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Single-cell proteins polyhydroxyalkanoates-rich microbial biomass from municipal and winery waste as potential additive for aquafeeds

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ABSTRACT

This study evaluated single-cell protein production from PHA-rich mixed microbial cultures obtained from fermentation and subsequent PHA storage, using urban (namely food waste and municipal sewage sludge; FW-MSS) and agricultural waste (namely wine lees; WL) streams as substrates. FW-MSS fermentation achieved stable short-chain fatty acid (SCFA) production and a high COD_{SCFA}/COD_{SOL} ratio of 0.77 ± 0.01, which allowed to select a mixed microbial culture (MMC) with intracellular PHA content of 15.1 wt%, which aligns with fish dietary standards and yielded a MMC biomass with a protein level of 55.1 wt% and a balanced essential amino acid (EAA) profile. In contrast, WL fermentation showed lower SCFA content and stability, yielding a MMC with 45.8 wt% of protein along with a high non-conformance rate (53.65 %), and 7.2 wt% PHA, making the resulting MMC more suited as a supplemental protein source. Distinct microbial communities developed in the two SBRs due to different feedstocks, influencing the abundance of PHA-storing bacteria, with no known fish pathogens detected in either sample. Statistical analysis confirmed FW-MSS's superior product consistency, supporting its potential as a good quality SCP for aquafeed, especially for rainbow trout, as confirmed by its high essential amino acid index (EAAI).

Introduction

In the context of the circular economy the valorisation of organic waste is a critical priority, as established by the 2030 goals [1]. Among the most pressing environmental challenges is the management of waste streams with high organic carbon content, such as municipal sewage sludge (MSS), food waste (FW), and agricultural waste (for example wine lees; WL). These streams, while abundant and ubiquitous, are typically not valorised, leading to significant environmental impacts. However, they hold great potential as feedstocks to produce bioactive molecules, including polyhydroxyalkanoates (PHA), a biodegradable polymer stored in bacterial cells [2]. PHAs can be produced from short-chain fatty acid (SCFA)-rich streams obtained via waste fermentation. The primary approach for synthesizing PHA through microbial mixed culture (MMC) involves a multi-step process with both anaerobic and aerobic phases. It begins with dark acidogenic fermentation of waste to produce a stream rich in SCFA (direct precursors for PHA synthesis), followed by an initial sequencing batch reactor (SBR) for MMC selection/enrichment and a second fed-batch reactor for PHA accumulation within cellular walls [3–7].

Traditionally, research on PHAs has centred on their application as precursors for bioplastic production. However, a new frontier has emerged, focusing on the potential of PHA-producing microorganisms as single-cell proteins (SCPs), an emerging alternative protein source. Notably rich in proteins, vitamins, and lipids, SCPs hold promise as supplements for both human and animal diets [8]. SCP have the advantage of using little land and resources compared to other food sources, making them efficient and environmentally sustainable [9]. Moreover, this innovation aligns with the growing demands of the aquaculture sector, which, according to the recent FAO report [10], surpassed capture fishing in seafood production in 2024. This livestock production system represents the fastest-growing sector in protein production, and in 2021 aquatic foods contributed at least 20 % of the per capita protein intake from all animal sources for 3.2 billion people [10].

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Thus, the primary challenge in aquaculture lies in providing cost-effective and environmentally sustainable ingredients for feed production with a high protein content, especially for carnivorous fish [11,12].

This research proposes a dual-benefit strategy: transforming waste into a resource while addressing two critical challenges in aquaculture, namely feed sustainability and disease management. PHA-producing SCPs not only offer an eco-friendly alternative to traditional fish feed production but also provide bioactive properties. PHA has been shown to promote growth [13] and enhance the immune system of aquatic animals, offering increased resistance to pathogens [14,15]. This latter property of the SCP microorganism may be useful to address the long-standing challenges associated with aquaculture, particularly the prevalence of diseases caused by viral and bacterial pathogens [16]. Antibiotics, commonly used to tackle such diseases, pose risks of antibiotic resistance and environmental threats [17], whereas vaccines, though effective, suffer from time-consuming development and high costs [18].

In response to these challenges, employing PHA as a natural bioactive compound for aquafeed has raised research attraction [19–21], as PHA degrades into SCFAs, known for their antimicrobial properties [17]. However, the practical application of SCFAs in aquaculture faces challenges due to their water-soluble nature, leading to potential leaching and ineffective delivery [17]. In contrast, PHA, being water-insoluble, presents a more promising solution.

Moreover, while PHAs have been traditionally tested in powder form, recent interest has shifted towards utilizing whole cells in aquaculture feed, minimizing extraction costs and energy consumption [22], while also providing additional proteins deriving from the microbial cell itself [9]. To reduce costs and environmental impact by aligning the circular economy principles, these biopolymers can be obtained from waste, thus contributing to a sustainable and environmentally friendly aquaculture system perspective. In addition, if produced from fermented waste (generally characterized by a wide spectrum of SCFAs), the intracellular PHA can have amorphous characteristics with improved biotic and abiotic degradability compared to the commercial crystalline PHA (usually poly 3-hydroxybutyrate; P-3HB).

The feedstock and fermentation conditions significantly influence PHA composition, nutritional richness, and potential probiotic characteristics. Municipal sewage sludge (MSS) and food waste (FW) have been extensively investigated both individually and in co-fermentations, yielding promising outcomes and realistic opportunities for biorefinery development [23]. Previous studies on sewage sludge have demonstrated the technical feasibility of recovering PHA-rich biomass under high and low oxygen availability [24,25] with similar PHA content (> 50 % w/w). Investigations into FW have revealed the capability of MMC to reach PHA accumulation potential equal to or higher (> 70 % w/w) than MSS fermentation liquids [26,27]. In terms of feedstock conversion into PHA, generally indicated as overall PHA yield, the co-fermentation of MSS and FW has proven to enhance overall waste fermentation performance, attributed to factors such as heightened buffer capacity, balanced macronutrients and micronutrients, dilution of toxic compounds, and the promotion of a more diverse microbial community [28,29]. In prior research, MSS and FW co-fermentation enabled stable SCFA production without the need for external chemical agents for process control, leading to a final overall PHA yield of around 100 g PHA/kg VS [30]. Conversely, winery wastewaters have been less frequently employed in these processes, and the literature provides only a few examples, typically involving pure culture systems, with PHA accumulations ranging from 40 % [31] to 71.3 % w/w [32].

Overall, this research has two main aims: to evaluate the production of PHA-enriched MMC in SBR using urban (a mixture of FW and MSS) and agricultural (wine lees, WL) waste; and to analyze the chemical composition of these MMCs, with a focus on their protein content and amino acid profile, related to a possible use as fish-feed additives.

Materials and methods

Feedstock and process scheme

FW and MSS were sourced weekly from the Treviso municipal wastewater treatment plant (WWTP) in northeast Italy. Combined in equal parts, these streams mimic the influent typically treated at the nearby full-scale anaerobic digestion facility. MSS was obtained from the WWTP's static thickener, while FW came from the organic fraction of source-separated municipal solid waste, collected from over 50 districts within Treviso province. After screw-press squeezing and homogenization pre-treatments, the FW was sent to the full-scale WWTP.

The wine lees (WL) were gathered from a cellar in northeast Italy. The WL are byproducts of wine clarification prior to bottling, commonly carried on using bentonite to promote the aggregation and settling of suspended solids in the raw wine.

