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Valorisation of agricultural residues into *Thauera* sp. Sel9 microbial proteins for aquaculture

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ABSTRACT

Unconventional protein sources are necessary to tackle the increasing demand for food. Microbial proteins (MP) are an alternative source of proteins for feed or food, suitable as feed for aquaculture. Substituting fishmeal with MP obtained from agricultural wastes could reduce the environmental burden of aquaculture and help with waste management. In this study, pure culture MP from the PHA-producer *Thauera* sp. Sel9 were obtained from agricultural residues (agro-zootechnical digestate and pasta industry leftovers). The produced MP was used in feeding tests with the model fish zebrafish (*Danio rerio*) to assess potential toxic effects and evaluate overall fish health. The obtained MP was rich in protein (59.5 % w/w over TS) and PHAs (15.0 %) and comprised all fish essential amino acids. The chemical scores and essential amino acid index confirmed the excellent quality of the MP. The feeding tests with 50 % feed substitution with MP resulted in survival rates (80–88 %) comparable to the control group (78 %), with only 100 % MP showing increased mortality. *Thauera* MP obtained from agricultural residues has the potential to become a partial fishmeal substitute in fish-farming.

1. Introduction

Overpopulation is the root cause of many of the world's environmental problems. The constantly increasing number of humans on Earth - 10 billion are estimated by 2050 (Ezeh et al., 2012; UN World Population Prospects, 2022) - is putting a strain on the available resources and is aggravating the issue of waste disposal. Because of the increasing population, the demand for water, food, materials, energy, and transportation is also growing, hence contributing to deforestation, reduced biodiversity, increased pollution, drought and famine. When considering only the demand for food, production needs to grow by 70 % before 2050 to be able to meet the demand of the growing population (Godfray et al., 2010), with animal-derived protein request doubling in the same timeframe due to rising incomes and urbanisation levels (Henchion et al., 2017).

All of this considered, it is evident that alternatives to traditional protein sources are urgently needed to avoid further impacts on the ecosystems. Indeed, the production of proteins from animals and plants has a high carbon footprint, requires large amounts of water and land, and contributes to soil, water, and atmosphere pollution (Oonincx and Boer, 2012).

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Microbial proteins (MP), also called single-cell proteins (SCP), are a mean of producing proteins for feed or food without the use of animals or plants (Litchfield, 1983). The protein sources are microorganisms, such as bacteria, fungi, yeasts, or algae, which are obtained from the fermentation of carbon- and nitrogen-rich substrates in controlled environments, and dehydrated before use (Matassa et al., 2016). These microorganisms are rich in proteins because they intake carbon and nutrients and turn them into microbial biomass rich in proteins and other cell constituents such as carbohydrates, lipids, vitamins, minerals and pigments (Ravindra, 2000; Overland and Tauson, 2010; Areniello et al., 2023). The production of MP has long been established, particularly when obtained from yeasts or fungi, which are already commercialised by different companies, though with varying levels of success (Banks et al., 2022; Ritala et al., 2017). The choice of feedstock is essential to make production cheaper and guarantee a higher chance of survival for MP-producing companies. Traditional "first-generation" MP are produced from non-renewable fossil-fuel-based feedstocks or from dedicated crops (Areniello et al., 2023); however, these feedstocks can be expensive, and they can put MP production in direct competition with the food market or chemical industries (Ciani et al., 2021). The use of waste streams (e.g., food waste, agricultural residues, industrial wastewater) or byproducts from the production of other goods (e.g., cheese whey, brewers' spent grain, sugarcane bagasse, seafood shells) (Yadav et al., 2020; Abdullahi et al., 2021; Martínez et al., 2015; Mondal et al., 2012; Milala et al., 2018; Vethathirri et al., 2021; Koukoumaki et al., 2024), means not only a reduction in wastes in need of disposal, but also the availability of renewable and inexpensive biomass for MP production (Matassa et al., 2022). However, these "second-generation" MP produced from renewable or recovered feedstocks are not devoid of problems, since the possibility of carrying biological or chemical contaminants through the production chain limits their use in many countries, in particular for their use in the food market (Ukaegbu-Obi, 2016). Furthermore, issues such as availability, variability, seasonality and transportation costs of the renewable feedstocks (Junghare et al., 2023) also need to be taken into account.

Algae, fungi and yeasts microbial proteins can be used as human food because they are valuable sources of proteins, lipids, vitamins and pigments, while bacteria are employed only for the production of animal feed, due to the legal and safety issues involved in the use of bacteria and the general public's aversion to such practices (Nyyssölä et al., 2022). Bacteria-based MP have been tested on animals such as ruminants, poultry and pigs, showing similar or better feed intake and conversion, growth performances and overall health when compared to animals fed with their normal diet (Matassa et al., 2016; Overland and Tauson, 2010). However, their most promising market is fish-farming, which is rapidly expanding, representing almost 50 % of all fish consumed worldwide, and is predicted to grow to the number one source of fish by 2030 (FAO, 2022). The rapid expansion of aquaculture is encouraging, since fish are among the most efficient protein types in terms of protein and energy retention, feed conversion ratio, water consumption and carbon footprint (Sheeshka and Murkin, 2002; Fry et al., 2018; Jones et al., 2020), resulting in a reduced impact on the environment. On the other hand, aquaculture is highly reliant on fishmeal (a flour rich in proteins obtained from fish milling), which is produced mainly from wild-catch and therefore contributes to overfishing when produced at the quantities required by the expanding fish-farming sector (Henchion et al., 2017; Matassa et al., 2016). Indeed, in 2020 the production of fishmeal accounted for 20 % of the fish captured at a global scale, 86 % of which was employed in the aquaculture sector (FAO, 2022), weighing considerably on global fish production and therefore being unsustainable on a long-term scale.

The use of MP as fish feed – partially substituting fishmeal in carefully designed formulas - has been shown to be successful for some commercial species such as rainbow trout, Atlantic halibuts, Japanese yellowtail, Atlantic salmon, Asian sea bass and Mozambique tilapia (Overland and Tauson, 2010; Jones et al., 2020; Sharif et al., 2021; Biswas et al., 2020; Delamare-Deboutteville et al., 2019; Suguna et al., 2014). Recently, improved growth survival has been recorded when the MP was enriched with polyhydroxyalkanoates (PHAs), biopolymers produced by the microorganisms and used as energy storage (Raza et al., 2018). Most of the work on this subject shows the beneficial effects of PHAs when they are added to the feed mixture (Suguna et al., 2014; Najdegerami et al., 2012; De Schryver et al., 2010; Situmorang et al., 2016), with one publication only showing how it is better to produce PHA-accumulating MP and use these directly to feed the fish, therefore providing proteins as well as PHAs (Pesante et al., 2022). In comparison to the typical PHA valorisation pathway as substitutes for bioplastics, their use for fish feed when still inside the bacterial cells would overcome the high costs associated with their extraction and purification. The beneficial effects of PHAs are linked to their probiotic action, enriching the intestinal microbiota and, therefore, stimulating the immune system, with the consequent effect of improving the health of the fish (Suguna et al., 2014). The immune system is probably stimulated by the digestive system's degradation of PHAs into their precursors β-hydroxy short-chain fatty acid monomers, which have been shown to improve fish gastrointestinal health in light of their bacteriostatic properties (Defoirdt et al., 2009).

