a parent fermenter producing CA from wine lees (Dessì et al., 2024) and was routinely maintained at 30 °C in a medium containing EtOH (10 g/L) and AA (2 g/L). Before inoculation, the culture was harvested by centrifugation (6000 rpm, 10 min) and resuspended in the respective CW fermentation medium. The bottles were regularly sampled for pH and metabolite analysis.

2.4 Bioreactor configurations for caproic acid production

Experiments were performed in 2 L glass fermenters (**Fig. S1**), equipped with temperature and pH control systems. The temperature was controlled at 35 °C with a recirculating water bath (Fisherbrand Isotemp 4100, USA) connected to the fermenter jacket. The pH was controlled at a set point of 6.8 via a control unit consisting of a pH probe (WTW, UK), a relay controller (Hanna Instruments BL931700) and a peristaltic pump (Velp Scientifica

SP311, Italy) that automatically dosed 3 M NaOH. Mixing was provided by a magnetic stirrer and by recirculating the culture broth at 170 mL/min using a peristaltic pump (Masterflex L/S, USA) through an external loop containing the pH probe and the NaOH dosing point.

In the first batch fermentation (Ferm3), the fermenter was filled with a 950 mL working volume of CW fermentate containing approximately 12 g/L LA and 8 g/L EtOH, obtained by inoculating 10% (v/v) *K. marxianus* NS62 into non-sterile CW. After sparging the vessel with N_2, 50 mL (5% v/v) of the chain elongator mixed culture was added. The inoculum was prepared by centrifuging the stock culture (6000 rpm, 10 min) followed by cell pellet resuspension in the same CW fermentate. The reactor was operated in batch mode for 15 days, with samples collected every 1-2 days.

The second experiment (Ferm4) was conducted using a previously developed membrane-assisted fermenter setup (Dessì et al., 2024). Briefly, a silicone tube coil (2 mm internal diameter, 1 mm wall, and 6.66 m length) was installed inside the fermenter in a 3-D printed support. The fermenter was filled with 1140 mL of a mineral medium containing (in g/L): KH_2PO_4 (0.1), NaCl (0.8), NH_4Cl (1.0), $MgCl_2 \cdot 6H_2O$ (0.2), KCl (0.1), $CaCl_2 \cdot 2H_2O$ (0.02), MES buffer (1.95), and Wolfe's trace metal solution (1 mL/L). After N_2 sparging, the reactor was inoculated with 60 mL (5 % *v/v*) of the chain-elongating inoculum, resuspended in the same mineral medium. A separate 800 mL volume of sterile CW fermented by *K. marxianus*, containing EtOH (17 g/L) as the main fermentation product, was continuously recirculated at 21 mL/min from an external bottle through the lumen of the silicone tubing. This allowed for the gradual diffusion of EtOH from the fermentate into the mineral medium, serving as the substrate for chain elongation. The reactor was operated for 21 days, and samples were taken every 1-2 days for pH and metabolite analysis.

2.5 PHA production using simulated cheese whey fermentate

2.5.1 Inoculum preparation for PHA production

The PHA-producing strain *C. necator* H16 was used for these experiments. A stock culture from -80 ˚C was revived on a nutrient agar plate (30 ˚C, 24 h). A single colony was transferred to 20 mL of nutrient broth in a 100 mL flask and incubated at 30 °C and 200 rpm for 12 h (pre-culture 1). Subsequently, 5 mL (10 % *v/v*) of pre-culture 1 was inoculated into 45 mL of mineral medium in a 250 mL flask, which was incubated at 30 °C and 200 rpm for 16 h (pre-culture 2). This pre-culture served as the final inoculum for the PHA biosynthesis experiments. The composition of all media is detailed in the Supplementary Information (**Section S1**).

2.5.2 Fed-batch cultivation for PHA biosynthesis

PHA biosynthesis was evaluated in fed-batch mode using four different simulated fermentates mimicking the carboxylic acid and alcohol concentrations from the serum bottles (SFerm1 and SFerm2) and batch fermenters (SFerm3 and SFerm4). Analytic grade carboxylic acids and ethanol were added to a growth medium containing micro- and macronutrients as

detailed in the Supplementary Information (**Section S1**). The use of simulated fermentates was intended to isolate the metabolic effects of substrate composition on PHA synthesis by *C. necator*, which would otherwise be affected by the complex and variable background matrix of real fermentate. However, a key limitation of this approach is that it does not account for potential matrix effects encountered under real-world conditions. The experimental fermentation conditions and the detailed composition of the simulated media are provided in the Supplementary Information (**Tables S2 and S3**). The trials were conducted in 250 mL flasks with an initial working volume of either 40 mL (for SFerm4) or 70 mL (for SFerm1-3). The initial culture medium contained 10 g/L of LA and was inoculated with 10% v/v of pre-culture 2 to achieve a high cell density (OD_{600} > 9.0). After an initial growth phase of 24 h on LA, a fed-batch strategy was initiated. The simulated fermentates were added in pulses at 24, 36, and 48 h. The 48-h feeding was omitted for the SFerm4 condition to prevent excessive culture dilution. Feeding was ceased for all groups (SFerm1-3) after 48 h to avoid substrate accumulation and potential inhibition, as the residual total acid concentration was ~6 g/L. The detailed feeding scheme is presented in the Supplementary Information (**Table S4**).

2.6 Analytical methods

2.6.1 Analysis of cheese whey and fermentation samples

For initial CW characterization, chemical oxygen demand (total and soluble COD) was measured according to the APHA standard methods and total protein content was determined by the Lowry method. TOC was quantified using a TOC-LCSN analyzer (Shimadzu, Kyoto, Japan) as previously reported (Marino et al., 2025). Total nitrogen (TN) was measured with the same instrument equipped with a chemiluminescence gas analyzer. Anions and cations were analyzed by ion chromatography (930 Compact Ion Chromatograph Flex, Metrohm, Switzerland) equipped with both an anion exchange column (A supp 19-150/4.0) and a cation exchange column (Metrosep C6 250/4.0). The operating temperature was 30 °C and the eluent flow rate was 1 mL/min. Sample pH and conductivity were measured by a pH and conductivity meter (Mettler Toledo, USA). Concentrations of sugars (lactose, glucose, and galactose), carboxylic acids (LA, AA, PA, BA, VA, and CA) and EtOH were determined by a high-performance liquid chromatograph (1260 Infinity III, Agilent, USA) equipped with a Hi-Plex H column (300 × 7.7 mm) at 60 °C and a refractive index detector at 55 °C, using 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min. The calibration range of each compound spanned from 100 to 800 mg/L.