In both cases, the feedstocks were collected fresh during the experimental period, stored at 4°C, and used shortly thereafter.

The physical-chemical features of the feedstock mixture (FW-MSS) and WL are listed in Table 1.

As described in previous studies [23], the process involved using a fermentation reactor to produce a SCFAs-rich stream, which was then centrifuged before being fed to the first aerobic reactor (SBR). Here, the PHA storage response was stimulated in the initial activated sludge (inoculum) by applying the so-called feast-famine conditions.

Acidogenic fermentation of urban and agricultural waste

Mesophilic acidogenic fermentation was performed in a 5 L semi-continuous stirred tank reactor (CSTR), mechanically stirred and under temperature control (37°C) using a thermostatic jacket. The hydraulic retention time (HRT) was set at 5 days (equal to sludge retention time, SRT). The pH (5.5) was externally controlled with a peristaltic pump connected to a software designed by Idea Bioprocess Technology Srl. The applied OLR was different among the two feedstocks: 7.6 and 8.8 g VS/(L d) for the FW-MSS mixture and WL, respectively. Before being used as feed for the aerobic PHA line (in both SBR and fed-batch reactor), the fermented SCFA-rich stream was centrifuged (Heraeus Megafuge 40, Swinging Bucket Rotor, maximum radius 195 mm, minimum radius 83 mm; Thermo Fisher Scientific, Waltham, MA, United States) for 15 min at 4700 rpm, to remove the solid fraction from the fermentation broth.

Biomass enrichment in SBR

The MMC was grown in an SBR with a 4 L working volume, initially inoculated with activated sludge from the Treviso WWTP. Two separate SBR runs were performed under identical process conditions, with the only difference being the feedstock used. The overall cycle duration was set to 12 h, with the feed phase lasting 15 min.

No settling phase was performed, so all excess biomass was removed with the mixed liquor within 1 min, right before each cycle ended. As a result, both the SRT and HRT were maintained at 1 day. The reactor's temperature was kept constant at 25°C using a thermostatic jacket, while membrane compressors provided aeration and a mechanical

Table 1
Physical-chemical features of the waste streams.

Parameter	FW-MSS	WL
Total Solids (TS, g/kg)	45 ± 4	67 ± 4
Volatile Solids (VS, g/kg)	38 ± 2	44 ± 2
COD_{SOL} (g/L)	23 ± 3	27 ± 3
COD_{SCFA} (g/L)	3.1 ± 0.2	-
Total Kjeldahl Nitrogen (TKN; g N /kg TS)	28 ± 3	30 ± 9
Phosphorus (P; g P/kg TS)	2.8 ± 0.4	6 ± 1

impeller stirred the mixture at 80 rpm. The dissolved oxygen (DO) was monitored continuously and was kept above 2 mg/L throughout the entire cycle. The organic loading rate (OLR) was maintained at medium-high value, approximately 7.0–7.5 g COD_{SOL}/(L d) by diluting the fermented feedstock with tap water. Control of the cycle length, feeding, withdrawal times, and aeration was managed by software developed by Idea Bioprocess Technology Srls.

To evaluate the SBR performance, biomass concentration (as volatile suspended solids, VSS) and PHA content were measured at the end of both the feast phase and the full cycle. Each SBR run lasted up to 75 days. Biomass was collected daily by harvesting it after mixed liquor withdrawal, followed by centrifugation (10 min at 4700 rpm). The biomass was then stored at −20°C before being air-dried in a commercial food dryer for around 2 days at 45°C. Once dried, the biomass was processed in a mixer to create a homogeneous fine powder, which was then used for nutritional value analysis.

Analytical methods and microbial community analysis

The FW-MSS mixture and WL were characterized weekly after each collection. Analysis was conducted to measure TS, VS, TKN, P, COD_{SOL}, and SCFAs. The same approach was applied to fermentation effluents, though with more frequent sampling (at least twice a week). In the aerobic reactors, parameters such as total and volatile suspended solids (TSS and VSS), COD_{SOL}, SCFAs and PHA were quantified. In the SBR, mixed liquor samples were taken at the end of the cycle for COD_{SOL} and PHA measurements and at the end of the feast phase for TSS, VSS, COD_{SOL}, and PHA analysis. All analyses, except for SCFAs and PHA, were performed according to standard methods [33].

SCFAs were analyzed using an Agilent 6890 N gas chromatograph (GC) equipped with a flame ionization detector (FID) set to 250 °C. A fused silica capillary column, Agilent J&W DB-WAX (60 m length, 0.53 µm ID, 1.0 µm film), served as the stationary phase, with hydrogen (HyGen 200, Claind, hydrogen generator) as the carrier. The inlet was set with a split ratio of 6:1 and ramped from 40 °C (held for 2 min before increasing) to 160 °C at a rate of 22 °C/min, maintaining the final temperature for 15 min. Prior to GC analysis, each sample was centrifuged (10 min, 4700 rpm) and filtered (0.2 µm; Whatman acetate-cellulose filters).

For PHA analysis, 5.0 mL of mixed liquor was treated with 1.0 mL of NaClO solution (5 % active Cl_2) and subsequently analyzed following the method described in Moretto et al. [23]. The PHA was extracted, hydrolyzed, and converted into 3-hydroxyacyl methyl esters, which were then quantified via the GC method. The relative abundances of 3-hydroxybutyric (3HB) and 3-hydroxyvaleric (3HV) monomers were determined using P(3HB-*co*-3HV) Sigma-Aldrich standard polymer (5 wt % 3HV).

Mixed liquor samples were taken in both SBRs at the end of operation (the steady state was fully achieved and maintained) and stored at −20°C for microbial community analysis. The extraction of DNA and 16S rRNA gene high-throughput sequencing from the samples was carried out as an external service (https://www.bmr-genomics.it/).

Calculations

All the parameters characterizing reactors' performances were calculated when the steady state (or pseudo-steady state in SBR) was achieved.

In the acidogenic fermentation process, the steady state was recognized from the stability of SCFA concentration and pH values. Fermentation yield (Y_{SCFA}) was quantified according to the VS level of the unfermented feedstock.

An additional parameter used to assess the acidogenic performance is the COD_{SCFA}/COD_{SOL} ratio, calculated as the SCFAs (in COD units) produced at a given time point divided by the soluble COD measured at the same time point. This ratio provides insight into the extent to which solubilized organic matter is converted into SCFAs.

In the SBR, the achievement of the pseudo-steady state was defined according to the trend of the feast phase length and only when it remained approximately constant (with 5 % deviation from the average) for at least 10 consecutive days [23]. In each SBR cycle, the DO was continuously monitored to identify the SCFAs depletion (end of feast phase), indicated by a sudden increase in the DO concentration. The active biomass (X_A) was calculated as the difference between VSS and PHA concentrations: X_A = VSS − PHA. The biomass PHA content (g PHA/g VSS) was defined as the ratio between the PHA and VSS concentrations. For yield calculation, the mass values of X_A, PHA and VFA were converted into COD units by using the relative conversion factor from oxidation stoichiometry. The PHA storage yield was calculated according to Eq. 1 defined by the ratio between the produced PHA and the consumed SCFAs in the feast phase, both expressed as COD equivalent:

$$Y_{P/S}^{feast} = \frac{\Delta PHA}{\Delta SCFA} \quad (1)$$

The observed yield was calculated according to Eq. 2:

$$Y_{OBS}^{SBR} = \frac{VSS}{OLR \cdot HRT} \quad (2)$$

The specific PHA storage rate (r_{PHA}) was calculated according to the difference between the maximum and the minimum PHA values (at the end of feast and end of famine respectively) and considering "t" as feast phase length and the active biomass (X_A) at the end of the feast phase:

$$Y_{PHA}^{feast} = \frac{\Delta PHA}{Xa \cdot t} \quad (3)$$

Nutritional value of the PHA-rich MMC

The crude lipids content and amino acid analyses were performed by an external laboratory. Amino acid results were expressed as g 100 g^{-1} protein. Moisture and ash contents were determined gravimetrically according to standard methods [33]. Crude protein amounts were quantified according to the total nitrogen content with the Dumas method [34] and then multiplying the result by the conversion factor of 6.25 [35].