MP can be obtained from mixed microbial cultures (MMC) or pure strains, both methods having advantages and disadvantages. MMC are generally more productive and more stable to changes, and they have higher protein content because of the presence of many different types of interacting bacteria in the consortium (Alloul et al., 2021; Santillan et al., 2019). They are also cheaper and easier to maintain because sterility is unnecessary (Vethathirri et al., 2021). However, pure cultures are preferred when the aim is to obtain a product of known composition and characteristics – given the same growth conditions - and for which toxicity and pathogenicity can be excluded because the only strain used has been certified as safe (Nyyssölä et al., 2022).

Considering all the aspects described above, and with a circular economy model in mind, our research aimed to produce microbial proteins to be used as fish feed in aquaculture, using unutilised agricultural residues as starting feedstock. A local agricultural cooperative provided with a two-stage anaerobic digester and a pilot plant for the production of PHA was used for the conversion of the cooperative's crop and animal wastes into a volatile fatty acids-rich liquid (Righetti et al., 2020), which was used as a culture medium for the production of MP. An axenic culture was chosen to produce MP, in order to obtain a safe product with a stable biomass composition. A strain able to produce PHAs (*Thauera* sp. Sel9) was favoured to take advantage of the immunostimulant effects of PHAs on fish; the genus *Thauera* was selected because it was found to be one of the most common PHA-producers among MMC grown on volatile fatty acids obtained from the acidogenic fermentation of organic wastes (Andreolli et al., 2022). *Thauera* sp. Sel9 has recently

been isolated and characterised from MMC, and its genome has been published (Andreolli et al., 2022), highlighting its potential exploitation as MP for feed, following a detailed analysis of its protein content. However, this is the first time that any strain belonging to the genus *Thauera* has been used for producing MP, starting from agricultural residues and ending with tests on fish through structured feeding trials. Analysis of the composition and nutritional value of the bacterial biomass was conducted to evaluate its suitability as fish feed. Feeding tests were performed on the model fish zebrafish (*Danio rerio*) to gather preliminary information on the safe use of *Thauera* sp. Sel9 as an MP and on the effect of the presence of PHAs inside the bacterial cell, or externally added. PHA-containing MP have previously been used for the production of fish feed and used for feeding trials only using either MMC (Pesante et al., 2022) or pure cultures of two strains of the genus *Bacillus* (Suguna et al., 2014; Umesh et al., 2017), therefore this work extends the results to a different group of bacteria.

2. Materials and methods

2.1. Experimental set-up

The work aimed to upconvert agricultural residues into PHA-containing microbial proteins to be tested for their use as fish feed in aquaculture.

The experimental work consisted of four phases (Fig. 1).

In phase 1 agricultural residues were treated at the agricultural cooperative "La Torre" located in Isola della Scala, Verona, Italy (details in Section 2.3), where an anaerobic digester and a pilot-plant for the production of PHA-accumulating bacteria are strategically located (Righetti et al., 2020) and were available to be used by our research group (LabICAB Laboratory of Chemical Engineering for the Environment and Bio-processes Department of Biotechnology, University of Verona, Italy). The feedstock was mixed, homogenised and loaded into an acidogenic fermenter inoculated with mixed microbial cultures. The volatile fatty acids (VFA)-rich liquid phase obtained from the fermenter's effluent was used as a culture medium for bacterial growth in phase 2, after filtration using a 0.2 μm pore size filter in order to remove all the initial suspended solids and potential source of biological contamination for the pure culture.

Phase 2 was performed in LabICAB's laboratories at the University of Biotechnology of Verona aiming to produce PHA-containing microbial proteins for phase 3 in a fed-batch mode (Section 2.4).

In phase 3, the dry biomass (hereafter named MP for Microbial Proteins) obtained at the end of phase 2 was first analysed to confirm the purity of the culture, according to the protocol described in Section 2.2. The MP was then characterised for its proximate and aminoacidic composition and nutritional value (Section 2.5) before being used for the feeding trials with fish (phase 4).

Phase 4. Fish-feeding trials were performed with the aim of assessing the actual nutritional value of the produced MP. The trials took place in the Zebrafish facility of the University of Verona, Italy, and are described in detail in Section 2.7.

2.2. Bacterial strain and 16 S rRNA gene sequencing

The bacterial strain used to produce microbial proteins was *Thauera* sp. Sel9. This strain belongs to a genus of aerobic, heterotrophic gram-negative bacteria in the family *Zoogloeaceae*, order *Rhodocyclales*, class *Betaproteobacteria* (Heider and Fuchs, 2015), but it has not formally been described yet. It was originally isolated from mixed microbial cultures used to accumulate PHAs in a sequencing batch reactor (Conca et al., 2020; Frison et al., 2021). The strain was identified by 16 S rRNA gene sequencing as having 99 % similarity to *Thauera butanivorans* NBRC 103042 T (Botturi et al., 2020), which belongs to a genus of known PHA producers (Sabapathy et al., 2020). The analysis of its genome confirmed the presence of all the genes involved in PHA synthesis, regulation and

