Arch Biol Sci. 2024;76(2):191-203 https://doi.org/10.2298/ABS240403013G

Use of bacterial-based single-cell protein MRD-Pro® in diets for Nile tilapia (*Oreochromis niloticus*) fry

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Received: April 3, 2024; **Revised**: April 18, 2024; **Accepted**: April 19, 2024; **Published online**:May 10, 2024

Abstract: The research assessed the inclusion of MRD-Pro®, a bacterial-derived single-cell protein (SCP), in the diets of Nile tilapia (*Oreochromis niloticus*) fry with an initial weight of 0.12 grams. Using a diet composed of 45% crude protein and 14% crude fat, with an initial fishmeal content of 8.0% (designated as Diet 0.00% SCP, the control), SCP replaced 50% and 100% of the fishmeal on a protein basis, incorporated at levels of 4.25% and 8.50%, respectively. In addition, two more diets were prepared with higher levels of SCP, 14.50% and 21.00%. All diets were isoproteic and isolipidic. Weight gains of fish fed with the control diet (27.26 g) and the 4.25% SCP diet (21.61 g) were statistically comparable among themselves but were significantly greater than those of fish fed the 8.50% SCP (10.45 g), 14.50% SCP (11.54 g), or 21.00% SCP (7.28 g) diets, a trend observed across all growth and feed utilization indices. Increasing dietary SCP significantly reduced the crude fat and dry matter content in fish muscle tissue, while minimal changes in the amino acid profile of fish muscle tissue were observed. The bacterial-based SCP MRD-Pro® is a nutritious feed additive that can be effectively incorporated, within limits, into the diet of tilapia fry.

Keywords: bacterial-based single-cell protein, Nile tilapia, fry, fishmeal replacement

Abbreviations: single cell protein (SCP), soybean meal (SBM), fishmeal (FM), specific growth rate (SGR), feed intake (FI), feed conversion ratio (FCR), protein efficiency ratio (PER), Fulton's condition factor (K), crude protein (CP), amino acid (AA), ortho-phthalaldehyde (OPA), analysis of variance (ANOVA)

INTRODUCTION

The cultivation of tilapia (*Oreochromis niloticus*) is increasing, with production reaching 4.4 million metric tons in 2020; it is currently in third position among the top ten farmed finfish species globally [1]. Unlike carnivorous finfish species, for which fishmeal and fish oil are used to prepare feeds [2], tilapia is a lower-trophic level species [3]. This has contributed to the success of commercial tilapia cultivation, as it exhibits adaptability to various sources of dietary protein, e.g., plant and animal sources, terrestrial animal by-products, and a range of unconventional protein sources [4-6]. Nevertheless, low levels of fishmeal (3 to 10%) are still incorporated into tilapia diets to ensure optimal growth [3]. The need for fishmeal is heightened in commercial diets for tilapia larvae and fry since the earlier stages have higher nutritional requirements for protein (35-50% or higher) compared to juveniles (30-40%) or adults (20-30%) [4]. As with other finfish species, reducing reliance on fishmeal in the tilapia diet, especially in the earlier stages, remains an ongoing challenge. A variety of unconventional protein feedstuffs have been tested as fishmeal replacements in order to accomplish this goal. Among them, meals derived from dead and dry microbial whole cells or extracts, collectively known as single-cell protein (SCP), are emerging as attractive alternatives [7]. In particular, bacterial-based SCPs stand out with their high protein (50-80% of the dry weight) and low-fat content, good digestibility, and comprehensive essential amino acid, vitamin, and mineral content. With short bacterial generation times, this type of SCP can be produced using a variety of low-cost substrates, such as agricultural by-products and wastes, and hydrocarbon substrates, e.g., methane and methanol [7-11).