Based on the amino acid composition, the chemical score (CS) values of the SCP (the PHA-rich MMCs) were calculated by dividing the content of each essential amino acid (EAAi) of the proteins from the MMC by one of the two selected standards. The first is the herring fish meal (herring fish meal 5–02–000; [36]), as it is considered the ideal protein source [37] for aquafeed, while the second is the soybean meal (soybean meal 5–04–612; [36]) as it is one of the most used vegetable protein sources [38]. Additionally, the CS for each EAA of the PHA-rich MMCs was also compared to the EAA protein requirement for three freshwater fish species with differing feeding habits: rainbow trout (*Oncorhynchus mykiss*) carnivorous, common carp (*Cyprinus carpio*) and tilapia (*Oreochromis* spp.) omnivores and herbivores [36]. The lowest value of each ratio indicates the first limiting essential amino acid of the MMC protein source, and therefore its CS value.

The Essential amino acid index (EAAI) was calculated by Eq. 4:

$$EAAI = \sqrt[n]{(aa1/AA1)\cdot(aa2/AA2) \quad \ldots \quad (aan/AAn)} \quad (4)$$

EAAI is the n root of the ratio of essential amino acids in the MMC (aa) to those in a reference protein (AA). This ratio is determined by considering the two ingredients [36] instead of chicken eggs [39] or the EAA protein requirements for the three fish species.

Statistical tools for product's quality

The quality analysis of the process was achieved with the Shewhart Control Chart, for individual observations along with the capability

analysis of the production. Shewhart Control Charts were used to check the statistical control of the process. For this purpose, the Three Sigma limits approach was adopted, and the Moving Range (MR) was applied to estimate the variability of the process [40]. For this analysis, an out-of-control condition has been indicated when individual (or multiple) points plots outside the control limits, in the x or MR charts, according to Eqs. 5, 6 and 7.

$$MR_i = |x_i - x_{i-1}| \tag{5}$$

$$UCL = 3\frac{\overline{MR}}{1.128};\ LCL = -3\frac{\overline{MR}}{1.128} \tag{6}$$

$$UCL_{MR} = 3.267 \bullet \overline{MR} \tag{7}$$

The capability analysis was used to assess the capable of the process of producing conforming units within specified requirements. The Upper Specification Limit (USL) and Lower Specification Limit (LSL) were chosen to optimize the PHA production. According to Montgomery [40], Potential Capability (CP) and Actual Capability (CPK) were calculated by Eqs. 8 and 9, respectively.

$$C_P = \frac{USL - LSL}{6\sigma} \tag{8}$$

$$C_{PK} = \min\left\{\frac{USL - \pi}{3\sigma}, \frac{\pi - LSL}{3\sigma}\right\} \tag{9}$$

The statistics μ and δ describe the average value and the process standard deviation, respectively.

Finally, the cumulative normal distribution (Eq. 10) was used for estimation of the fraction of nonconforming products from each process.

$$P\{x \le a\} = \Phi\left(\frac{a - \mu}{\sigma}\right) \tag{10}$$

Where φ (.) is the cumulative distribution function of the standard normal distribution (mean = 0, standard deviation =1).

Results and discussion

Acidogenic fermentation of FW-MSS and of WL

As mentioned in the literature, the acidogenic fermentation process aims to achieve a sufficiently high SCFA concentration (according to their utilization) and, most importantly, the highest COD_{SCFA}/COD_{SOL} ratio possible [41]. This parameter serves as a key indicator of acidogenic efficiency, reflecting the balance between solubilization and acidification. A higher ratio suggests a more effective conversion of solubilized organics into SCFAs, which not only implies a successful fermentation process but is also a key requisite for the following MMC selection process, especially when a fermentation broth is particularly rich in macronutrients (nitrogen and phosphorus) [42]. The FW-MSS mixture displayed a markedly more stable and efficient fermentation performance compared to WL. The fermentation process of FW-MSS mixture showed stable performance after one week. The pH control helped to maintain the desired process conditions, minimizing the perturbances typically observed in the acidogenic fermentation of these putrescible wastes, such as variations of SCFA distribution and peaks of SCFA production compared to the average. In the frame of this stable fermentation activity, the average value of SCFA concentration was 21.3 ± 0.4 g COD/L. The COD_{SCFA}/COD_{SOL} ratio ranged between 0.65 and 0.92, with an average value of 0.77 ± 0.01 COD/COD. These parameters indicated a successful SCFAs accumulation from the FW-MSS mixture. The average fermentation yield (Y_{SCFA}; 0.44 ± 0.02 g $COD_{SCFA}/g\ VS_0$) was in line with the values obtained with similar feedstock found in the literature [3]. In terms of composition, butyric and acetic acids (respectively 43 % and 31 % COD/COD) represented more than 70 % of the total COD_{SCFA}.

WL was also fermented under the same operating conditions (except for the OLR). However, the acidification performances were quite different and largely lower than those obtained with FW-MSS feedstock. Despite the pH control, the WL fermentation was characterized by heavy fluctuations of SCFA concentration and COD_{SCFA}/COD_{SOL} ratio. The maximum SCFA concentration was 24 g COD/L, but the average value was abundantly lower and equal to 14.7 ± 0.9 g COD/L; the COD_{SCFA}/COD_{SOL} ratio was 0.55 ± 0.02 COD/COD, meaning that a high fraction of the organic matter remained unconverted.

As WL is typically tested only for biogas production, there is sparse information regarding the optimization of SCFA production from this waste available in the literature. Thermophilic temperature (55°C) and high OLR (up to 70 g COD/L d) were preferred to exploit WL as substrate for SCFAs production, and the only reported fermentation yield was equal to 0.25 g $COD_{SCFA}/g\ VS_0$ [43,44].

In terms of SCFA composition, compared to the fermented FW-MSS mixture, propionic acid was present at 24.6 % COD/COD, after butyric (33.2 %) and acetic acid (37.1 %).

For the aim of this study, the FW-MSS feedstock allowed to obtain a SCFA-rich stream amenable for the subsequent PHA-MMC selection process, achieving higher SCFA concentrations and, most importantly, higher COD_{SCFA}/COD_{SOL} ratio than WL feedstock. In fact, a fermented stream particularly rich in organic matter different from SCFAs is usually not ideal for maximizing the selection of PHA-producing microorganisms, potentially creating more risks of contamination from non-PHA-storing bacteria, that can grow on available organics, independently from the feast-famine approach [45]. The poorer performance of the WL fermentation, highlighted by the relatively low COD_{SCFA}/COD_{SOL} ratio, might be related to the presence of polyphenols in this substrate [3], which are known recalcitrant species that might hinder the fermentation process. To tackle this issue an upstream step aimed to remove part of these compounds, thus creating a more suitable environment for the microorganisms, would be necessary.