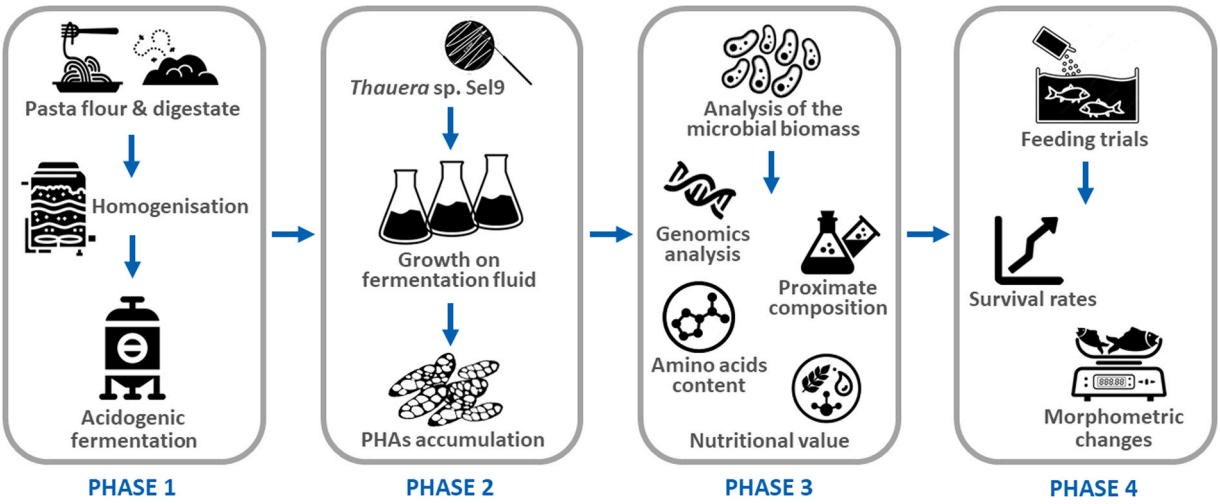

Fig. 1. Schematic representation of the four phases of the experimental set-up.

degradation (Andreolli et al., 2022). Thus, *Thauera* sp. Sel9 was selected for its ability to grow and accumulate PHAs by using different VFAs (mainly acetate and butyrate) as the sole sources of carbon and energy (Andreolli et al., 2022). Moreover, previous studies suggested the possible use of this PHAs-storing strain as a feed supplement for fish (Botturi et al., 2020; Critelli et al., 2022). Despite Hydrogen-Oxidizing Bacteria (HOB) and Methane-Oxidizing Bacteria (MOB) being at present promising microorganisms as MP for their high protein content and for their ability to use low-cost and renewable substrates such as CH_4 and H_2(Areniello et al., 2023; El Abbadi and Criddle, 2019), the heterotroph *Thauera* sp Sel9 can also use renewable substrates without competing with the human food chain, such as acetate produced by ethanol or bioelectrosynthesis (Deutzmann and Spormann, 2017), or generated by fermentation of syngas (CO and H_2) (Abubackar et al., 2015) or by degradation and fermentation of lignocellulosic waste streams (Strazzera et al., 2021).

Glycerol stocks of the pure strain were plated on nutrient broth agar (Oxoid, Milan, IT), and a single colony was used to inoculate a modified Brunner's mineral medium (Critelli et al., 2022), which was kept at 30°C and 180 rpm for 3 days. 100 mL of this inoculum were used to inoculate the fed-batch cultures described in phase 2 of the experimental set-up. Samples of the cultured bacteria were taken at the beginning of phases 2 and 3, to ensure culture purity and confirm the identity of the grown strain. This was done by centrifuging 2 mL of the culture at maximum speed for 2 min and sending the bacterial pellet to BMR genomics (www.bmr-genomics.it/) for 16 S rRNA gene sequencing analysis. Details on the sequencing protocol can be found at the link www.bmr-genomics.it/servizi/servizi-ricerca/ngs-next-generation/sequencing/ampliconi ngs/16 s-ngs/). Briefly, DNA was extracted from the bacterial pellet using the Dneasy® 96 PowerSoil® Pro QIAcube® HT Kit (Qiagen) and purified with the Qiacube HT kit (Qiagen). The V4 rRNA gene region was amplified by PCR using universal primers for bacteria and archaea (Takahashi et al., 2014), and the DNA was purified with Thermolabile Exonuclease I (NEB). The library was prepared with the Nextera XT DNA Library Preparation kit (Illumina), normalised, multiplexed and sequenced with an Illumina MiSeq Sequencer. The quality was checked using the PASTQC software, and the taxonomic analysis was performed on a QIIME2 pipeline against the Greengenes 8_13, Silva 132 and RDP databases.

2.3. Acidogenic fermentation of agricultural residues

The agricultural residues used in phase 1 consisted of leftover flour from a local pasta-making factory and solid digestate from a two-stage anaerobic digestion plant. This feedstock was mixed and diluted with water to keep the solid contents below 8 %, homogenised in a storage tank, and loaded into an acidogenic fermenter inoculated with mixed microbial cultures (Righetti et al., 2020). The fermentation process had a hydraulic retention time of 4 days and an organic loading rate of 18 Kg COD/m^3d, allowing the conversion of particulate COD into fatty acids, producing a FF rich in VFAs (15.1 gCOD/L). Details on the AD plant, with its components and their working parameters, are reported in previous papers (Pesante et al., 2022; Righetti et al., 2020). The fermenter effluent was separated into solid and liquid phases by means of a screw press (SEPBIOH11502, WAMGROUP) with a capacity of 5 m^3/h. The liquid phase (fermentation fluid, FF) was mechanically filtered at 0.22 μm with a Juice Clarification System pump (model MMSL LAB from Ju.cla.s Srl, Italy), its pH was adjusted to 7.0 with 1 M NaOH and a COD/N (COD = Chemical Oxygen Demand) ratio of 10 (for optimal growth of *Thauera* sp. Sel9 (Andreolli et al., 2022)) was reached by adding 20 g/L ammonium bicarbonate. This medium is suitable for bacterial growth and is particularly indicated when the aim is to produce PHAs with pure cultures (Khatami et al., 2022), since VFAs are their direct precursors (Szacherska et al., 2021). The growth medium was autoclaved for sterilisation, cooled at room temperature and 2 mL/L of trace elements (Critelli et al., 2022) were added to provide microelements potentially lacking in the fermentation fluid (Table S1).

2.4. Production of microbial proteins

In order to produce microbial proteins to be used as feed for fish, six 1 L flasks were filled with 450 mL fluid composed of *Thauera* sp. Sel9 inoculum and the FF previously described, diluted with distilled water to reach the final concentration of VFAs expressed as soluble COD ($sCOD_{VFA}$) of 1 g/L. After growing for six days, during which fresh FF was added daily to maintain the $sCODF_{VFA}$ concentration of 1 g/L, PHA accumulation was performed with a multi-spike feeding strategy (Valentino et al., 2019). Five spikes of 15 mL of FF at C/N 20 were provided at 50-min intervals. The biomass obtained from the accumulation process was centrifuged at 3900 rpm for 20 min, resuspended in deionised water to remove any remaining culture medium, and centrifuged again. The obtained pellet was frozen at 80 °C for 2 h and then lyophilised for 48 h with a Lio 5 P freeze-dryer (5 Pa Srl, Italy) in order to obtain the MP to use for the fish-feeding trials. This was further ground with a mortar and pestle and milled with a mixer mill (Mixer Mill MM400, Retsch, Germany) to reduce the particle size to the same dimension of those of the CF (80–200 μm according to the manufacturer). Images of both the MP and the CF were taken with a stereomicroscope (MZ 16 F, Leika, Germany) to compare granulometry.