Numerous recent studies have investigated the use of bacterial-derived SCPs as substitutes for fishmeal in the diets of various food fish species, yielding predominantly positive outcomes, including salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), turbot (*Scophthalmus maximus*), halibut (*Hippoglossus hippoglossus*), yellow tail (*Seriola quinqueradiata*), and barramundi (*Lates calcarifer*), among others [12-19]. For Nile tilapia (*O. niloticus*), 50% of dietary fishmeal was successfully replaced by a bacterial protein meal (*Methylophilus methylotrophus*) [20]. However, reduced growth was reported when dietary SCP derived from *Micrococcus glutamicus* exceeded a 10% inclusion level [21]. Statistically comparable diet digestibility and growth performance were observed in tilapia fed with diets containing 0 or 15% SCP (a mixture of *Lactobacillus* and brewer's yeast) in place of fishmeal [22]. Satisfactory growth and improved immune response of tilapia were reported after replacing dietary fishmeal with bacterial SCP (*Methylococcus capsulatus*) at levels ranging from 0 to 8.5% [23]. Finally, incorporating 0, 5, 10, 15, and 20% of bacterial SCP (*Clostridium autoethanogenum*) in the diet of tilapia did not alter growth. However, it did result in impaired intestinal histology, particularly noticeable at or above dietary SCP levels of 10% [24,25]. The studies mentioned above focused on juveniles with an initial weight of 0.7 to 55 g. Bearing in mind that fishmeal is still being used in commercial diets for young tilapia fry, which are nutritionally more demanding than older fish in terms of both the quantity and quality of dietary protein, the use of bacterial-based SCP, such as MRD-Pro®, to replace fishmeal during this phase is of interest. MRD-Pro® is a bacterial-based SCP produced by the proprietary MTech SCP Process (Meridian Biotech, LLC, The Woodlands, Texas, USA), a hybrid of fermentation and advanced wastewater treatment technologies utilizing proprietary mixtures of bacteria. The technology is uniquely adaptable to utilizing multiple organic coproducts and waste stream feedstocks. MRD-Pro® is a promising alternative to enhance the sustainability, nutrition, and productivity of aquaculture operations. However, the use of MRD-Pro® in diets for Nile tilapia fry has not been explored. Therefore, the present investigation aimed at evaluating the effects of incremental levels of dietary MRD-Pro® on growth, feed utilization, and proximate and amino acid composition of muscle tissue of Nile tilapia (*O. niloticus*) fry.

MATERIALS AND METHODS

Ethics statement

The authors attest that procedures for the farming and handling of organisms complied with the Official Mexican Norm (NOM-062-ZOO-1999) on the Technical Specifications for the Production, Care, and Use of Laboratory Animals [26] and the National Research Council's Guide for the Care and Use of Laboratory Animals [27].

Experimental fish and culture system

Monosex (all-male) Nile tilapia (*O. niloticus*) fry purchased from the hatchery CRILAP S.P.R. de R.L., San Pedro el Saucito, Sonora, Mexico, were transported to the Wet Laboratory of Aquaculture Nutrition of the Kino Bay Experiment Station, University of Sonora at Kino Bay, Sonora, Mexico. Fish were randomly placed in a recirculating aquaculture system consisting of twenty-four 250-L circular tanks, each filled with 150 L of filtered freshwater. Water from a 1,100-liter sump tank was circulated using a 1.5-HP pump (Jacuzzi, Model 150MFT, Little Rock, AR, USA). The circulation process involved passing through a sand filter (Jacuzzi, Model L-190-7, Little Rock, AR, USA), a biofilter, a 1500-W inline heater (Model DE-6115, Aquatic Ecosystems, Apopka, FL, USA), a 120-W inline ultraviolet light chamber (Rainbow Lifeguard, Model UV97, El Monte, CA, USA), before being directed into the culture tanks. Finally, the water returned to the sump tank. Tanks were individually provided with continuous aeration using a 1.0-HP blower (Fuji, Model VFC40, Saddle Brook, NJ, USA) and submerged air stones. Tilapia fry with an initial wet body weight of 0.12±0.00 g (mean±standard error of the mean, SEM) were randomly stocked into tanks at a rate of 10 fish/tank. Uneaten feed and feces were siphoned out of the tanks each morning. Daily water quality monitoring involved measuring dissolved oxygen concentration and temperature using a multifunction oxygen meter (YSI, Model Pro2030, Yellow Springs, OH, USA). Weekly measurements were made of the concentrations of total ammonia nitrogen and nitrite using a Hach spectrophotometer (Model DR3900, Loveland, CO, USA), and pH, using a benchtop meter (pH/ISE, Thermo Scientific, Model Orion 4-Star pH/ISE, Beverly, MD, USA). Mean values for these

measurements were 5.61±0.07 mg O2/L, 29.05±0.09°C, 0.08±0.03 mg NH4-N/L, 0.05±0.02 mg NO2-N/L, and 8.07±0.21, respectively, all within adequate levels for Nile tilapia culture [28].