Table 2 summarizes the average parameters and performances of the two sCSTR.

Enrichment of PHA-storing biomass with FW-MSS and WL fermentation liquids

A long-term SBR operation (138 days; 69 HRTs) was conducted to confirm the enrichment of PHA-storing biomass and to evaluate the performance of the high-rate selection strategy. Substrate depletion (feast phase) was observed after two days (or HRTs), and a stable feast-famine cycle was established within two weeks, with an average feast phase length of 95 min, accounting for 13.2 ± 0.7 % of the total cycle time. This result aligns with other studies that report this duration as effective for enhancing PHA accumulation in mixed microbial cultures. Generally, a feast phase length of 20 % is considered optimal, regardless of the applied OLR [46]. In previous studies using fermented FW-MSS broth, a feast phase length of 12 % or lower was reported [23,30], with an OLR of 4.0 g COD/(L d). The fermentation process in this case had robust performance (COD_{SCFA}/COD_{SOL} ratio of 0.77 ± 0.01), ensuring adequate selective pressure and limiting the growth of non-PHA-storing biomass. However, the OLR applied was too high to maximize the enrichment of PHA-accumulating bacteria, thus the specific storage rate and yield were found to be 200 mg PHA/(g X_A h) and 0.22 COD/COD, respectively. As a result, the biomass produced might not be sufficient for bioplastic production due to the low final PHA content, which may not meet the requirements for sustainable processing [47]. On the other hand, the high OLR allowed for greater waste treatment capacity, with the resulting biomass (with lower PHA content) being in line with the purpose of this research. The PHA content in the withdrawn biomass at the end of the feast phase was 15.1 ± 0.4 % g PHA/g VSS. If used as a fish-feed supplement, this biomass can be used as probiotic SCP at 20 % of the total fish-feed diet, achieving a final PHA content in the diet slightly higher than 3.0 wt%, in agreement with

Table 2
Parameters and main performances of the acidogenic fermentation and biomass production reactors with municipal and winery waste.

Parameter	Unit	Acidogenic fermentation reactor		Microbial biomass production reactor (SBR)	
		FW-MSS	WL	FW-MSS	WL
COD_{SOL}	g COD/L	14.0 ± 0.3	14.9 ± 0.4	-	-
COD_{SCFA}	g COD/L	11.7 ± 0.4	8.3 ± 0.2	-	-
COD_{SCFA}/COD_{SOL}	COD/COD	0.77 ± 0.01	0.56 ± 0.03	-	-
N-NH_4^+	mg/L	680 ± 26	759 ± 2	-	-
P-PO_4^{3-}	mg/L	250 ± 12	108 ± 2	-	-
VSS (end of feast)	mg/L	-	-	2592 ± 36	2483 ± 25
PHA (end of cycle)	mg/L	-	-	67 ± 5	56 ± 5
PHA (end of feast)	mg/L	-	-	428 ± 10	210 ± 6
PHA content (end of feast)	g/g wt%	-	-	15.1 ± 0.4	7.2 ± 0.3
Y_{OBS}^{SBR}	COD/COD	-	-	0.53 ± 0.02	0.48 ± 0.01
$Y_{P/S}^{feast}$	COD/COD	-	-	0.22 ± 0.01	0.13 ± 0.01
feast/cycle length ratio	h/h (%)	-	-	13.2 ± 0.7	22.3 ± 0.9
r_{PHA}^{feast}	mg/(g h)	-	-	200 ± 12	44 ± 3
PHA composition	g_{3HB}/g_{3HV} wt %	-	-	90.0 ± 0.4 / 10.0 ± 0.4	92.8 ± 0.2 / 7.2 ± 0.1
Biomass Productivity	g/L/d	-	-	1.30 ± 0.01	1.24 ± 0.01

Hülsen et al. [48], who suggested PHA inclusion levels between 0.2 and 5.0 wt%.

In contrast, the WL fermentation broth was less effective in promoting the selection of PHA-storing organisms. Notably, the WL broth contained a higher proportion of non-VFA COD_{SOL}, which accounted for 45 % of the soluble organic matter. This affected the selection quality, leading to a longer average feast phase of 160 min (22.3 ± 0.9 %), higher than the values obtained with FW-MSS fermentation and generally higher than those observed in previous studies conducted with similar OLR [49]. Both the specific storage rate and yield were negatively impacted by the low acidification of the WL, with values of 44 mg PHA/(g X_A h) and 0.13 COD/COD, respectively.

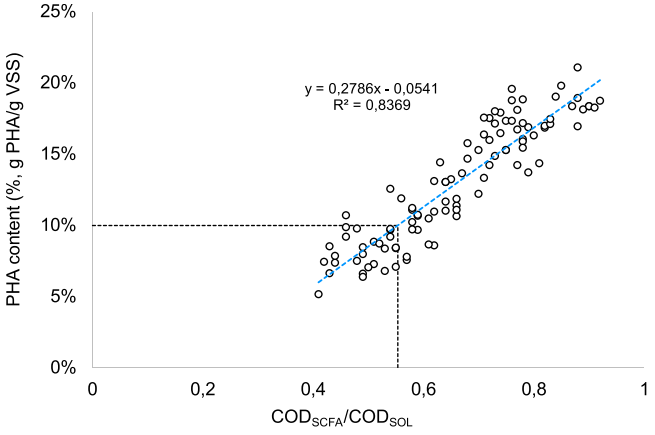

Fig. 1. Correlation between the PHA content in the biomass (end of feast) and the COD_{SCFA}/COD_{SOL} ratio of the fermentation liquids.

The influence of the COD_{SCFA}/COD_{SOL} ratio on the PHA storage capacity of the biomass is illustrated in Fig. 1. Data obtained from experiments using FW – MSS and WL as substrates revealed a significant linear correlation ($R^2 = 0.837$; F – statistic, p-value < 0.05) between the two parameters, as described by the equation reported below (Eq. 11).

$$PHA = 0.278565 \left(\frac{COD_{SCFA}}{COD_{SOL}}\right) - 0.054145 \quad (11)$$

The quality of the carbon source is known to significantly influence the efficiency of the selection and accumulation processes. The high ethanol content in the WL fermentation broth (approximately 20 % COD/COD) probably did not contribute to the development of the high-kinetic metabolism routes strictly connected to the VFA presence [46]. Due to the low PHA accumulation efficiency, WL was not considered optimal for achieving high PHA content in biomass, with a final PHA content of 7.2 ± 0.3 % g PHA/g VSS at the end of the feast phase. Without additional process steps, such as reducing nutrient levels (especially ammonia) in the WL fermentation liquid to enhance PHA storage, this substrate may not be suitable for achieving a target PHA content (at least 35–40 % as suggested by Werker et al. [47]). Nevertheless, the WL-derived biomass could still serve as a viable supplement for fish feed. Moreover, as previously hypothesized in relation to the limited fermentation efficiency, part of the unconverted COD_{SOL} in the WL stream may have consisted of inhibitory compounds such as polyphenols, which could have also impacted the PHA biosynthesis pathways. This might explain why, despite the higher proportion of propionic acid in the SCFA profile, the 3HV content in the resulting PHA was not higher, as would typically be expected from odd-numbered carbon precursors.