2.5. Composition and nutritional value of the bacterial biomass

The MP was characterised for its proximate and aminoacidic composition before being used for the feeding trials with zebrafish (phase 4), with the same protocols described previously and here reported (Pesante et al., 2022). Moisture and ash contents were determined gravimetrically according to standard methods (APHA, 1998; IRSA-CNR, 2003). Crude lipids, oils and cellulose were quantified by Chelab Srl laboratories - Merieux NutriSciences (Resana, Treviso, Italy, www.merieuxnutrisciences.com/it/) using internal methods subject to copyright and therefore not disclosable. Nitrogen-free compounds were obtained by subtracting the other obtained values from 100, which are presented as g/100 g. Crude protein amounts were determined by quantifying the total nitrogen content with the Dumas method (Saint-Denis and Goupy, 2004) and then multiplying the result by the conversion factor of 6.25

(IRSA-CNR, 2003); indeed, the use of this conversion factor is widely used for the calculation of crude protein amounts from both TN and TKN (Delamare-Deboutteville et al., 2019; Øverland et al., 2006; Schøyen et al., 2007), despite some debate is found in the literature on whether a different parameter should instead be used (Krul, 2019). We believe we could apply the 6.25 factor to our samples, despite a possible slight overestimation since not all the nitrogen in our samples comprised proteins, and because the Dumas method calculates not only organic nitrogen, but also ammoniacal nitrogen. The amino acid composition was determined in triplicates by oxidising and then hydrolysing the samples (or vice versa, depending on the amino acid under examination) and then determining each amino acid's presence and quantity by ion chromatography with post-column derivatisation with ninhydrin. Cystine and cysteine were both determined as cysteic acid and then calculated as a sum expressed as cysteine; methionine was determined as methionine sulfone and then calculated as methionine. Tryptophan was determined according to the AOAC method 2017.03 (Draher and White, 2018). Total carbohydrate contents were calculated by a two-step acid hydrolysis with sulfuric acid (72 % v/v) (Sluiter et al., 2008), followed by HPLC using a Jasco Extrema LC-4000 system equipped with a Rezex RoA H+ (Phenomenex) column.

Sugar content analysis (fructose, glucose, lactose, maltose, sucrose and total sugars) was also performed at Chelab Srl laboratories following a protocol that is not fully disclosable, which involved the extraction of the sugars from the samples with water and the analysis by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using internal standards.

The nutritional value of the MP was evaluated by calculating its chemical score (CS), essential amino acid index (EAAI) and energy content. The chemical score is a value that indicates whether each amino acid is found in quantities similar to a reference protein, highlighting limiting amino acids that could potentially reduce fish growth (Hepher, 1988). It was obtained with the formula:

$$\text{Chemical Score(CS)} \quad = \quad \left(\frac{\%\text{of a given EAA in MP protein}}{\%\text{of the same EAA in reference protein}}\right) x100$$

where EEA stands for essential amino acid (Bunda et al., 2015; Jauncey, 1998; Kirimi et al., 2020). A detailed protein requirement for zebrafish, inclusive of the required amino acid quantities, is not available, therefore the reference proteins to which the MP was compared were three: 1. the protein requirement of the common carp (*Cyprinus carpio*), a freshwater fish species belonging to the same family of zebrafish, *Cyprinidae* (Akiyama et al., 1997), and with similar eating habits; 2. the ideal zebrafish protein profile proposed by Kaushik and colleagues (Kaushik et al., 2011); 3. the amino acid composition of the commercial feed (CF) used in the feeding trials with zebrafish, to understand how the MP performed compared to the control group.

The essential amino acid index is a value used for the quantitative analysis of proteins, since it assesses and compares protein's quality biologically, using the geometrical mean of the ratio of all EAA of the tested protein in relation to a reference protein (Machado et al., 2020; Oser, 1959; Wilson and Halver, 1986). It was calculated with the formula:

$$\text{Essential amino acid index(EAAI)} \quad = \quad [\left(\frac{aa1}{AA1}\right) \times \quad \left(\frac{aa2}{AA2}\right) \times \ldots \times \left(\frac{aan}{AAn}\right)] \times 1 \Big/ n$$

where aa are the mg of a given essential amino acid found in the TS of the MP, AA the mg of the same essential amino acid in the reference protein, and n is the number of evaluated amino acids (Bunda et al., 2015; Kirimi et al., 2020; Oser, 1959), which in our case were all 10 EAA. The reference proteins were the same as those used for the CS, with the addition of the protein distribution of fishmeal, as reported in the literature (Schøyen et al., 2007).

The energy content is an important property of food, representing how much energy can be released from carbohydrates, fats and proteins, and used to sustain the metabolism. It was calculated according to the following equation (Manzi et al., 2001):

Energy (Kcal/g) = 4 x (g of protein + g of carbohydrate) + 9 x (g of fat)

where the g of proteins, carbohydrates and fat of a given amount of biomass are multiplied by appropriate conversion factors, and the total energy content is obtained by their sum. In this manuscript, the energy content was calculated for 100 g of TS.

2.6. Analytical methods

Soluble and total chemical oxygen demand (sCOD and tCOD), total and volatile solids (TS and VS) and ammoniacal total Kjeldahl nitrogen and total nitrogen (N-NH_4, TKN and TN) were determined according to standard protocols (APHA, 1998; IRSA-CNR, 2003), with analysis performed in duplicate. VFA concentration was determined for single VFAs (acetic, propionic, butyric, isobutyric, pentanoic, isopentanoic and heptanoic acids) and total VFA values were obtained as the sum of each acid. The method for VFA detection was ion-chromatography (Dionex ICS-1100, Thermo Fisher Scientific, USA) equipped with an IonPac ICE-AS1 column, as described in (Conca et al., 2020). PHA contents were determined by gas chromatography, as explained in (Pesante et al., 2022). The standards were of pure poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (Sigma, 403105) with a 8 % PHV content. The PHA amounts in the experimental samples (MP, FF or CF) were calculated as a percentage (w/w) of the TS.