Experimental treatments and diet preparation

Using soybean protein isolate and soybean meal (SBM) as the primary protein sources, a baseline diet (45% crude protein, 14% crude fat) was formulated to contain 8.0% fishmeal (FM) (Diet 0.00%, control). Then, 50 and 100% FM from the control diet were replaced, on a protein basis, by incorporating the bacterial-based SCP MRD-Pro® (Meridian Biotech, LLC, The Woodlands, Texas, USA) at levels of 4.25% (Diet 4.25%) and 8.50% (Diet 8.50%), respectively. Two more diets were prepared with higher levels of bacterial-based SCP, 14.50% (Diet 14.50%) and 21.00% (Diet 21.00%). All diets were isoproteic and isolipidic. The control diet was supplemented with methionine and lysine to meet the quantitative dietary requirements for tilapia [29,30], while the other diets were supplemented to match the calculated levels in the control diet (Supplementary Table S1). The moist dough of each diet was passed through the meat grinder attachment (3-mm die) of a Hobart mixer (Hobart Corporation, Model A-200, Troy, OH, USA) and oven-dried overnight at 40°C. These were then ground and kept at -20°C until used. Experimental treatments were randomly assigned to four replicate tanks. Fish were fed to apparent satiation (aided by visual cues to monitor consumed feed and adjust the ration) with pre-weighed daily feed rations, which were divided into three portions, administered at 08:30, 13:30, and 19:00 h.

Fish performance

After 42 days of feeding, fish were grouped, weighed, and counted by tank to determine the final weight (g). Weight gain (gr) was calculated as the difference between final weight (gr) and initial weight (gr). Daily weight gain (gr per day) was determined by dividing weight gain by the duration of time (in days). Percent weight gain (%) was computed as the ratio of weight gain to initial weight multiplied by 100. Specific growth rate (SGR, % per day) was calculated using the formula: (ln final weight - ln initial weight) / time (days) × 100. Survival rate (%) was determined by multiplying the final number of organisms by 100 and then dividing by the initial number of organisms. Feed utilization was evaluated through feed intake (FI), calculated as feed consumed (in grams) per fish per day; feed conversion ratio (FCR), calculated as feed consumed (gr) divided by weight gain (gr); and protein efficiency ratio (PER, gr per gr), calculated as weight gain (gr) divided by protein consumed (gr).

Proximate and amino acid composition

At the end of the feeding trial, selected fish were euthanized in chilled water (4°C) with an overdose of tricaine methasulfonate (MS-222, 300 mg/L, Sigma-Aldrich, St. Louis, MO, USA). Fish were individually weighed and measured to determine Fulton's condition factor K = (weight (g) / total length 3 (cm)) × 100 [31]. Then, the fillet was excised to determine the proximate and amino acid composition of composite muscle tissue samples, each composite sample consisting of muscle tissue from three fish randomly taken from each of 4 tanks per treatment, which were homogenized and stored at -20 °C until analysis. For diets, the proximate and amino acid composition was determined on triplicate samples. For the proximate composition analysis, crude protein (CP) was determined via combustion using a Dumas Nitrogen Analyzer (Model NDA 702, VELP® Scientifica, Usmate, Italy) and Dumas method (N factor = 6.25; method 968.06) [32]. Crude fat was determined using a gravimetric method [33]. Moisture and ash were determined by the standardized methods 930.15 and 942.05, respectively, of the Association of Official Analytical Chemists [32]. For diets only, the gross energy content was measured with an adiabatic bomb calorimeter (Model IKA C5003; IKA-Werke GmbH, Staufen, Germany) (Supplementary Table S1).