The average parameters and performances of the two SBR are also reported in Table 2.

Statistics of the process and product's quality

The importance of the COD_{SCFA}/COD_{SOL} ratio in the culture broth, and consequently the type of substrate used to feed the fermentation process, is reflected in the quality of the final product, as highlighted in this section.

The LSL (Lower Specification Limit) value of 0.55 COD_{SCFA}/COD_{SOL} (utilized for the capability analysis) has been estimated by the linear regression model (Eq. 11), considering 10 % by weight as the minimum value of PHA content in biomass. This value represents a sufficient threshold to achieve a certain PHA-storage response in the selected biomass. The value of 1.0 COD_{SCFA}/COD_{SOL}, which is the maximum, ideal and theoretical value of the ratio, has been selected as the USL (Upper Specification Limit).

Fig. 2 presents the results of the individual Shewhart control charts related to the SBR tests conducted with fermented FW-MSS and WL. Specifically, Figs. 2-a and 2-b show the control charts for FW-MSS and WL, respectively, while Figs. 2-c and 2-d show the moving range control charts for FW-MSS and WL. As highlighted, throughout the entire experimental period, no points in either the control charts or the moving range control charts were plotted outside the control limits (represented by the solid lines) in both tests.

Regarding the Capability Analysis, both the Potential Capability (CP) and Actual Capability (CPK) with the fermented FW-MSS as substrate, were equal to 1.21. These results highlighted that the process was exactly at the midpoint of the specifications (Fig. 2-a, USL and LSL are represented by the dashed lines) and uses less than 100 % of the tolerance band (6δ). Consequently, relatively few (about 0.14 %) non-conforming units will be produced by this process; hence, the substrate and the bioprocess conditions ensured a product with the required characteristics for almost the total production. For the fermentation process carried out with WL, the Potential Capability (CP) and Actual Capability (CPK) were recorded as 0.91 and −0.02 respectively. In

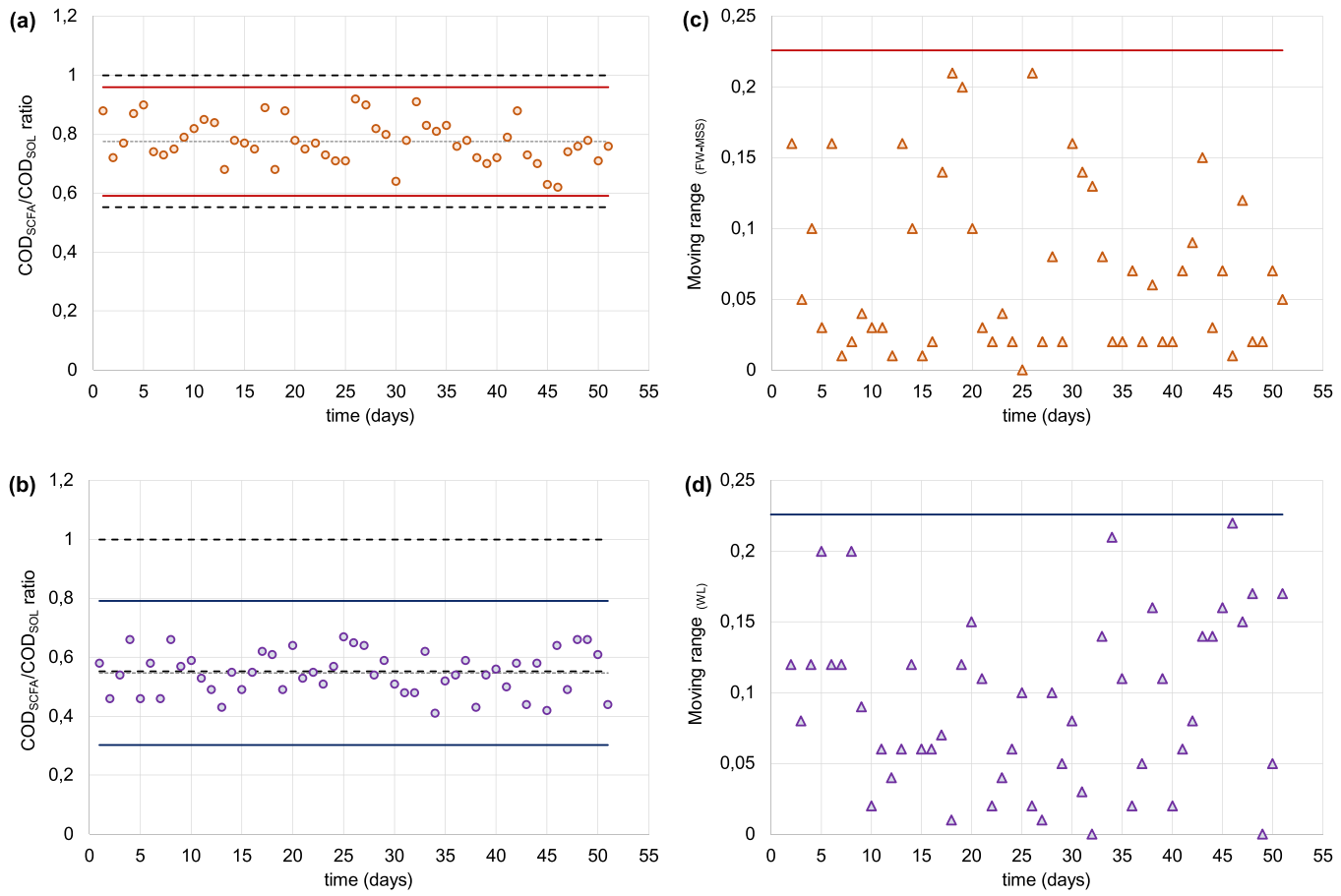

Fig. 2. Control limits (continuous lines; UCL and LCL) and specification limits (dashed lines; USL e LSL) for fermented FW-MSS (Fig. 2-a) and for fermented WL (Fig. 2-b); moving range for fermented FW-MSS (Fig. 2-c) and for fermented WL (Fig. 2-d).

contrast to the previous case, the process is not placed at the midpoint of the specifications (Fig. 2-b) and uses more than 100 % of the tolerance band (6δ). As a result, many non-conforming units (about 53.65 %) were produced by this process. Hence, the substrate and the bioprocess did not ensure a product with the required characteristics (more than 50 % of the product must be discharged) and some adjustments are needed to increase the percentage of conforming units (feedstock pretreatment to boost WL fermentability, among others).

Microbiome composition

The majority of the bacterial taxa obtained from the steady state in the two SBRs was mainly affiliated with phyla *Proteobacteria*, *Actinobacteria,* and *Bacteroidetes*. A certain abundance of *Firmicutes* was also found in the WL fed SBR. In general, *Proteobacteria* was the most abundant phylum in both runs, representing 62.4 % and 55.4 % of total reads in the FW-MSS and WL fed SBR, respectively. However, remarkable differences were noted between the two SBR runs, presumably due to the different utilized feedstock.

In the FW-MSS fed SBR, within *Betaproteobacteria*, sequences affiliated with genera *Hydrogenophaga* showed the highest relative abundance of 28.5 % (Fig. 3), followed by *Thauera* genus (4.2 %). Members of *Alphaproteobacteria* were also observed, and they were affiliated with *Brevundimonas* (9.7 %), *Rhodobacter* (7.1 %), and *Paracoccus* (4.4 %). More than 10 % of the total reads belong to *Actinobacteria* phyla, with the predominance of the genus *Leucobacter* (11.6 %). Individually, *Bacteroidetes*, *Gammaproteobacteria,* and *Firmicutes* represented less than 5 % of the total reads.