2.6.2 Analysis of PHA production samples

For PHA production samples, EtOH and carboxylic acids were determined by gas chromatography (GC) (7890B, Agilent Technologies, USA) equipped with a flame ionization detector and a TG-WaxMS column (Thermo Fisher Scientific, USA). Before injection, samples were acidified by adding 97% formic acid (10 % v/v). LA concentration was measured separately by HPLC with an Aminex HPX-87H column (#1250140, Bio-Rad Laboratories, Inc., USA) and a RID. Calibration curves for all acids and ethanol were linear in the range of 10–2000 mg/L (R^2 > 0.995). Ammonium nitrogen (NH_4^+-N) was quantified using a flow injection analysis system (Lachat QuikChem 8500 Series 2, HACH Company, USA). Cell dry weight (CDW) was determined gravimetrically by taking a 10 mL aliquot of the culture broth.

After centrifugation at 5000 rpm for 10 min, the cell pellet was washed once with deionized water and freeze-dried at -70 °C overnight (Sentry 2.0, SP Scientific VirTis, USA).

2.6.3 PHA quantification

Intracellular PHA content was quantified using a modified acid-catalyzed methanolysis method (Comeau et al., 1988). Briefly, ~10 mg of finely crushed dried cells were digested in a solution of 1 mL of chloroform and 2 mL of acidified methanol (3 % v/v H_2SO_4) at 100 °C for 4 h. After cooling, phase separation was induced by adding 1 mL of water and 1 mL of chloroform. An aliquot of the bottom chloroform phase, containing the resulting fatty acid methyl esters, was then filtered with a 0.22-µm PTFE filter and analyzed by GC-FID (7890B, Agilent Technologies, USA) using a TG-WAXMS column (Thermo Scientific, USA) to determine PHA monomer concentration and composition.

2.7 Data analysis and calculations

For the fermentation experiments, production rates (g/L/d) were calculated as the change in concentration over a specific time interval. Lactose conversion efficiency (%) was calculated as the proportion of lactose consumed during each experimental run. Carboxylic acid yields on substrate (g C/g C lactose) were calculated as the total carbon mass of the carboxylic acid products formed, divided by the carbon mass of lactose consumed. Product selectivity (%) was calculated as the carbon mass of the target product divided by the total carbon mass of all measured products.

For the PHA production experiments, the biomass yield on substrate (Y_X, g biomass/g *C*) was defined according to Eq. 1:

$$Y_{X/S} = \frac{x - x_0}{s_{cons}} \qquad \text{Eq. 1}$$

where x and x_0 are the final and initial biomass concentrations (g/L), and s_{cons} is the consumed substrate (g C/L).

The PHA yield on substrate ($Y_{PHA/S}$, g PHA/g C) was calculated as:

$$Y_{PHA/S} = \frac{c - c_0}{S_{cons}} \qquad \text{Eq. 2}$$

where c and c_0 are the final and initial PHA concentrations (g/L), and s_{cons} is the consumed substrate (g C/L).

One-way ANOVA was used to assess statistical significance among the experimental groups, where *p*-values < 0.05 were considered significant.

3. Results and discussion

3.1 Two-step caproic acid production from cheese whey in serum bottles

The strategy for CA production from CW involved two steps: (i) an initial lactose fermentation by indigenous bacteria and inoculated yeasts to a mixture of EtOH and LA, and (ii) a secondary fermentation for the conversion of these precursors to CA by a mixed culture containing chain-elongating microorganisms. Without yeast inoculation, spontaneous whey fermentation mainly produces LA (Asunis et al., 2019). A key aspect of the strategy adopted in this study was the promotion of EtOH production through the inoculation of *K. marxianus*. This approach offers several advantages compared to the direct production of CA from whey. Firstly, EtOH is a more efficient and selective electron donor for CA synthesis than LA (Wang & Yin, 2022). Furthermore, in open culture systems, a proportion of LA is often converted to propionic acid (PA) via the acrylate pathway (Akedo et al., 1983), resulting in product diversification. Another important advantage arises from the ability of *K. marxianus* to compete with lactic acid bacteria (LAB) naturally occurring in CW (Christensen et al., 2011) which could reduce process time and improve substrate conversion. Moreover, since LA does not diffuse through the silicone membrane (Dessì et al., 2020), EtOH is the only suitable electron donor for membrane-based fermentation.

A preliminary experiment was conducted to determine the most suitable *K. marxianus* inoculum level (0, 3, 5, or 10% v/v), to promote EtOH and LA production for subsequent chain elongation (Arhin et al., 2025; Wu et al., 2022). In all cases, lactose fermentation started immediately after inoculation. In the control (no yeast added), lactose was initially consumed at a rate of 10.8 g/L/d, but after only one day the metabolism decreased due to rapid acidification, with the pH dropping below 4 (**Fig. S2** in the Supplementary Information). Conversely, in the yeast-inoculated trials, a sustained lactose consumption rate of 12-13 g/L/d was observed for the first two days. This suggests that the acid-tolerant *K. marxianus* strain can thrive and effectively produce EtOH from CW at a pH below 4 (Christensen et al., 2011). The EtOH concentration increased proportionally with the yeast inoculum (3, 5, and 10%), particularly in the early days. The highest EtOH production rate of 3.6 g/L/d was achieved with the highest yeast inoculum (10%). Interestingly, EtOH was also produced in the control from day 6 onwards, suggesting the contribution of indigenous yeasts/bacteria occurring in the non-sterile CW (Martini et al., 2021). LA was produced at similar rates in the range of 3.3-3.8 g/L/d on day 1, regardless of the yeast inoculum, suggesting that LAB and *K. marxianus* engaged in exploitative competition, each utilizing a fraction of the available lactose without direct interaction (Sun et al., 2023). AA was produced in all cases from day 6 onwards but did not exceed 2 g/L concentration. The trial with 10% yeast was the only one characterized by an EtOH-LA molar ratio approaching 2 (on days 1-3), while in trials with 5% and 3% yeast it only reached 1.5 and 1.3, respectively. Hence, a 10% yeast inoculum was selected for subsequent experiments.