The amino acid (AA) composition was analyzed by high-performance liquid chromatography (HPLC) [34]. After the gravimetric lipid extraction [33], dried ground muscle samples (3.0 mg) were digested in 3 mL 6 N HCl with 3.0 mg of sodium thioglycolate (Cat. 106691, Merck, Darmstadt, Germany) for 6 h at 150°C in 6-mL vacuum hydrolysis tubes (Cat. 29,751, Thermo Scientific, Rockford, IL, USA). The hydrolyzed samples were evaporated under a vacuum in a rotary evaporator (IKA HB 10 Digital, IKA Works Inc., Wilmington, NC, USA) at 65°C, rinsed twice with 3

mL HPLC water and reconstituted in 1 mL of sodium citrate buffer (pH 2.2). A 200-µL aliquot of each sample was transferred into a 1-mL volumetric flask with 40 µL of internal standard (α-aminobutyric acid) and brought to 1 mL with sodium citrate buffer. Finally, subsamples of 250 µL were derivatized with 250 µL of ortho-phthalaldehyde (OPA) solution, filtered through a 0.22-µm nylon syringe filter, and a 10 µL aliquot injected into high-performance liquid chromatography (HPLC Varian 9012, Walnut Creek, CA, USA) equipped with a Microsorb 100 C18 column (Agilent Technologies, Middleburg, Netherlands) coupled to a fluorescence detector (Varian Pro-Star Fluorescence Detector). Excitation and emission were set at 340 and 455 nm, respectively. Identification of AAs was performed by comparison of retention times to a known AA standard solution (amino acid Standard H, Cat. 20088, Thermo Scientific Pierce, Bothell, WA, USA), and quantification was performed by computation of the areas against the internal standard. Values were expressed as g/100 g of dry tissue for muscle samples and as g/100 g dry diet for feeds (Supplementary Table S2).

Statistical analysis

Fish performance (growth, survival, feed utilization), proximate and amino acid composition data were analyzed by one-way analysis of variance (ANOVA), with a significance level of $P<0.05$ (prior confirmation of homocedasticity and normality by Bartlett's and Shapiro-Wilk's tests, respectively). Survival data were transformed by arcsine square root before analysis, but untransformed data are presented. Tukey's honestly significant difference test was employed as the mean separation procedure when significant differences were detected. Orthogonal polynomial contrasts analysis was performed to thoroughly examine the relationship between the dietary level of bacterial-based SCP and the various response variables. All statistical analyses were performed using the Statistical Analysis System software (SAS Institute Inc., 2013, Software Release 9.4, Cary, NC, USA).

RESULTS

Fish fed with the control diet (devoid of SCP, 0.00%) and the diet containing 4.25% SCP (replacing 50% FM) had statistically comparable growth performances. In contrast, the growth performances of both of these treatments were significantly better, based on one-way ANOVA, than those of fish fed higher levels of SCP (8.50%, 14.50%, and 21.00%). For instance, fish fed the 0.00% and 4.25% SCP diets exhibited significantly higher ($P<0.0001$) weight gains of 27.26 g and 21.61 g, respectively, compared to those fed the 8.50% (10.45 g), 14.50% (11.54 g), and 21.00% SCP (7.28 g) diets. Also, fish fed both the 0.00% and 4.25% SCP diets (1.84 and 1.76 g/g, respectively) exhibited a significantly greater ($P<0.0001$) protein efficiency ratio than fish receiving the diets 8.50%, 14.50%, and 21.00% SCP (1.13, 1.14, and 0.93 g/g, respectively) (Table 1). A similar trend of better performance of fish fed the control diet 0.00% and 4.25% SCP vs. 8.50%, 14.50%, or 21.00% SCP, was also observed across feed utilization indices. For the condition factor, K, fish fed the control diet had a significantly greater value (2.15) than the rest of the treatments, but 4.25% SCP (1.93) was also statistically greater than 8.50%, 14.50, and 21.00% SCP (1.71, 1.72, and 1.68, respectively). Based on orthogonal polynomial contrasts analysis, there was a linear decrease in all the growth indices, as well as feed intake, protein efficiency ratio, K, and survival, with the increment in the dietary level of SCP, while FCR exhibited a linear increase (Table 1). Quadratic and/or quartic trends were also detected for some of the growth performance variables; however, the P values for the linear trends were statistically more significant (Table 1).