2.7. Feeding experiments

Feeding trials were performed on the freshwater species *Danio rerio* (zebrafish), a vertebrate model for biomedical and translational research, as well as developmental, genetic and toxicological studies. This species was chosen for practical reasons (zebrafish are small, robust, cheap to raise, and require small quantities of food) and because it can give a reasonable indication of feed quality, which

would later be used for tests with commercial fish species, the final target of this research. Husbandry and experimental procedures complied with the European Legislation for the Protection of Animals used for Scientific Purposes (Directive 2010/63/EU) and with the Italian law on animal experimentation (D.L. 4 March 2014, n.26), and were performed according to the research permit n° 715/2022-PR (issued in accordance with art. 31 of D.lgs. 26/2014). At five days post-fertilization (dpf), larvae were transferred into 10.5 $\times$15.0 $\times$ 6.0 cm tanks containing the formulated water, and the day after they started to be daily fed until 15 dpf with food (0.005 $\pm$ 0.0004 g) differently formulated depending on the experimental group they belonged to (Table 1). The same density of fish was applied in each tank to avoid the impact of food availability on growth. Detailed procedures for producing and caring for fish eggs and larvae and for determining fish total length are described in (Pesante et al., 2022). Survival rates (percentage of animals in a feeding group that are still alive at the end of the experimental period) were calculated from 5 to 15 dpf because this first-feeding phase represents the most crucial period in larviculture (Best et al., 2010) (Monteiro et al., 2018), providing valuable indications of the nutritional value of a feed.

Since the CF contained 13 % oils (according to the manufacturer), and previous research indicated that the MP had no oils but that their addition is beneficial to fish survival, (Pesante et al., 2022; Zuliani, 2021), fish oil powder was added to groups B, C, D and E to reach the same concentration of the CF; however, this hypothesis was later found not to be accurate, since the MP contained some lipids and oils, resulting in a final oil concentration in the feeding group ranging from 13.0 % to 16.7 %. PHAs were added to groups C and D to evaluate the effects of added PHAs to the diet; in particular, group D was designed to test the addition of external PHAs to the CF and to compare the results to the control group that had no PHAs in the diet. The CF composition and the characteristics of the PHAs and Omega 3 fish oil powder used to supplement the diet of some groups, have also been described previously (Pesante et al., 2022). Comparisons between experimental and control groups were evaluated with the Kruskal-Wallis test, followed by Dunn's *post-hoc* test.

3. Results and discussion

3.1. Feedstock and fermentation fluid composition

The feedstock used for acidogenic fermentation was composed of 300 kg of leftover flour, 100 L of digestate and 700 L of water. The composition of the obtained FF is listed in Table 2. The COD was mostly soluble (97.0 % of the tCOD), and VFAs contributed to almost 30.0 % of the sCOD of the FF, with butyric acid being the most abundant of the VFAs (58.8 %), followed by acetic acid (33.8 %) and by small quantities of propionic and formic acid (5.0 and 2.4 % respectively). The presence of VFAs contributed to the low pH (4.7) of the FF, while the total volatile solids constituted most (73.0 %) of the content of total solids. Ammoniacal nitrogen was 80.0 % of the TKN, therefore organic nitrogen was only 20.8 % and most likely was a small part of the TN. As expected, PHAs were not present in the FF.

3.2. 16 S rRNA gene sequencing

Genomic analysis by next-generation sequencing of the 16 S rRNA gene was conducted at the beginning of the fed-batch experiment of phase 2, just after inoculating the flasks with *Thauera* sp. Sel9, to confirm the identity and purity of the bacterial strain. The same analysis was performed at the beginning of phase 3, to ensure that aseptic conditions were maintained and that only *Thauera* sp. Sel9 was present in the biomass obtained from the fed-batch experiment and destined for the fish-feeding trials. The analysis of both samples revealed the presence of only one strain with 100 % similarity (e-value 3e-127) to *Thauera* sp. strain Sel9 (accession number OP279920.1), confirming the purity of the bacterial culture and the MP used for the fish trials.

3.3. Composition and nutritional value of the bacterial biomass

The fed-batch experiment coupled with multi-spike feeding of phase 2 lasted 10 days and allowed the production of 5 g of dried biomass to be used as feed for the fish. Before proceeding with the feeding tests, the bacterial biomass was analysed for its composition and energy value, to understand if it could be a suitable substitute for commercial feed (Table 3).

Ash represented 20.7 % (w/w on a TS basis) of the MP's total solids, representing a potential source of minerals and trace elements for the fish. The remaining TS mainly comprised of proteins (59.5 %), a value well above 37.6 % and 44.8 %, which are the

Table 1
Composition of the diets used for the feeding trials of zebrafish. "Added values" indicate the mg added of each ingredient, while "final quantities" refer to the resulting final concentration in the diet, considering that oils and PHAs were already present in the CF and in the MP. Since the total amount considered is 100 mg, these values also represent overall percentages (w/w). CF = commercial feed, MP = microbial proteins.

Name	Description	Added quantities (mg)				Final quantities (mg)		
		CF	MP	oils	PHAs	Total oils	Total PHAs	Total proteins (from CF/MP)
Group A	**CF (control group)**	100.0	0.0	0.0	0.0	13.0	0.0	55.0 (55.0/0.0)
Group B	**CF + MP + oils**	50.0	43.5	6.5	0.0	14.9	7.5	53.4 (27.5/25.9)
Group C	**CF + MP + oils + PHAs**	50.0	38.5	6.5	5.0	14.6	10.8	50.4 (27.5/22.9)
Group D	**CF + oils + PHAs**	83.9	0.0	2.1	14.0	13.0	14.0	46.1 (46.1/0.0)
Group E	**MP + oils**	0.0	87.0	13.0	0.0	16.7	13.0	51.8 (0/51.8)

Table 2
Characterisation of the FF obtained from the acidogenic fermentation of the agricultural residues.

Parameter	Unit	Value
pH	-	4.7
TS	%	7.7
TVS	%	5.6
sCOD	g/L	51.3
tCOD	g/L	52.9
$N\text{-}NH_4$	g/L	1.7
TKN	g/L	2.2
Total VFAs	g/L	15.1
Formic acid	g/L	0.4
Acetic acid	g/L	5.1
Propionic acid	g/L	0.7
Butyric acid	g/L	8.9
PHAs	g/L	0.0

Table 3
Centesimal composition, nutritional and caloric value of the fermentation fluid (FF), microbial biomass (MP) and commercial feed (CF). The values are relative to 100 g of TS. Different methods were used to detect some of the compounds, therefore the sum of all values does not reach 100. <LoQ = <0.10 g/100 g of sample.