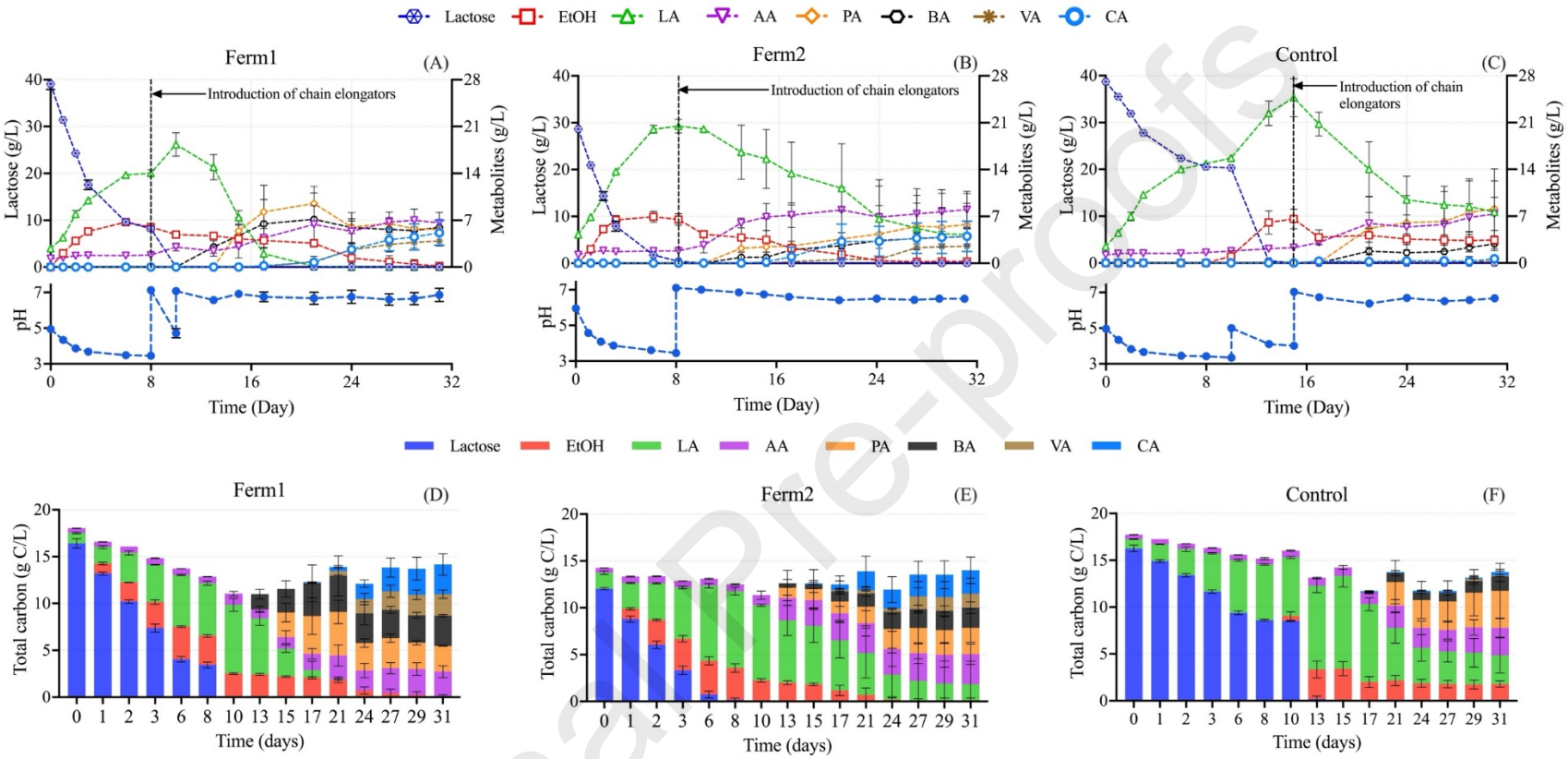

Fig. 1. Time-course profiles of lactose consumption, soluble metabolite concentration, pH, and corresponding carbon content, from batch cheese whey (CW) fermentation in serum bottles. The experiment compares unbuffered CW with 10% yeast (A, D), buffered CW with 10% yeast (B, E), and a control without yeast (C, F). A chain-elongating culture was introduced on day 8 (A, B) or on day 15 (C) after pH adjustment to 7 and sparging with N_2. EtOH: Ethanol; LA: Lactic acid; AA: Acetic acid; PA: Propionic acid; BA: Butyric acid; VA: Valeric acid; and CA: Caproic acid. Error bar = Standard deviation (n = 3).

A second batch experiment was conducted to evaluate CA production from CW under three conditions (**Fig. 1**): (i) Control: raw CW, (ii) Ferm1: CW inoculated with 10% *K. marxianus* (unbuffered), and (iii) Ferm2: buffered CW inoculated with 10% *K. marxianus*. Yeast addition resulted in a substantially faster lactose consumption on days 0-3, 7.4 and 6.5 g/L/d in the unbuffered and buffered trials, respectively, compared to 3.9 g/L/d in the control. In the buffered, yeast-inoculated CW, lactose was 97% depleted by day 8, favoured by the slower pH drop. On the same day, lactose was consumed by 78% and 49% in the unbuffered incubations with and without yeast, respectively, suggesting that both the indigenous LAB and *K. marxianus* were inhibited by pH below 3.5. In both cases, lactose consumption resumed once the pH was manually adjusted, resulting in complete lactose depletion.

EtOH production in the yeast-inoculated trials was not significantly impacted by buffering. Its concentration steadily increased from day 0 and then plateaued at 6.7-6.9 g/L on day 6, before slightly decreasing by day 8. LA was produced along with EtOH in the yeast-inoculated trials and was the only major metabolite detected in the control. LA reached 14.6-15.0 g/L in the unbuffered CW, regardless of yeast inoculation, while its concentration was significantly higher (21.6 g/L) in the buffered CW, likely due to the more gradual pH decrease. After the pH was adjusted to 5 on day 8, EtOH was produced in the control, confirming the presence of indigenous solventogenic bacteria and/or yeasts in the CW. In the yeast-inoculated trials, CA was detected 3-5 days after inoculation with the chain-elongating culture on day 8 (at pH 7). CA production was sustained until EtOH was completely depleted, reaching concentrations of 5.1 ± 1.9 and 4.0 ± 2.3 g/L by day 31 in the unbuffered and buffered trials, respectively. In the control, despite the favorable pH for chain elongation, EtOH was only consumed by about 50%, resulting in lower CA concentrations of 0.6 ± 0.5 g/L. This low CA production was likely due to the high LA concentration (24.7 ± 2.8 g/L), which could potentially exert an inhibitory effect on the chain-elongating microbial community.