The crude fat content in the muscle tissue of fish decreased linearly ($P<0.0001$) with the increasing level of incorporation of the bacterial SCP, as detected by orthogonal polynomial contrasts analysis (Table 2). Such decrement, as shown by one-way ANOVA, was due to crude fat contents of 4.59% and 4.48% for fish fed the 0.00% and 4.25% SCP diets, respectively, which did not significantly differ from each other. However, they were both significantly higher ($P=0.0001$) than those of fish fed the 8.50% (3.46%), 14.50% (3.41%), and 21.00% SCP (3.12%) diets. In addition, a significant ($P=0.0003$) linear decrement in the dry matter content of muscle was observed as the level of dietary SCP increased. Fish fed the control 0.00% and 4.25% SCP diets had the highest dry matter contents (24.64 and 23.38%, respectively) and were both significantly higher ($P = 0.0011$) than those of fish fed the 8.50% (22.33%), 14.50% (22.71%), or 21.00% SCP (22.51%) diets (Table 2). The contents of crude protein and

Table 1. Growth performance and feed utilization indices of Nile tilapia fry fed increasing levels of bacterial-based single cell protein meal MRD-Pro®

	Dietary levels of SCP					Orthogonal polynomial contrasts (P>F)				
	0% (Control)	4.25%	8.50%	14.50%	21.00%	ANOVA P>F	Linear	Quadratic	Cubic	Quartic
Initial weight (g)	0.12±0.00	0.12±0.00	0.12±0.00	0.12±0.00	0.12±0.00	0.3445	0.9227	0.1003	0.7712	0.2114
Final weight (g)	27.38^{a}±1.08	21.73^{a}±2.71	10.57^{b}±0.63	11.66^{b}±1.84	7.40^{b}±0.72	< 0.0001	< 0.0001	0.0240	0.9752	0.0185
Weight gain (g)	27.26^{a}±1.08	21.61^{a}±2.71	10.45^{b}±0.63	11.54^{b}±1.84	7.28^{b}±0.72	< 0.0001	< 0.0001	0.0239	0.9760	0.0185
Daily weight gain (g/day)	0.65^{a}±0.03	0.52^{a}±0.06	0.25^{b}±0.01	0.28^{b}±0.04	0.18^{b}±0.02	< 0.0001	< 0.0001	0.0249	0.9512	0.0190
Percent weight gain (%)	22720.54^{a}±891.04	18032.80^{a}±2223.76	8858.58^{b}±551.97	9658.97^{b}±1552.81	6063.92^{b}±630.65	< 0.0001	< 0.0001	0.0274	0.9830	0.0205
Specific growth rate (%/day)	12.92^{a}±0.09	12.33^{a}±0.30	10.69^{b}±0.15	10.80^{b}±0.43	9.78^{b}±0.24	< 0.0001	< 0.0001	0.3912	0.9177	0.0246
Survival (%)	95.00±5.00	93.00±4.79	73.00±8.54	73.00±11.81	73.00±6.29	0.1099	0.0181	0.4019	0.4859	0.3890
Feed intake (g/fish/day)	0.78^{a}±0.04	0.64^{ab}±0.06	0.50^{ab}±0.06	0.56^{ab}±0.12	0.43^{b}±0.03	0.0264	0.0029	0.4168	0.4139	0.3474
Feed conversion ratio	1.20^{c}±0.01	1.26^{bc}±0.05	1.99^{a}±0.24	1.93^{ab}±0.13	2.38^{a}±0.24	0.0001	< 0.0001	0.9808	0.7402	0.0641
Protein efficiency ratio (g/g)	1.84^{a}±0.02	1.76^{a}±0.06	1.13^{b}±0.14	1.14^{b}±0.09	0.93^{b}±0.12	< 0.0001	< 0.0001	0.3224	0.3004	0.0239
Condition factor	2.15^{a}±0.03	1.93^{b}±0.06	1.71^{c}±0.04	1.72^{c}±0.01	1.68^{c}±0.04	< 0.0001	< 0.0001	0.0013	0.6430	0.1570

Values are means±SEM of four replicate tanks. Means with different superscripts within the same row are significantly different (P<0.05). SCP – single-cell protein.

Table 2. Determined proximate composition (% of wet weight) of muscle tissue of Nile tilapia fry fed increasing levels of bacterial-based single-cell protein meal MRD-Pro®.