Additionally, the WL fed SBR showed a high abundance of *Betaproteobacteria*, with the main sequence represented by *Thauera* (18.6 %), which showed a remarkable increase compared to the previously discussed SBR; on the contrary, the genus *Hydrogenophaga* decreased to 4.3 % of total reads. In this SBR, *Alphaproteobacteria* were comparable to *Betaproteobacteria*; they were mainly affiliated with *Paracoccus* (11.2 %), *Mesorhizobium* (6.7 %) and *Brevundimonas* (4.2 %) genera. Roughly 10 % of the total reads belong to *Bacteroidetes* phyla, which showed a fragmented presence of genera; *Persicitalea* was the most abundant genus with a relative abundance of 4.5 %. Within the *Actinobacteria* phyla, the *Leucobacter* genus was the only one detected (9.0 %). It has to be also highlighted the non-negligible presence of *Firmucutes* phyla, mainly affiliated with genera *Lactobacillus* (6.9 %), *Acetoanaerobium* (3.2 %), and a lower abundance of *Planococcus* (1.3 %).

Despite the clear difference in the microbiome composition between the two SBRs, the relative abundance of putative PHA-storing bacteria selected with the two different feedstock was more than satisfying for the scope of the work and in line with values detected in the literature [50]. The highest relative abundance of sequences affiliated with PHA-storing bacteria was found in FW-MSS fed SBR (close to 60 %). The WL fed SBR was instead characterized by 38.4 % of PHA-storing bacteria. Given the similar operating conditions and inoculum adopted in the two SBRs, the differing abundance of genera identified as PHA-storing organisms was likely attributable to different feedstock compositions and the resulting selective pressure (as discussed in paragraph 3.2). As previously hypothesized, the lower COD_{SCFA}/COD_{SOL} ratio and the potential presence of inhibitory compounds such as polyphenols may have hindered the establishment and enrichment of PHA-storing bacteria, ultimately impacting the microbial community structure.

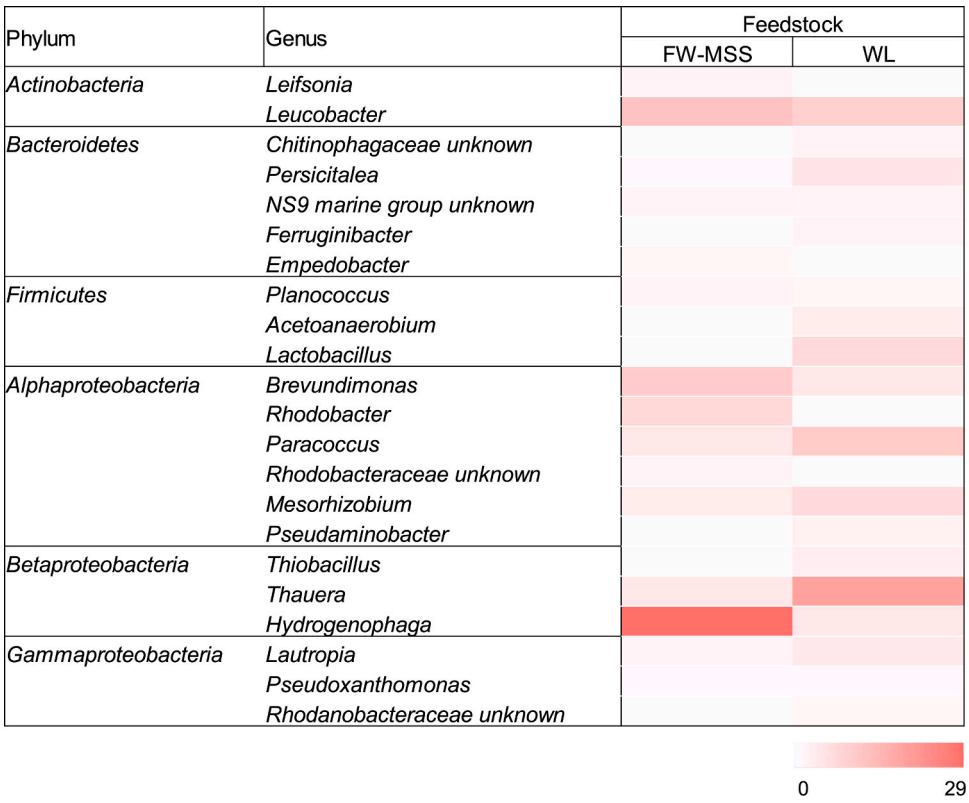

Fig. 3. Frequency heat-map of bacterial communities at genus level: the color intensity in each cell shows the relative abundance (data not reported for relative abundance <1 %). Samples taken at end of feast phase in both SBRs.

In particular, *Hydrogenophaga*, *Brevundimonas*, and *Rhodobacter* were enriched in the SBR fed with fermented FW-MSS characterized by a higher PHA-storage response, whereas *Thauera* and *Paracoccus* were the main PHA-storing genera in the WL fed SBR, where the microbiome was more fragmented and with a lower PHA production ability.

In terms of potential aquafeed application, none of the identified genera have been reported as bacterial fish pathogens net of 15 % of the total reads (roughly), which remained unclassified. In a recent review, among others, *Aeromonas* and *Edwardsiella* are commonly reported as pathogens for tilapia, rainbow trout, carp, salmon, and red sea bream, whereas *Streptococcus* is the source of streptococcosis disease in salmon, golden shiner and mullet [51]. *Lactococcus* is recognized as a pathogen for rainbow trout, yellowtail and Nile tilapia; while *Shewanella* for zebrafish and other freshwater farmed fish [51,52]. Despite the lack of pathogens in both analyzed samples, this remains a pivotal aspect for the safe utilization of waste-derived SCP in aquaculture in accordance with the standard requirements of a marketed product. Based on this, the necessity of an additional sterilization stage cannot be excluded, which consequently may lead to an increase in the cost of the final product within the biotechnological waste-derived value chain.

Proximate composition

Proximate analysis of the dry PHA-rich MMC biomass was performed on samples routinely taken in the steady-state period, which better represented the operating conditions of the production process at an industrial scale. The analysis of the dry biomass revealed a protein content of 55.1 % and 45.8 % for the microbial biomass recovered from FW-MSS and WL, respectively (Table 3). The range of protein content of other microbial SCP shows values ranging from 50 % to 83 % [9], higher than that obtained here from WL. However, the crude protein content of the PHA-rich MMC is comparable to those of conventional plant protein ingredients commonly used in aquafeed [53], and even higher than the

Table 3
Proximate composition of the PHA-rich MMC produced in the SBR fed with fermented FW-MSS and WL; values calculated as percentage of the dry samples (w/w).