On a carbon basis, the unbuffered and buffered yeast-inoculated trials yielded a similar amount of volatile fatty acids (0.79-0.85 g C/g C lactose), with a slightly different CA share (22.6 and 20.5%, respectively). The remaining carbon was detected as LA (in the buffered trials only), along with traces of residual EtOH. The unaccounted carbon fraction was likely released as CO_2, especially during fermentation, or assimilated into microbial biomass. PA and AA, common LA fermentation products under neutral pH, were likely produced by indigenous CW bacteria through the acrylate pathway (Kucek et al., 2016). BA can also be produced from LA, mostly by *Clostridium* sp. (Wu et al., 2023), likely present in the CW or added with the mixed chain elongation inoculum. VA was found at significant concentrations (up to 16% selectivity in the yeast-inoculated trials). Similarly to CA, VA can be produced by reverse *β*-oxidation using EtOH as the reducing equivalent and acetyl-CoA source, and PA as the odd-chain precursor (Candry et al., 2020). Thus, yeast addition is essential to shift fermentation towards EtOH, providing the necessary electron donor for the chain elongation culture to produce not only CA, but also valuable VA, which is a key precursor of PHV. In contrast, phosphate buffering did not significantly affect the product spectrum and was therefore omitted in the following experiments.

3.2 Caproic acid production from cheese whey fermentate in bioreactor

A first fermenter run evaluated CA production from a yeast-assisted CW fermentate, containing 11.7 g/L of LA and 8.3 g/L of EtOH, under controlled pH (> 6.8) (**Fig. 2A, C**). On days 0-3, AA, PA, and BA were detected, suggesting the rapid onset of LA fermentation and

EtOH oxidation pathways. Both EtOH and LA were completely depleted by the end of the experiment. The degradation of additional substrates, such as proteins or fats, likely contributed to fermentation, as the carbon recovered in the final products exceeded that of the main substrates by 16% (**Fig. 2C**). Chain elongation began on day 3. CA was produced at a maximum rate of 2.0 g/L/d on days 8-10 and then plateaued after day 10, when EtOH was completely depleted, reaching a final concentration of 6.7 g/L on day 15. Higher CA concentrations (10.2 g/L) were obtained in a two-step process where expired dairy and beverage waste were first fermented to LA (24-25 g/L), which was continuously fed as a carbon and electron source for chain elongation at an organic loading rate of 19.2 g COD/L/d (Bian et al., 2024). In this study, the CA selectivity reached 39.3% on a carbon basis (**Fig. 2C**), significantly higher than in the serum bottle batches, suggesting that controlled pH is important for efficient CA production. SCCAs (C_2-C_5) were all produced with selectivity ranging from 12.8% to 17.3%. Such selectivity is in line with a previous study in which CW was co-digested with sewage sludge in fed-batch mode at organic loading rates ranging from 0.98 to 1.81 g COD/L/d (Iglesias-Iglesias et al., 2021).

A second fermentation run was carried out to assess CA production from a pre-sterilized, yeast-based CW fermentate, containing EtOH (17.1 g/L) as the main metabolite (92.8% of total carbon) (**Fig. 2B, D**). Since such a high EtOH concentration might inhibit chain elongators (Lonkar et al., 2016), this experiment was conducted in a previously developed silicone membrane-assisted fermenter, which allows for slow EtOH diffusion from the feedstock towards the chain elongators (**Fig. S3**) (Dessì et al., 2024). This approach enabled a CA production selectivity of up to 95.6%, the highest reported to date from CW, although both the production rate (0.7 g/L/d) and final concentration (5.2 g/L) were lower than those obtained in the presence of LA. Similar results were obtained when using EtOH-containing wine lees as feedstock in the membrane-assisted fermenter, where 94% CA selectivity and 1.0 g/L/d production rate were achieved (Dessì et al., 2024). This suggests that membrane-based EtOH delivery can allow for high CA purity. The presence of SCCAs, such as AA and BA, as chain initiators can enhance production rates. In CW systems, these compounds may originate from LA fermentation, though this pathway typically occurs at the expense of product selectivity.

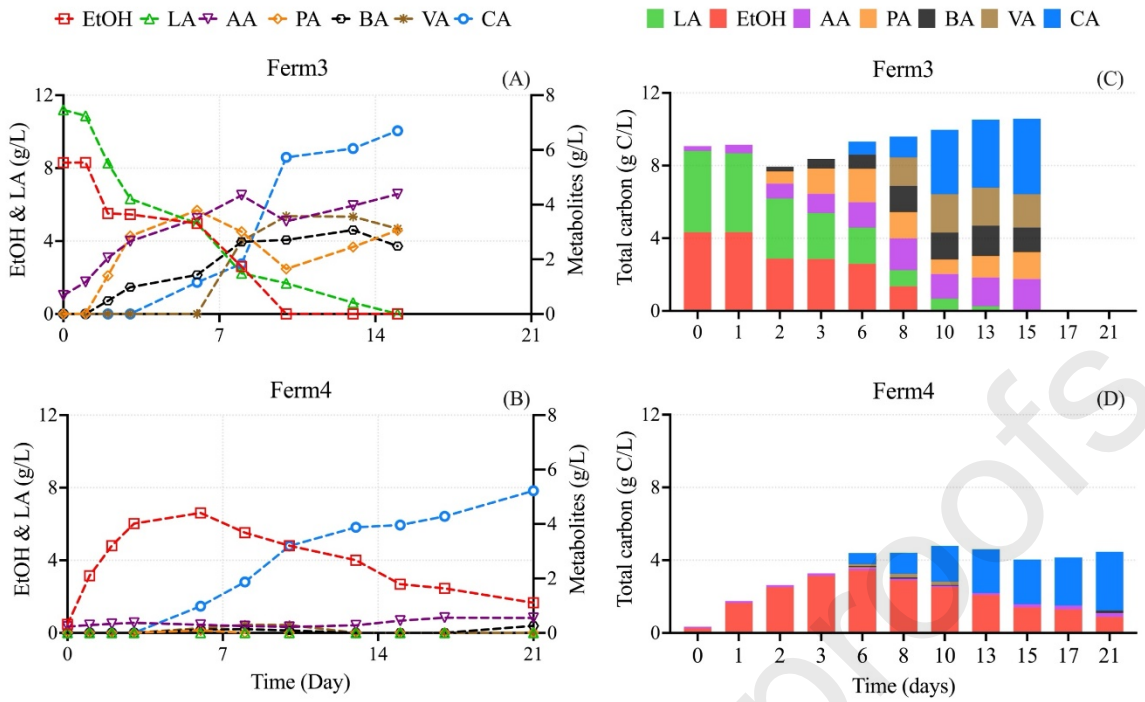

Fig. 2. Carboxylic acids production profiles and corresponding carbon content in two bench-scale fermenter configurations. (A, C) A conventional fermenter (Ferm3) fed with a mix of EtOH and lactic acid. (B, D) A membrane-assisted fermenter (Ferm4) fed with EtOH. EtOH: Ethanol; LA: Lactic acid; AA: Acetic acid; PA: Propionic acid; BA: Butyric acid; VA: Valeric acid; and CA: Caproic acid.