	FF (g)	MP (g)	CF (g)
Ash	0.0	20.7	10.6
Crude lipids and oils	0.0	4.3	10.7
Crude proteins	17.6	59.5	64.0
Carbohydrates (total)	0.0	4.5	0.0
Glucose	0.0	4.5	0.0
Fructose	0.0	<LoQ	0.0
Lactose	0.0	<LoQ	0.0
Saccharose	0.0	<LoQ	0.0
Maltose	0.0	<LoQ	0.0
Crude cellulose	0.0	<LoQ	0.0
Nitrogen-free extracts	82.4	15.5	14.7
PHAs	0.00	15.5	0.0
Energy (kcal)	70.6	280.7	356.7

recommended protein content for zebrafish diets for weight gain and maximum protein retention, respectively (Fernandes et al., 2016). When diets were prepared for the different groups of the feeding trials, CF (which has a protein content of 55.0 % as reported by the manufacturers) was mixed with MP, oils, and PHAs, making the protein content never lower than 46.1 % (Table 1), still above the recommendations. The MP protein content was in the range of that of the MP of bacterial origin, which is reported to be approximately 50–83 % (Matassa et al., 2016; Overland and Tauson, 2010). It was higher than that reported from the same strain of *Thauera* sp. Sel9 (41 % on MLSS), which was produced in a continuous stirred-tank reactor (CSTR) (Botturi et al., 2020) rather than in flasks in fed-batch mode and is therefore not directly comparable. However, comparison of the protein content with this strain or with other species of the genus *Thauera* is not possible, since no literature on the subject is available apart from the above cited CSTR-based research.

Carbohydrates made up 4.5 % (w/w) of the TS of the MP and were constituted entirely of glucose, with the other tested carbohydrates (fructose, lactose, saccharose and maltose) resulting below the level of quantification. The recommendation for zebrafish diets is to have at least 5 % carbohydrates (Robison et al., 2008), since they are a source of energy and their presence allows the utilisation of proteins for building body mass (weight gain) rather than as an energy source (Watts and D'Abramo, 2021).

Crude lipids and oils constituted 4.3 % (w/w) of the TS. Fish do not have a specific requirement for lipids; however, their presence is advised as a source of metabolisable energy and fatty acids. Fish belonging to cyprinids – the same family of zebrafish – living in similar habitats and having comparable diets have dietary requirements of lipids in the range of 10–18 % (Watts and D'Abramo, 2021). The recommended level for zebrafish is at least 12 % of the dry matter to meet energy and fatty acid requirements (Watts and D'Abramo, 2021). The MP was, therefore, short in lipids and oils, and the addition of external lipid sources was required before the fish feeding trials and in all the groups' diets it was set to equal the amount found in the CF (13 % as reported by manufacturers) for easier comparison of the effects of the different diets.

Micronutrients such as minerals and vitamins were not studied in the MP, due to the complexity of the analysis and the limited quantities of MP available.

The PHA content of the MP after the multi-spike feeding strategy was 15.5 % (w/w of the TS). This amount is lower than expected, since results with the same bacterial strain in CSTR mode reported a concentration of 41 % (w/w of mixed liquor suspended solids) (Botturi et al., 2020) and values up to 90 % of cell dry weight have been found for other pure cultures feed with waste materials

(Khatami et al., 2022).

The comparison with the FF highlights how *Thauera* sp. Sel9 bacteria could use the VFAs, transforming them into PHAs (from 0 % w/w of the TS in the FF to 15.5 % in the MP) and proteins (from 17.6 % to 59.9 % w/w of the TS). The increment in protein content is probably more pronounced than these values show, since protein amounts were calculated from the TN value using the conversion factor of 6.25 (Elgar et al., 2016). However, the TN of the FF was primarily made up of ammonia and therefore the protein content was low, while in the MP nitrogen was mostly found in an organic form, which is mainly composed of proteins. The growth of the bacteria also allowed the increase of the energetic value from 70.6 kcal of the FF to 280.7 kcal in the MP, nearly a quadruplication. Lipids, oils and carbohydrates were also obtained by the bacteria from conversion of the nutrients found in the FF, since none of these constituents were found in the FF.

A comparison of the MP to the CF highlights a higher ash content in the TS for the MP (20.7 % w/w of the TS compared to 10.6 %), lower lipid and oil levels (4.3 % w/w of the TS vz 10.7 %), similar quantities of nitrogen-free extracts (15.5 % w/w of the TS vs 14.7 %) and not too dissimilar amounts of proteins (59.5 % w/w of the TS vs 64.0 %), making the MP a good substitute for the protein fraction of the commercial feed.

The protein quality found in the MP was first assessed by its amino acid profile (Fig. 2, dark blue bars). All ten essential amino acids (EAA), which are those that the fish are not able to synthesise from metabolic intermediates and have to be present in the diet, were found in the MP; leucine and lysine were the most abundant of these and tryptophan, histidine and methionine were the least abundant, comparable to what was previously reported from PHA-containing MP used for fish feed (Pesante et al., 2022) and according to the abundance of amino acids in proteins (Krick et al., 2014). The presence and amount of EAAs are important because they have to be part of the diet to achieve optimal growth, otherwise lower growth rates are a consequence (Watts and D'Abramo, 2021). Glutamic acid, alanine, aspartic acid and leucine together made up the majority of all the amino acids (40.2 % w/w of total amino acids) of the MP, with methionine, tryptophan and cysteine being instead at the lowest end (3.9 %). Lysine, a limiting amino acid whose concentration affects the feed's biological value (Jarmołowicz and Zakęś, 2014), was the 5th most abundant. When the MP's amino acid profile is compared to the ideal zebrafish protein profile proposed by Kaushik and colleagues (Kaushik et al., 2011) and to the commercial feed (Fig. 2, light blue and grey bars, respectively), the MP shows a very similar trend, with the same most and least abundant amino acids and only very few amino acids being present in different proportions; this is a good indication of how the produced bacterial biomass could be suitable to use as zebrafish feed.

The proportional amount of each EAA is also important, since amino acids in limiting quantities can potentially reduce fish growth (Hepher, 1988). The chemical score is a value that compares each amino acid quantity to that of a reference protein, further indicating the quality of a given protein (Bunda et al., 2015). Fig. 3 reports the EAA chemical score of *Thauera* sp Sel9 MP when compared to the ideal zebrafish protein profile, the recommended diet of the common carp and the commercial feed used in the feeding trials.