		Dietary levels of SCP					Orthogonal polynomial contrasts (P>F)				
	Initial fish	0% (Control)	4.25%	8.50%	14.50%	21.00%	ANOVA P>F	Linear	Quadratic	Cubic	Quartic
Dry matter (%)	30.36	24.64^{a}±0.11	23.38^{ab}±0.51	22.33^{b}±0.31	22.71^{b}±0.39	22.51^{b}±0.17	0.0011	0.0003	0.0117	0.4689	0.2555
Crude protein (%)	23.81	23.03±0.46	22.95±0.22	23.19±0.50	23.01±0.29	23.37±0.22	0.9208	0.5272	0.7313	0.8592	0.5788
Crude fat (%)	4.06	4.59^{a}±0.19	4.48^{a}±0.16	3.46^{b}±0.19	3.41^{b}±0.18	3.12^{b}±0.06	0.0001	< 0.0001	0.2619	0.5273	0.1261
Ash (%)	1.14	1.18±0.01	1.12±0.04	1.09±0.04	1.14±0.02	1.15±0.04	0.3873	0.6392	0.0905	0.5069	0.4978

Values are means±SEM of four composite samples, each consisting of tissues from three fish per tank. Means with different superscripts within the same row are significantly different (P<0.05). The initial fish sample consisted of one large sample of initial fish, ca. 100 organisms. SCP – single-cell protein.

ash in muscle tissue were not affected by the level of incorporation of bacterial SCP (Table 2).

A significant (P=0.0018) linearly reduced content of glutamic acid was observed as dietary SCP increased. As shown by one-way ANOVA, fish fed the control 0.00% and 4.25% SCP diets had significantly higher (P=0.0199) contents (9.62 and 9.34 g/100 g dry tissue, respectively) than fish fed the 21.00% SCP diet (6.71 g/100 g dry tissue), but did not display significantly higher levels than fish fed the 8.50% or the 14.50% SCP diets (8.81 and 7.89 g/100 g dry tissue, respectively). The contents of all other AAs, essential or non-essential, were not statistically affected by the diet (Table 3).

DISCUSSION

The present study demonstrated that 50% of the protein provided by fishmeal in a control diet, with a baseline fishmeal content of 8.0%, could be substituted with the bacterial-based SCP MRD-Pro® without significantly altering growth, feed utilization, or survival of tilapia fry. Because fishmeal was replaced on a protein basis, this represents an actual level of incorporation of SCP of 4.25%. Conversely, fish performance was significantly depressed as dietary SCP was progressively increased to levels of 8.50%, 14.50%, or 21.00%. These results concur with the notion, supported by previous studies of tilapia and other fish species, that bacterial SCP represents a nutritious feed ingredient that can be effectively incorporated, within limits, into the diet. Surprisingly, despite its economic significance, the body of research conducted on this topic with tilapia is unexpectedly limited. In earlier research, up to 50% of fishmeal could be successfully replaced by SCP derived from *M. methylotrophus* [20], while the growth of tilapia (1.4 g) was adversely impacted when levels of SCP (*M. glutamicus*) exceeded 10% of the diet [21]. Using a mixture of *Lactobacillus* sp. and brewer's yeast, added at levels of 0% (control) and 15% of the diet, adequate growth of tilapia (ca. 55 g) was observed [22]. Recently, it was reported that tilapia (3 g) exhibited good growth and enhanced immune response when bacterial SCP (*M. capsulatus*) replaced fishmeal at levels ranging from 0 to 8.5% of the diet. [23]. In turn, incorporating up to 20% SCP (*C. autoethanogenum*) did not affect the growth of tilapia (0.7 g); however, intestinal histology damage was observed with SCP substitution at or above 10% [24,25]. In the only other study identified in which tilapia fry was used (0.078 g), improved growth was observed after feeding bacterial SCP (*Rfhodovulum sulfidophilum*) [35]. However, the diet design was somewhat unorthodox, consisting of (i) a commercial feed (control diet) and (ii) a combination of SCP:commercial feed, pelleted together at a 1:2 ratio (w/w). Consequently, the diets were neither isoproteic nor isolipidic and had different contents of crude fiber. For this reason, the results may not be conclusively attributed solely to the effects of dietary SCP, and comparison with the results of the present study would be inaccurate. Overall, the level of SCP incorporation that provided sufficient growth of tilapia in this study, 4.25% of the diet, appears relatively modest compared to findings from previous research. This result is likely associated with the size of the fish employed, 0.12 g in this study vs. 0.7-55.0 g for fish in the previous studies (excluding the study using tilapia fry [35]). However, considering the protein requirements for tilapia larvae and fry (35-50% or higher) are higher than those for juveniles (30-40%) and adults (20-30%) [4], it is unsurprising that tilapia fry in this study, requiring higher quantities and quality of the dietary protein, exhibited relatively lower tolerance to bacterial SCP in the diet compared to larger fish in previous studies. Despite the relatively modest substitution level, the results are significant from an environmental perspective, considering the possibility of halving the expensive and ecologically unsustainable inclusion of fishmeal [36] by using bacterial SCP, an ingredient with a substantially reduced environmental footprint [37].