3.3 Evaluation of PHA production from simulated CW fermentate

3.3.1 Substrate consumption profiles on different simulated CW fermentate composition

Four synthetic solutions simulating the CW fermentates were used to evaluate PHA biosynthesis. *C. necator* H16 was first grown on LA for 24 h to achieve high cell density, consuming 7.4-7.5 g/L of LA at a rate of 0.31 g/L/h. After 24 h, the four simulated CW fermentates were added as carbon source for cell growth and PHA accumulation. In the presence of multiple acids, *C. necator* H16 preferentially consumed MCCAs over SCCAs (**Fig. 3**). CA showed the highest consumption rate (46.5-94.6 mg/L/h) in all trials, while SCCAs, such as PA and AA, tended to accumulate and were only consumed once longer-chain acids were depleted (**Fig. 3**). In the presence of other acids, the LA consumption rate was substantially lower than in the first 24 h, where this acid was the only carbon source. The observed substrate utilization ranking was: CA (C6) > BA (C4) > VA (C5) > LA (C3) > PA (C3) > AA (C2) > EtOH (C2). This pattern is consistent with previous findings indicating that *C. necator* H16 preferentially utilizes BA, LA, and PA over AA and EtOH (Hernandez-Herreros et al., 2025; Wen et al., 2025).

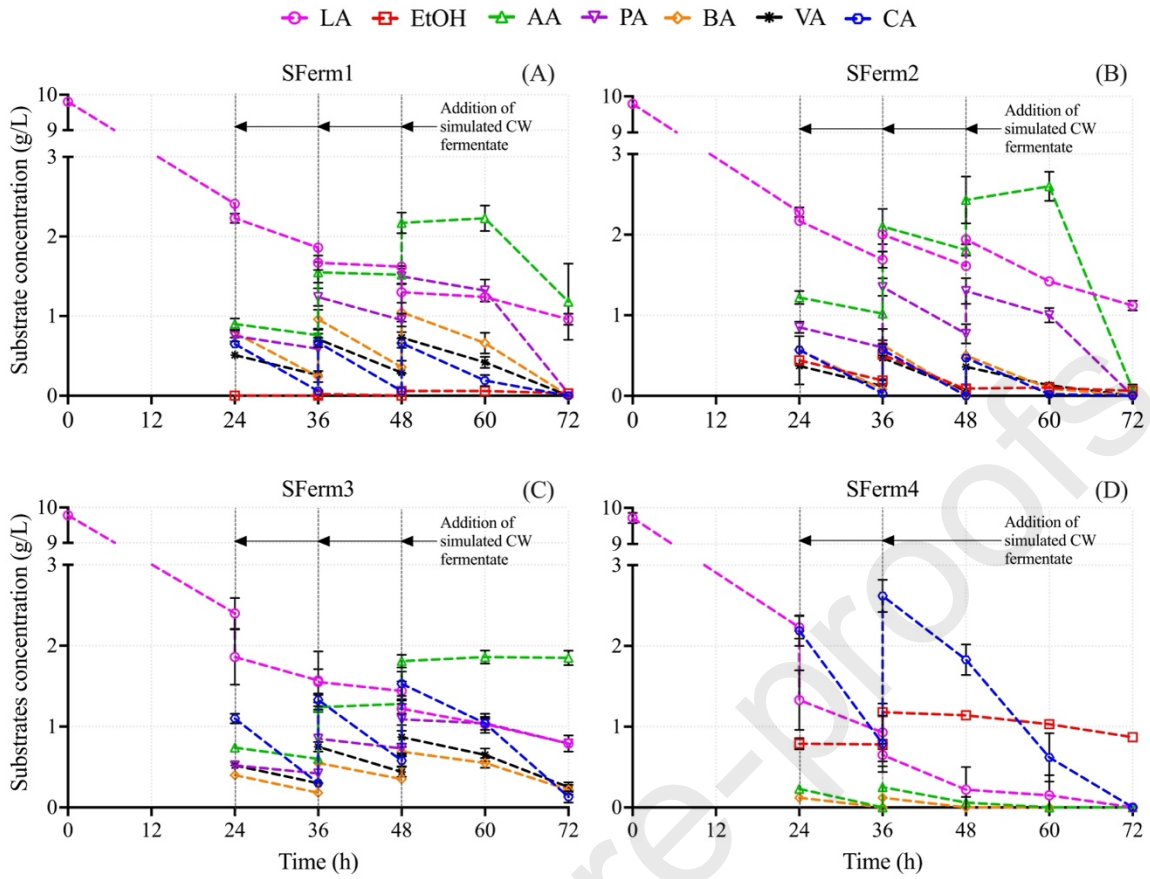

Fig. 3. Time-course of substrate consumption by *C. necator* H16 in flask-fed-batch cultures. In the initial growth phase (0-24 h), lactic acid (LA) was the sole carbon source. Subsequently, simulated cheese whey fermentates were added at 24, 36, and 48 h (indicated by arrows) for groups (A) SFerm1, (B) SFerm2, and (C) SFerm3, and at 24 and 36 h for trial (D) SFerm4. EtOH: Ethanol; AA: Acetic acid; PA: Propionic acid; BA: Butyric acid; VA: Valeric acid; and CA: Caproic acid. Error bar = Standard deviation (n = 3).