Proximate composition (%)	FW-MSS*	WL*
Moisture	5.3	7.6
Crude Proteins	55.1	45.8
Lipids	8.0	12.3
Carbohydrates**	12.6	18.0
Ash	4.5	6.1

* fermented feedstock

suggested protein requirement for omnivorous (ranging from 25 % to 35 % of the diet) and carnivorous fish species (40–55 % of the diet, [12, 54]). Lipids and carbohydrates were in the same range of values (8.0 – 12.3 % and 12.6 – 18.0 % for lipids and carbohydrates, respectively) of other SCP of bacterial origin reported in the literature [55,56]. As previously shown, the PHA content was different among the two bacterial cultures: 15.1 vs 7.2 g/g wt% for biomass grown on fermented FW-MSS and WL, respectively (Table 2). However, this parameter is difficult to discuss since no precise threshold or boundary conditions are available in the literature regarding the required PHA content in fish-feed supplements. It is also noteworthy that a strong increase in PHA content could lead to a decrease in protein levels in the bacterial cell, which may not be ideal for SCP utilization for fish feed. Thus, an excessive PHA accumulation may not be essential for the effective use of SCP, and the process modifications required to increase PHA levels, such as additional accumulation steps, could significantly raise production costs.

Amino acid composition, chemical score and essential amino acid index

To evaluate the protein quality of the PHA-rich MMC and understand the amino acid balance of the microbial biomass, an amino acid profile

analysis was conducted (Fig. 4). It is well known that proteins vary in their amino acid profiles, making it crucial to select protein sources with a well-defined chemical composition to create a balanced, high-quality diet. For example, fish require ten out of 21 essential amino acids (EAA) from their diet: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine because they cannot synthesize these on their own. It remains uncertain whether non-essential amino acids (non-EAA) are also necessary to meet the metabolic needs of fish. The most abundant non-EAA amino acids in the PHA-rich MMC were glutamic and aspartic acids (9.5 and 8.2 % in WL-derived SCP; 8.4 % and 7.1 % in FW-MSS-derived SCP). Among the EAA, methionine, tryptophan and cysteine were as the least abundant, having a related content in the narrow range of 0.1–1.1 % for both types of SCP. Lysine is considered a reference amino acid since is frequently present in small amounts, making it a limiting amino acid in fish nutrition [57] and, in addition, it is a protein building block, even though not involved in any other metabolic pathways in fish [58]. In this study, the lysine content was relatively low in the SCP obtained from fermented WL (1.3 % of protein) but higher in the fermented FW-MSS derived SCP (3.2 %). Therefore, the latter appeared more promising for enhancing the overall biological value of such microbial biomass as a feed additive. The PHA-rich MMC produced from fermented FW-MSS contained EAA accounting for 32 % of the total amino acid content, similar to a mixed microbial culture in a previous study [9]. In contrast, the PHA-rich MMC from fermented WL had a lower crude protein content (45.8 wt%) and a smaller proportion of EAA, making up only 22 % of the total amino acids.

The chemical score (CS) for each EAA in the PHA-rich MMC derived from WL (Fig. 5a) and FW-MSS (Fig. 5b) was calculated to assess whether each amino acid was present in amounts comparable to a reference protein, helping to identify limiting amino acids that could impact fish growth [59]. Both PHA-rich MMCs were compared with the amino acid profile of fishmeal (the golden standard ingredient for aquaculture), soybean meal (the most common fishmeal substitute, [38, 53]), and the protein EAA requirements of three freshwater fish species. The comparison showed that the WL microbial biomass had a CS below the threshold of 100 for Trp, Phe+Tyr, Met+Cys, Lys, His and Arg when compared with the fish meal or the soybean meal. When compared to the EAA protein requirements of the three fish species, the CS was below the threshold for all amino acids, except Leu in common carp (Fig. 5a).

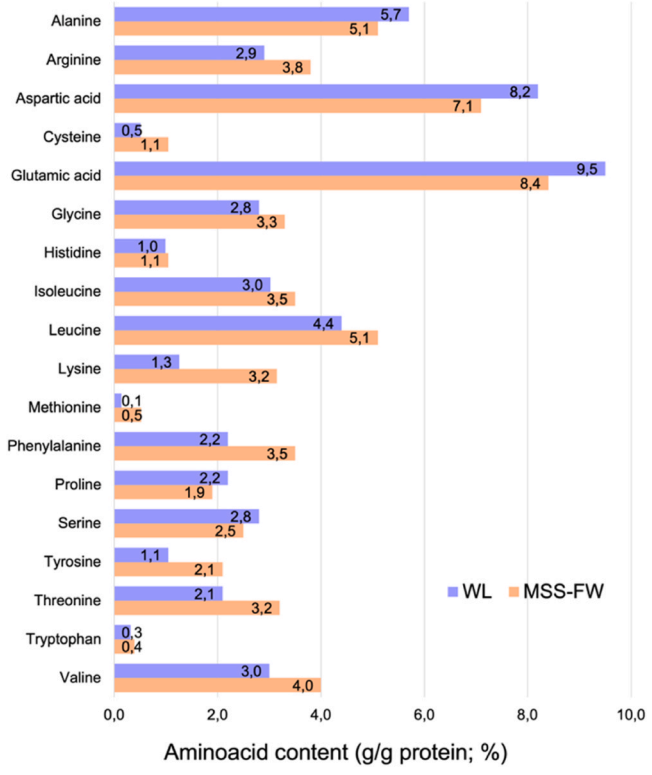

Fig. 4. Amino acid profile (g in 100 g of protein) of the SCP derived from fermented MSS-FW and WL.

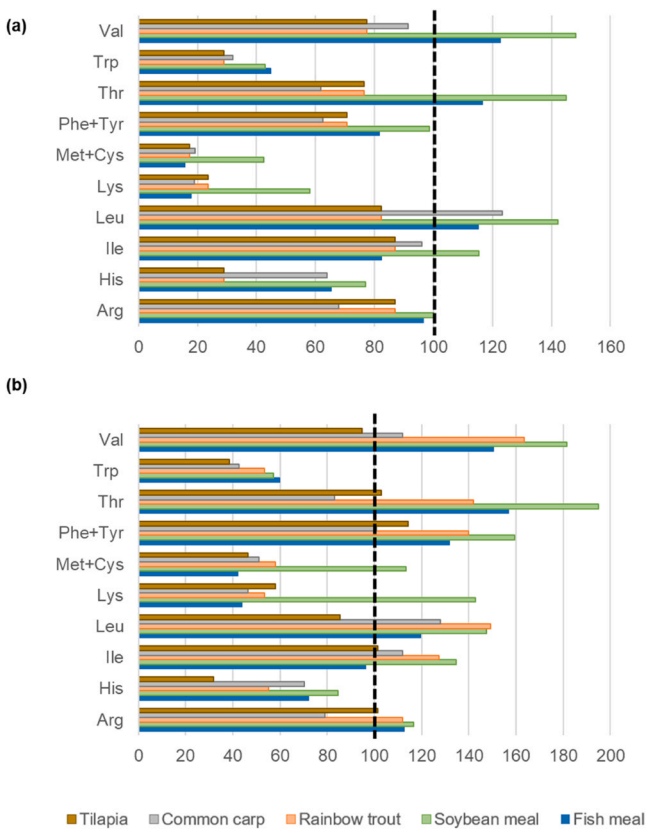

Fig. 5. Chemical scores (CS) of the essential amino acids within the microbial biomass, obtained from the SBR fed with either fermented Whole Loaf (WL) (a) or Municipal Solid Waste - Food Waste (MSS-FW) (b) determined by comparing the EAAI composition of the microbial biomass against two sets of standards aquafeed ingredients: fishmeal and soybean meal [36] or known EAA requirements in aquaculture species: rainbow trout (Oncorhynchus mykiss), common carp (Cyprinus carpio), and tilapia (Oreochromis spp.) [36]. The dashed line, set at 100, functions as a threshold to highlight whether the essential amino acid levels in the microbial biomass meet, exceed, or fall below those of the reference protein or fish requirements.