The interest in the use of bacterial-based SCP as fishmeal replacement has spread to food fish species; thus, for salmon (*S. salar*), dietary levels of SCP from 4.0 to 36.0% have been used with good results [12,13,38,39]; for rainbow trout (*O. mykiss*), bacterial SCPs have been used successfully at levels ranging from 4.0 to 28.0% of the diet [14,18,40,41]. The flatfish, halibut (*H. hippoglossus*) and turbot (*S. maximus*), have shown good growth at maximum levels of SCP of 9.0% [15] and 18.37% [19,42] of the diet, respectively. Other species for which some level of bacterial-based SCP has successfully been incorporated into the diet include Jian carp (*Cyprinus carpio*), largemouth bass (*Micropterus salmoides*), yellowtail (*S. quinqueradiata*), barramundi (*Lates calcarifer*), and black sea bream (*Acanthopagrus schlegelii*)

Table 3. Determined amino acid composition (g/100 g of dry tissue) of muscle tissue of Nile tilapia fry fed increasing levels of bacterial-based single cell protein meal MRD-Pro®.

		Dietary levels of SCP					Orthogonal polynomial contrasts (P>F)				
	Initial fish	0% (Control)	4.25%	8.50%	14.50%	21.00%	ANOVA P>F	Linear	Quadratic	Cubic	Quartic
Essential amino acids											
Arginine	15.52	12.57±1.46	14.05±2.39	14.30±1.53	15.47±0.11	14.43±1.07	0.8126	0.3568	0.5214	0.8597	0.7064
Histidine	6.85	6.87±1.11	6.49±1.00	5.99±0.86	6.62±0.99	6.83±0.26	0.9427	0.9845	0.4905	0.9247	0.7171
Isoleucine	2.43	3.01±0.63	2.90±0.21	2.84±0.01	3.35±0.02	3.74±0.80	0.8401	0.3453	0.5322	0.9462	0.8484
Leucine	4.86	6.34±1.20	6.12±1.39	5.58±0.27	5.90±0.77	6.68±0.94	0.9476	0.8828	0.4832	0.8260	0.8730
Lysine	1.87	2.18±0.05	2.05±0.02	1.77±0.05	1.95±0.02	1.95±0.35	0.8111	0.4665	0.4264	0.9810	0.5081
Methionine	1.89	2.69±0.58	2.73±0.07	2.79±0.07	2.89±0.02	3.03±0.47	0.9812	0.5645	0.8892	0.9955	0.9998
Phenylalanine	1.53	4.26±0.34	3.16±0.43	2.14±0.27	3.30±0.03	2.88±1.11	0.1143	0.1567	0.1061	0.3927	0.2298
Threonine	13.84	6.17±0.63	8.28±1.53	10.00±1.09	9.13±0.28	9.29±1.19	0.2014	0.1154	0.1924	0.7395	0.5703
Valine	3.76	5.63±1.23	5.43±0.45	4.77±0.10	5.32±0.59	5.26±0.81	0.9407	0.7263	0.6136	0.9572	0.6083
Non-essential amino acids											
Alanine	7.51	6.06±0.50	6.13±0.33	6.18±0.56	7.42±0.55	7.53±1.41	0.3629	0.0746	0.6299	0.6057	0.5327
Aspartic acid	3.53	4.24±0.50	3.82±1.22	3.64±0.10	3.86±0.40	3.75±0.41	0.8972	0.5330	0.5590	0.7389	0.8372
Glutamic acid	7.13	9.62^{a}±0.97	9.34^{a}±0.72	8.81^{ab}±0.82	7.89^{ab}±0.25	6.71^{b}±0.14	0.0199	0.0018	0.3974	0.9965	0.9591
Glycine	6.47	8.08±1.05	6.39±1.43	6.28±1.15	5.83±1.55	5.56±1.60	0.7124	0.2328	0.6567	0.7695	0.8584
Serine	2.53	4.15±0.78	2.60±0.30	2.42±0.12	3.48±0.25	3.52±0.76	0.1638	0.8402	0.0500	0.2331	0.6562
Taurine	7.28	8.20±0.32	9.18±0.58	9.48±0.65	8.13±0.89	7.45±0.11	0.2892	0.2627	0.0788	0.5572	0.5801
Tyrosine	4.11	4.60±1.03	5.28±0.31	5.45±1.10	3.45±0.19	2.71±0.42	0.1462	0.0603	0.1295	0.5356	0.4682