3.3.2 Effects of simulated CW fermentate composition on cell growth and PHA production

The effects of different acid mixtures on cell growth and PHA synthesis were investigated. The time courses of CDW, PHA, and NH_4^+-N concentrations in each experimental group are shown in **Fig. 4,** while key results on biomass and PHA yield and composition are summarized in **Table 1**. SFerm2 exhibited the highest CDW of 6.54 ± 0.17 g/L. The biomass yield ($Y_{X/S}$) was highest in SFerm1 (0.79 ± 0.06 g biomass/g C consumed), containing high concentrations of VA, BA, and PA, which are preferred substrates for *C. necator* H16 (Hernandez-Herreros et al., 2025). This preference is metabolically driven, as longer-chain acids can be efficiently channelled into the β-oxidation pathway to generate more acetyl-CoA and propionyl-CoA with fewer enzymatic steps compared to shorter-chain acids (Zhang et al., 2023). Conversely, SFerm4 exhibited the lowest biomass yield (0.45 ± 0.04 g biomass/g C consumed), likely because of its limited organic acid diversity, with CA as the main substrate (**Fig. 3**). PHA concentration and yield scaled proportionally with CDW under comparable PHA content (**Fig. 4**). The highest PHA concentration was obtained in the SFerm2 trials (2.89 ± 0.13 g/L), followed by SFerm1 (2.87 ± 0.03 g/L), SFerm3 (1.86 ± 0.12 g/L), and

SFerm4 (0.98 ± 0.22 g/L). The maximum PHA content obtained in this study was 46% of CDW, achieved despite constraints such as limited aeration and high ammonia levels. This outcome is comparable to findings from studies on PHA production using CW-derived feedstocks (**Table 1**). PHA contents of 71% have been obtained by *C. necator* from CW-derived VFAs concentrated by electrodialysis, although it should be noted that such an approach significantly increases the operational costs (Domingos et al., 2018).

Table 1. Comparison between the present study and other research employing CW-originated mixed carboxylic acid as a substrate for PHA production. Values represent the mean ± standard deviation (n = 3).

Substrate	**Pretreatment**	**Inoculum**	**CDW (g/L)**	**PHA content (% of CDW)**	**PHA type**	**PHV fraction (% w/w)**	**$Y_{PHA/S}$**	**Reference**
CW-SFerm1	Acidogenic fermentation	*C. necator* H16	6.26 ± 0.16	45.94 ± 0.90	PHBV	27.56 ± 1.80	0.36 ± 0.03 g_{PHA}/g_C (0.21 ± 0.01 $gCOD_{PHA}/gCOD_{acids}$)	This study
CW-SFerm2			6.54 ± 0.17	44.21 ± 1.67	PHBV	21.97 ± 0.33	0.33 ± 0.02 g_{PHA}/g_C (0.19 ± 0.01 $gCOD_{PHA}/gCOD_{acids}$)	
CW-SFerm3			4.44 ± 0.09	41.75 ± 2.37	PHBV	21.91 ± 1.21	0.29 ± 0.02 g_{PHA}/g_C (0.16 ± 0.02 $gCOD_{PHA}/gCOD_{acids}$)	
CW-SFerm4			2.74 ± 0.29	35.30 ± 4.57	PHBV	0.47 ± 0.05	0.16 ± 0.03 g_{PHA}/g_C (0.08 ± 0.02 $gCOD_{PHA}/gCOD_{acids}$)	

CW	Acidogenic fermentation	MMCs	-	54.05	PHBV	15	0.40 $gCOD_{PHA}/gCOD_{acids}$	Valentino et al. (2015)
CW	Acidogenic fermentation	MMCs	2.7	65.9	PHB	0	0.24 $gCOD_{PHA}/gCOD_{acids}$	Colombo et al. (2016)
CW	Acidogenic fermentation	MMCs	2.98	53	PHBV	31	0.26 $mmolC_{PHA}/gCOD_{acids}$ (0.0096 $gCOD_{PHA}/gCOD_{acids}$*)	Otero-Logilde et al. (2025)
CW	Acidogenic fermentation	MMCs	1.39	37	PHBV	46	0.55 gC_{PHA}/gC (0.53 $gCOD_{PHA}/gCOD_{acids}$)*	Asunis et al. (2025)
CW	Acidogenic fermentation and electrodialysis	*C. necator* DSM545	14.96	71	PHBV	6	0.6 g_{PHA}/g_{acids} (0.53 $gCOD_{PHA}/gCOD_{acids}$)*	Domingos et al. (2018)

MMCs: Mixed microbial cultures; CDW: Cell dry weight; PHA: Polyhydroxyalkanoates; PHBV: Polyhydroxybutyrate-co-valerate; PHB: Polyhydroxybutyrate; $Y_{PHA/S}$: PHA yield per substrate consumed. *calculated from the reported values.

PHA composition is a crucial parameter that determines its properties and potential applications. The fractions of PHB and PHV produced in the different trials are shown in **Fig. 4**. The PHB fraction reached 99.53 ± 0.05 % (w/w) in the SFerm4 trial where EtOH and CA were the major substrates, indicating that these substrates exclusively induce PHB production. In contrast, both PHB and PHV monomers were detected in the SFerm1, SFerm2, and SFerm3 trials, where PA and VA were present. Both acids can be converted to 3-hydroxyvaleryl-CoA, the precursor of PHV monomers (Yin et al., 2023). A higher VA utilization was correlated with an increased PHV fraction. In the SFerm1 trial, the consumption of 1.40 g/L of VA alongside 1.92 g/L of PA, resulting in the highest PHV fraction (27.56 % w/w). In SFerm2, a higher PA consumption (2.13 g/L) but lower VA utilization (1.02 g/L) yielded a significantly lower PHV fraction (21.97 % w/w). While VA is directly converted to valeryl-CoA and subsequently to the 3HV precursor (3-hydroxyvaleryl-CoA), PA undergoes condensation via the acetyl-CoA and propionyl-CoA pathway, which depends on a second molecule (acetyl-CoA) (**Fig. 5**), reducing 3HV conversion efficiency (Khanna & Srivastava, 2007).