The first limiting EAA based on the chemical score, i.e. the ratio between EAAi in PHA-rich WL/EAAi in fish meal or soybean meal, was the Met+Cys (16 % or 43 %, respectively) followed by the Lys in the case of fishmeal (18 %) or Trp (43 %) in the case of soybean meal. On the other hand, the first limiting EAA based on the CS related to the amino acid requirement of the three fish species, resulted in the Met+Cys (17 %, 19 % and 17 %; respectively) followed by the Lys (24 %, 19 % and 24 %; respectively).

The FW-MSS microbial biomass (Fig. 5b) resulted in a CS below the threshold of 100 only for the Trp, and His when compared to the two reference protein sources or the three fish species EAA requirements. However, Val, Thr, Phe+Tyr, Leu and Arg were all higher than 100 compared to both reference protein sources, while the CS was higher than 100 for Met+Cys, Lys and Ile only compared to the soybean meal. Comparing the CS of the FW-MSS microbial biomass with fish meal, the most limiting EAAs were Met+Cys and Lys (42 % or 44 %, respectively), while comparing it with soybean meal, the most limiting was the Trp (57 %). The FW-MSS biomass showed better results in the CS calculation, especially for rainbow trout (Fig. 4b), with high scores for the EAA

Val, Thr, Phe+tyr, leu, Ile, Arg. These results were less significant for the other two fish species, indicating that the CS is species-specific, as observed by [9].

To complete the qualitative analysis of the proteins in the microbial biomass, the Essential Amino Acid Index (EAAI) was taken into account. This index is a widely used measure to evaluate the biological quality of a protein, providing a single numerical value that reflects how well a protein source meets the amino acid profile or requirements of a reference protein or organism [39,60]. To this end, the EAAI was calculated against fishmeal or soymeal as reference proteins, or to the essential amino acid requirements for three fish species selected according to their feeding habit. A protein source is considered of good quality if its EAAI is 0.90, useful if 0.80, and incomplete if below 0.70 or less [60]. The EAAI of the PHA-rich MMCs is shown in Table 4. In the case of the comparison with the soybean meal the EAAI score reached 1.08 or 1.29 for WL or MSS-FW microbial biomass, respectively; indicating that this bacterial protein can be consider a very good standard. On the other hand, when comparison was done against fishmeal, the EAAI score of the WL biomass reached 0.77 thus sortable incomplete, while the MSS-FW biomass useful (Table 4). The EAAI also revealed that WL biomass contains an imbalanced EAA profile for all three fish species considered, whereas MSS-FW biomass can be considered a high-quality protein source for rainbow trout but an incomplete source for common carp.

In the present study CS and the EAAI resulted in a valuable tool to get a clearer picture of each PHA-rich MMCs protein quality. The CS helps identify specific deficiencies that might need targeted supplementation, while the EAAI provides an overall sense of the protein's biological value based on its complete essential amino acid profile. However, nutritionists typically recommend combining protein sources to improve the overall amino acid balance. Hence, these two indices can be considered good preliminary indicators until protein biological value is assessed in *in vivo* digestibility/feeding trial.

Table 4
Essential amino acid index for the bacterial biomass obtained in the SBR fed with fermented WL or FW-MSS.

	EAAI	
Reference protein	WL	FW-MSS
Fish meal	0.77	0.88
Soybean meal	1.08	1.29
Rainbow trout	0.60	0.90
Common carp	0.50	0.75
Tilapia	0.46	0.68

Conclusions

This study evaluated the feasibility of producing PHA-enriched MMC from FW-MSS and WL waste streams for potential use as SCP in fish feed. The acidogenic fermentation of FW-MSS achieved a stable, high SCFA concentration and a favourable COD_{SCFA}/COD_{SOL} ratio, creating an ideal substrate for PHA-rich MMC selection, as confirmed by the strong positive correlation between this ratio and PHA content ($R^2 = 0.837$). In contrast, WL fermentation resulted in lower SCFA content and stability, requiring an improvement in the upstream operations and making it less suitable as a primary feedstock for PHA production. The resulting MMC biomass had a level of PHA of 15.1 and 7.2 g/g wt% for FW-MSS and WL, respectively, and thus could represent a promising fish feed additive. Capability analysis confirmed that FW-MSS fermentation ensured a product with the required characteristics for almost the total production with minimal non-conforming units (0.14 %), while WL fermentation yielded high non-conformance (53.65 %), indicating the need for process adjustments to enhance product quality. The crude protein content of the PHA-rich MMC derived from FW-MSS biomass reached 55.1 %, meeting the established aquafeed standards set according to the fish feeding habit, at least 25–35 % crude protein [61]. The relatively high protein content and the amino acid profile indicated that FW-MSS biomass could potentially match the protein requirements for omnivorous and carnivorous fish species. However, analysis of the CS and EAAI highlighted that the PHA-rich MMC alone may not provide a fully balanced EAA profile to meet all fish dietary needs. In particular, while FW-MSS biomass shows a relatively balanced EAA composition, the WL-derived biomass had a lower crude protein content of 45.8 % and reduced EAA proportions, thus proving a lower quality SCP but could serve as a supplementary protein source in fish feed. These findings indicate that, although both types of microbial biomass could be included in aquaculture feed, their use should consider specific fish species and their amino acid requirements.

The microbial composition in the two SBRs varied, primarily shaped by the distinct feedstocks used. Both systems were dominated by *Proteobacteria*, particularly *Betaproteobacteria* and *Alphaproteobacteria*, as well as *Actinobacteria*, though with differing prevalent genera, *Hydrogenophaga*, *Leucobacter* and *Brevundimonas* in the FW-MSS fed SBR, and *Thauera, Leucobacter* and *Paracoccus* in the WL fed SBR. The FW-MSS reactor exhibited a higher relative abundance of PHA-storing bacteria (~60 %) compared to the WL reactor (~38 %). Despite these differences, neither system presented genera reported as bacterial fish pathogens, although a small portion of unclassified reads remains. Future studies needs to be focused on investigating the potential presence of pathogens among the unclassified reads, possibly through complementary toxicological tests. However, to ensure biosafety for aquafeed use, further sterilization may be necessary, which could impact economic feasibility. Overall, to the authors' best knowledge, this study is among the first to demonstrate the potential use of microbial biomass derived from wine residues and urban organic waste as sustainable ingredients for fish feed, either as supplements or substitutes for conventional sources. While further in vivo studies on digestibility and palatability are needed, these results have shown that waste-derived MMCs like FW-MSS could become valuable and sustainable SCP resources in the aquafeed sector.

CRediT authorship contribution statement

Francesco Valentino: Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Writing – review & editing. **Giulia Pascon:** Investigation, Visualization. **Paolo Pavan:** Funding acquisition, Project administration. **Aditi Parmar Chitharanjan:** Conceptualization, Formal analysis, Visualization, Writing – original draft. **Gloriana Cardinaletti:** Investigation, Methodology, Writing – original draft, Writing – review & editing. **Giulia Adele Tuci:** Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Marco Gottardo:** Formal analysis, Validation, 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.

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Data availability

Data will be made available on request.

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