The CS expresses each EAA as a percentage of the amount of the same EAA in the reference diet, and therefore a value of 100 indicates the presence of the same quantity as in the tested protein. The results show that the MP has an EAA content similar to that of the common carp, with leucine, isoleucine and tryptophan being particularly abundant, while phenylalanine and methionine represent possible limiting amino acids. The comparison to the ideal zebrafish protein profile highlights only valine as a potential limiting EAA, while the other amino acids are very similar to the reference protein. Finally, the commercial feed seems to be richer in most of the EAAs (only phenylalanine and histidine scoring a CS of 100 or more), a result that was expected since commercial feeds are specially formulated to have the best possible nutritional value, mixing fishmeal, soybean meal and other highly nutritious ingredients, thus producing a complete protein mix challenging to find in nature. The highlighted presence of possible limiting EAAs, however, does not have to be taken as an indication of a protein with low nutritional value, since this is calculated based on the contribution of all EAA, due to the compensation that occurs from other amino acids (Bunda et al., 2015; Oser, 1959).

To assess the MP protein biological quality, the essential amino acid index was calculated using the same reference proteins used for the CS and the protein composition of the fishmeal, the standard diet in aquaculture (Fig. 4). The EAAI is used to evaluate the biological

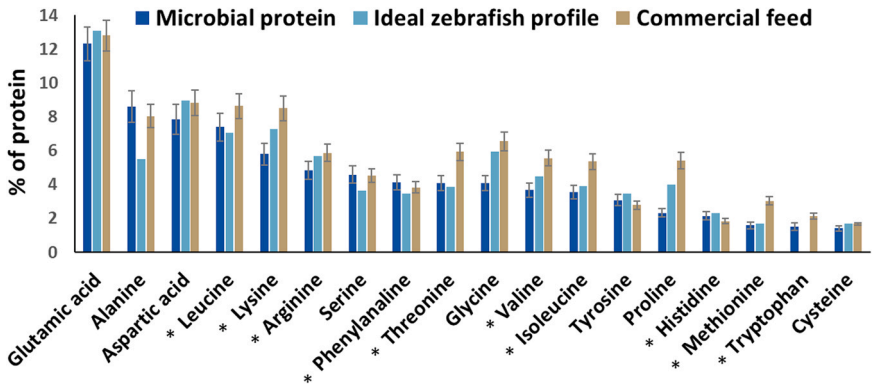

Fig. 2. Protein content and amino acid profile. Microbial biomass, ideal zebrafish protein profile and commercial feed are compared and essential amino acids are highlighted with an asterisk (*). Data for tryptophan and standard errors are not available for the ideal zebrafish profile, which was obtained from Kaushik *et al* (Kaushik et al., 2011).

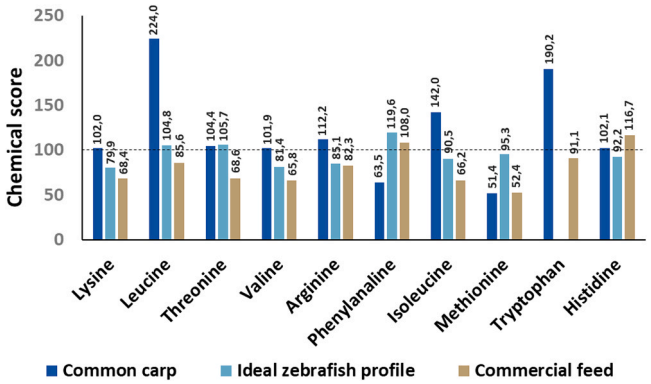

Fig. 3. Chemical score of the microbial biomass for the essential amino acids. The comparison was performed with the ideal zebrafish protein profile, the recommended diet of the common carp and the commercial feed used in the feeding trials. The dashed line represents the value of 100, at which the analysed protein has the same amino acid content as the reference protein. Data for tryptophan were not available for the ideal zebrafish profile.

quality of a protein (Machado et al., 2020; Oser, 1959; Wilson and Halver, 1986), with proteins of good quality scoring 0.9 or more, proteins defined functional as 0.8 and incomplete proteins having a score of 0.7 or less (Bunda et al., 2015). When the MP is compared to the common carp and to the ideal zebrafish protein profile, a good (0.95 for the ideal diet) or more (1.09 for the carp) protein quality is highlighted. When the evaluation was performed against commercial feed and fishmeal, the EAAI scores were lower (0.78 and 0.89, respectively); nevertheless, MP appeared to be of useful quality and a potential protein substitute for fish.

3.4. Feeding experiments

After evaluating composition, nutritional value and protein quality, the MP was tested as fish feed on zebrafish, to assess its actual effects on the animals' survival and growth rates and to highlight any possible toxic effects.

After a comprehensive 15-day trial, groups B and C exhibited a survival rate comparable to that of group A (CF-fed fish) whereas, the administration of MP enriched with oils (group E) led to a significant increase in mortality ($p<0.001$), as depicted in Fig. 5. The reduced survival of group E could be a consequence of a lack of micronutrient, as well as the effect of the heterogeneous particle size of the MP compared to the CF, which would reduce the digestibility of the feed. Indeed, pictures taken with a stereo microscope of both feeds (Figure S1) highlight that, despite grinding and milling of the MP, it wasn't possible to achieve the same homogeneous granulometry of the CF (80–200 μm) and particle of sizes bigger than 200 μm were present.

Notably, a survival rate higher than the reference group was achieved when zebrafish embryos received CF together with oils and PHAs (group D). The addition of PHAs to both the reference diet and the MP-CF diet showed contrasting results, since survival rates were significantly higher when PHAs were added to the commercial feed (91.2 % vs 78.0 %), while no significant difference – but a slight decrease (80.0 % vs 88.0 %) – was found for the diet with MP. However, the MP already contains 15.5 % of PHAs, which could

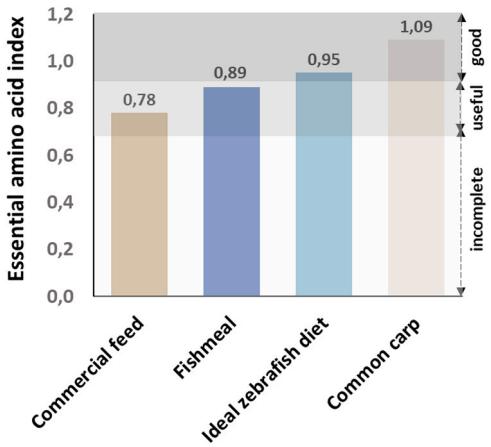

Fig. 4. Essential amino acid index of the microbial biomass. The comparison was performed with the ideal zebrafish protein profile, the recommended diet of the common carp and the commercial feed used in the feeding trials. The horizontal dashed lines indicate the range in which a protein is considered incomplete, useful or of good quality. Data for tryptophan were unavailable for the ideal zebrafish profile; therefore, the value was equal to that of the microbial protein.