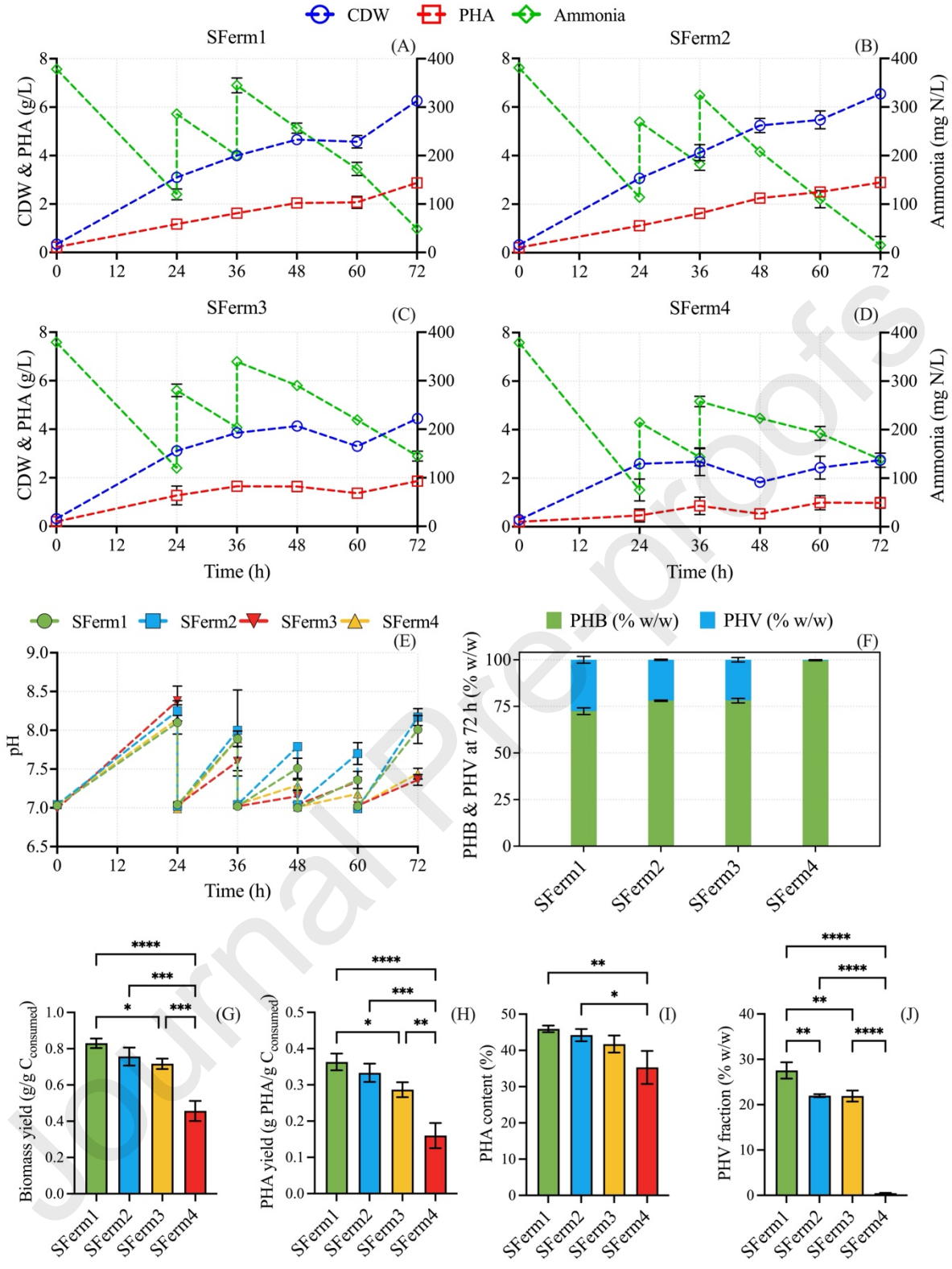

Fig. 4. Characterization of PHA biosynthesis by *C. necator* H16 using four simulated cheese whey fermentates (SFerm1-4). (A-E) Time-course profiles: (A-D) Cell dry weight (CDW), PHA concentration, and ammonium-nitrogen levels for each experimental group and (E) culture pH variation. (F-J) Endpoint analysis at 72 h: (F) Composition of the accumulated polymer, highlighting the polyhydroxyvalerate (PHV) fraction in relation to polyhydroxybutyrate (PHB) and (G-J) summary and statistical comparison of biomass yield,

PHA yield, PHA content, and PHV fraction at 72 h. Error bar = Standard deviation (n = 3). Asterisks represent significance at p-values ≤ 0.05 (*), ≤ 0.01 (**), ≤ 0.001 (***), and ≤ 0.0001 (****).

NH_4^+-N is an essential element for cell growth. To achieve high cell density before PHA accumulation, NH_4^+-N was supplemented at 0, 24, and 36 h to ensure sufficient nitrogen availability. After 36 h, NH_4^+-N addition was omitted to induce nitrogen-limited conditions for PHA accumulation. The NH_4^+-N profiles in each group are shown in **Fig. 4**. The SFerm1 and SFerm2 trials achieved higher CDW and exhibited higher NH_4^+-N consumption (641 mg N/L and 662 mg N/L, respectively). In contrast, the SFerm3 and SFerm4 trials showed lower consumption (495 mg N/L and 531 mg N/L, respectively), and higher residual nitrogen (139-145 mg N/L). This resulted in a lower PHA content (**Table 1**), as PHA accumulation occurs under stress conditions such as limited nitrogen availability (Troncoso-Castellanos et al., 2026). The feeding strategy resulted in a mixed growth and accumulation phase rather than a distinct two-stage process. The pH variation is shown in **Fig. 4**. The increase in culture pH was directly proportional to carboxylic acid consumption; the larger pH shifts observed in the SFerm1 and SFerm2 trials indicated greater overall acid utilization, consistent with previous studies (Huschner et al., 2015; Schwarz et al., 2018). This demonstrates that, despite performing best at neutral pH, *C. necator* can grow and accumulate PHA under alkaline conditions (Hernandez-Herreros et al., 2025).

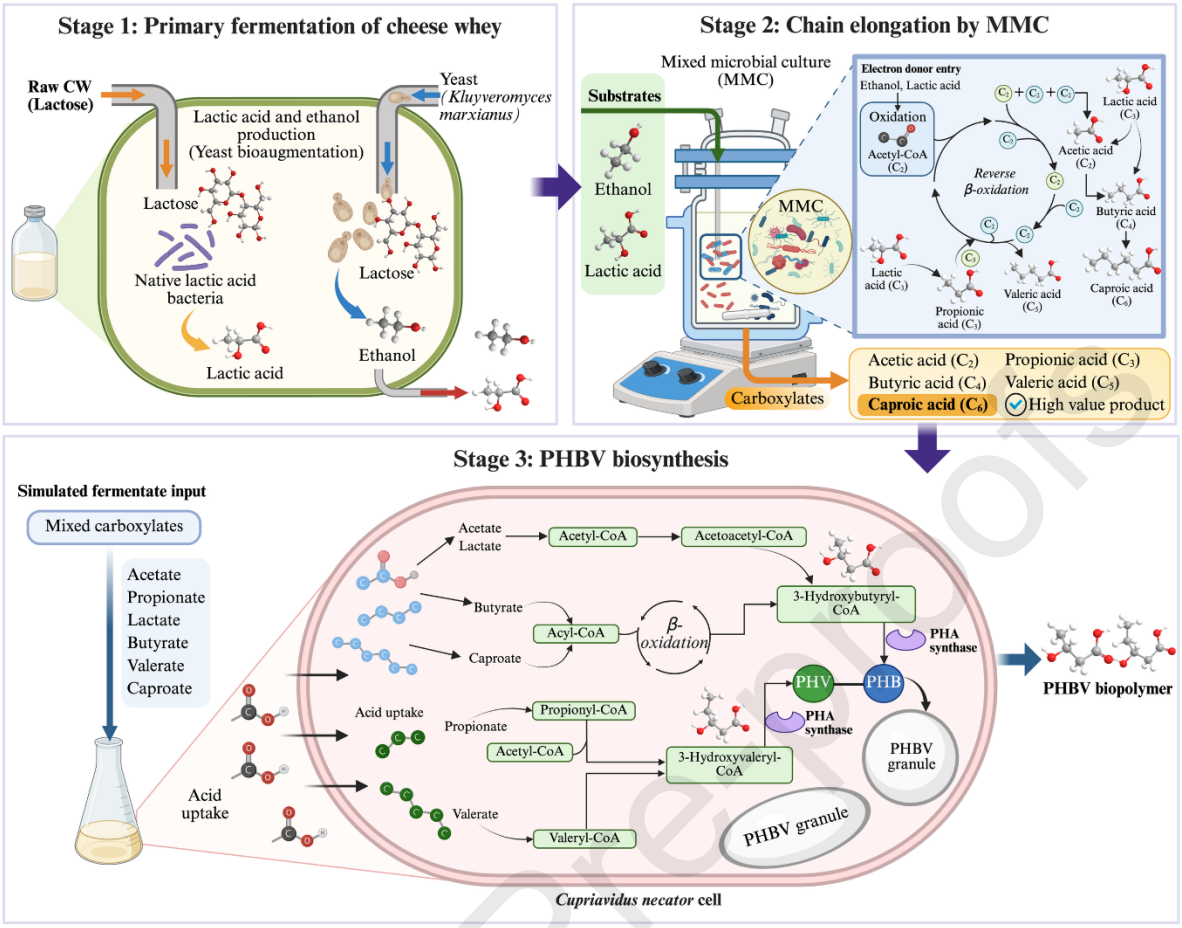

Fig. 5. Conceptual pathway for the three-stage conversion of cheese whey to poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), encompassing initial fermentation, chain elongation, and final polymerization. (Created with BioRender: Luo, L. (2026) *https://BioRender.com/drajv6j*).

3.4. Practical implications

CW is a promising and abundant feedstock for biotechnological applications such as MCCAs and PHA production. This study showed that the fermenter type (traditional *vs.* membrane-assisted) and operational parameters (yeast inoculation, CW pretreatment, and pH, among others) define the product spectrum attainable by CW fermentation. The CW fermentate composition, in turn, affects biomass growth as well as PHA yield and composition. The results suggest that the intermediate chain elongation step can act as a crucial control point. The ability to generate fermentates with predictable concentrations of odd-carbon acids (PA, VA) offers a potential route for influencing the PHV content in the final PHBV polymer. This strategy represents a step towards addressing the key bottleneck of inconsistent product quality and could help enable the production of tailor-made bioplastics suitable for an array of industrial applications. Furthermore, the highly selective CA produced in this study serves as a valuable intermediate, suitable not only for PHA production but also for applications such as biofuels, lubricants, fragrances, and environmentally friendly pesticides.

The proposed concept is illustrated in **Fig. 5**, which details how yeast-derived ethanol from CW drives an intermediate chain elongation step to produce the acid precursors for downstream PHBV synthesis. When considering the highest volatile fatty acid yield (0.85 g C/g C lactose) and the highest PHA yield (0.36 g PHA/g C) obtained in this study, a theoretical production of 5.9 g PHA/L CW can be expected. This is 25% higher than that obtained using sheep CW as substrate and a mixed culture inoculum (Asunis et al., 2025), further highlighting the potential of the hybrid culture strategy proposed in this study. Extrapolating to an industrial scale, a medium-sized cheese factory (e.g., producing 5,000 tons of cheese per year, which generates ~45,000 tonnes of whey) could theoretically produce up to 267 tonnes of PHA annually. This calculation does not account for the significant inefficiencies expected during scale-up and downstream processing. Nevertheless, this estimation highlights the potential scale of valorization, where the output could eventually cover a substantial portion of the company's needs for packaging (roughly 0.02-0.05 g plastic/g cheese). However, further research is needed to validate the PHA production results on real CW fermentate in bench- and pilot-scale trials, assessing the impact of the matrix on process performance. Further work must also address key bottlenecks, such as reducing costs related to biodegradable polymer production and extraction, to facilitate industrial-scale application (Thiele & Riedel, 2025).

4. Conclusions

This study demonstrated a multi-step valorization platform for producing high-value PHBV from CW, integrating (i) yeast-based fermentation, (ii) microbial chain elongation, and (iii) PHBV biosynthesis. Results revealed that *K. marxianus* inoculation of CW directs lactose fermentation towards EtOH production over lactic acid, positively impacting caproate productivity. Membrane-based fermentation enabled selective (95.6%) caproic acid production from an EtOH-containing CW fermentate. *C. necator* H16 preferentially consumed C4-C6 carboxylates (butyric, valeric, and caproic acids) over shorter-chain carboxylates and EtOH for PHA production. The CW-derived carboxylates were effectively converted into PHA, achieving polymer contents of up to 46% (w/w) and a yield of 0.36 g PHA/g C consumed, with a variable PHV fraction depending on the odd-chain carboxylic acid concentration in the feedstock. This integrated approach provides a promising framework for dairy waste stream valorization, establishing a proof-of-concept for a platform to produce high-value, tailor-made bioplastics.

Supplementary data

E-supplementary data for this work can be found in e-version of this paper online.

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