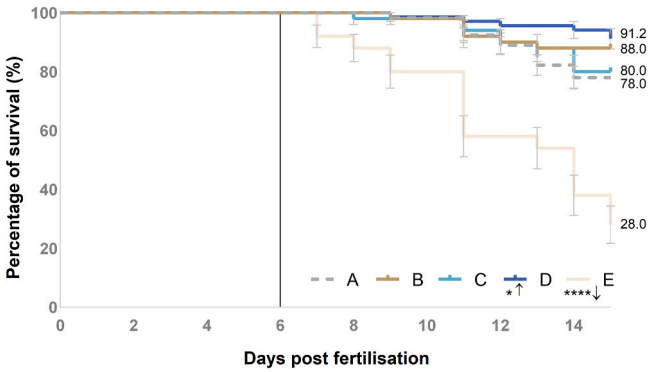

Fig. 5. Survival rate of zebrafish during the feeding trials. The mortality was recorded every day until 15 days post-fertilization, the vertical line shows the day when feeding of the larvae started. Group A (dashed line), whose fish were fed with commercial feed, was used as a control group, group B = 50 % CF and 50 % MP, group C = group B + PHAs, group D = group A + PHAs, group E = MP only. (Mantel-Cox test, *p≤0.05, ****p<0.001).

be the reason behind the reduce effect of the addition of commercial PHAs. Furthermore, PHAs still contained inside bacterial cells are found in amorphous form (Thai et al., 2014), which is easier to degrade than crystalline PHAs (Defoirdt et al., 2009).

Adding fish oil or PHAs improved fish survival to similar values to the reference group. Similar results were also obtained when measuring the fish's total body length and eye diameter (Fig. 6), even though results higher than the control group were never achieved for this morphological parameter. In particular, the growth rates in group A and D were found to overlap while in the other experimental groups (B, C, E) a reduced body length was observed compared to the reference group (A). It should be noted that these tests were performed on larvae, which have higher nutritional requirements than adults, as they are still under development (Watts and D'Abramo, 2021; Jarmołowicz and Zakęś, 2014). Therefore, adults having lower metabolic requirements could show better survival and growth rates than those obtained during these experiments with larval fish.

Further work is needed to assess the reason being the decrease in survival and growth rates of fish fed with MP only, which is likely to be attributed to limiting amounts of specific micronutrients which were not looked for in our analyses, in a reduced ingestion of the MP-based food due to incorrect granulometry, or in MP's digestibility issues (Sheeshka and Murkin, 2002). An improved assessment could be performed by formulating the feeds in a way that only the protein content would be different between the groups (MP for a group and CF for the other), with all the other ingredients being the same, allowing a direct comparison. Furthermore, preliminary digestibility tests for the feeds, and the evaluation of the amount of feed actually ingested by the fish would also help in assessment of the MP as fish feed.

The use of pure cultures and the lack of any toxic effect on the fish are an improvement towards the commercialisation of the final product – fish feed for aquaculture – when compared to similar products obtained from MMC (Pesante et al., 2022). A further step toward a possible commercial use of these MP is the performance of safety analyses (which include a precise taxonomic identity and the evaluation of antibiotics resistance, determinants, antimicrobial production and pathogenic traits) of *Thauera* sp. Sel9 for its inclusion in the qualified presumption of safety (QPS) list according to the European Food Safety Agency (EFSA, 2007; Rychen et al., 2018).

4. Conclusions

Agricultural residues provided an excellent feedstock for fermentation and bioconversion into microbial proteins by the pure strain *Thauera* sp. Sel9 . The bacterial biomass produced in our study had an improved energetic content and protein and amino acid profile when compared to the original fermentation fluid, and was also rich in PHAs, which are considered appealing food supplements in aquaculture for their antibacterial action. The good quality of the MP, particularly when compared to the recommended zebrafish and common carp diets, allowed its use as partial substitute for commercial feed with larval stages of zebrafish, showing similar performance to the reference diet, or even improved survival when PHAs were added. Further work is needed to assess the reason being the decrease in survival and growth rates of fish fed with MP only, which is likely to be attributed to limiting amounts of specific micronutrients that were not looked for in our analyses, to a reduced ingestion of the MP-based food due to incorrect granulometry, or to the MP's digestibility issues (Sheeshka and Murkin, 2002).

CRediT authorship contribution statement

Silvia Lampis: Writing – review & editing, Investigation, Data curation. **Andrea Vettori:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation. **Nicola Frison:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Giovanna Pesante:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Chiara Tesoriero:** Writing – review & editing,

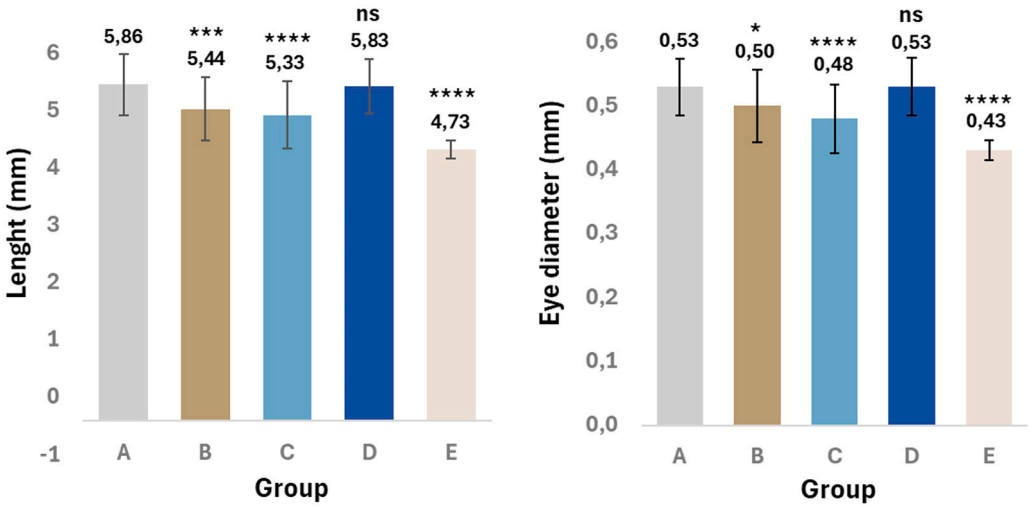

Fig. 6. Body length and eye diameter of zebrafish after the feeding trials. (Mantel-Cox test, *p≤0.05, ***p≤0.01, ****p<0.001, n =not significative).

Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation. **Emma Cadoria:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Marco Andreolli:** Writing – review & editing, Investigation, Data curation.

Declaration of Competing Interest

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

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.eti.2024.103772.

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