Values are means±SEM of four composite samples, each consisting of tissues from three fish per tank. Means with different superscripts within the same row are significantly different ($P < 0.05$). The initial fish sample consisted of one large sample of initial fish, ca. 100 organisms. SCP=single-cell protein.

[16,17,43,44,45,46,47]. Overall, these reports suggest that tolerance to dietary bacterial SCPs is species-specific. However, the species of bacteria and the culture substrate used to produce SCPs also affect their nutritional value for fish and help explain the variable results. While some bacteria can thrive on agricultural by-products and wastes, e.g., *Bacillus pumilis, Cellulomonas* sp., and *Prevotella* sp. [48,49,50], others can only grow on hydrocarbon substrates, such as methane and carbon monoxide, e.g., *M. capsulatus* and *C. autoethanogenum*, respectively [17,42]. This leads to varying nutritional attributes, including protein content, amino acid composition, crude fat, and vitamin contents. [11,51,52]. As an example of differing growth results for the same fish species, based solely on the bacterial species yielding the SCPs, in the case of rainbow trout (*O. mykiss*), replacing fishmeal by SCPs from either *Brevibacterium factofermentum* or *Bacterium glutamaticum*, each added at 0, 4, 8, or 16% of the diet, resulted in favorable growth in fish across all dietary levels of *B. factofermentum* SCP, but limited tolerance to more than 4% SCP from *B. glutamaticum* [41].

Nucleic acids have attracted the interest of researchers for their possible nutraceutical effects when added to fish diets at low levels, such as 500 mg/kg [53]. However, they are typically present in high quantities in bacterial SCP, raising concerns they may lead to increased plasma uric acid and precipitation, causing gout, kidney stones, and allergic reactions, especially in humans [54–57]. Indeed, bacterial-based SCP has the highest contents of nucleic acids (15 to 16%) compared to SCP from yeasts (7.1 to 12%) or microalgae (3 to 8%) [50,55]. Fortunately, the nucleic acid content can be reduced by hydrolysis or heat treatment, rendering bacterial

SCP fit for human consumption [58] but not without added production costs [7]. Another drawback, at least for some bacterial-based SCPs, is poor palatability [57,59]. Sightly reduced weight gain was observed in rainbow trout when 10% SCP (*Methylobacterium extorquens*) was added to the diet. This decrease was linked to reduced feed intake, with low palatability suspected as the cause [60]. This may have played a role in the present study since feed intake decreased significantly as dietary SCP increased. The problem could be overcome, as suggested earlier [60], by incorporating palatability enhancers. In addition to the high nucleic acid content and palatability issues of bacterial SCP, bacteria can produce endo- and exotoxins as secondary metabolites, which may cause mild to fatal reactions, depending on the bacterial species and the consumer [61,62.63]. Some exotoxins are thermolabile or can be denatured by organic solvents and acids [64], whereas endotoxins, as constituents of the bacterial cell, are more difficult to remove [54]. However, it should be emphasized that not all bacterial species are harmful [55]. Toxicological tests are recommended to verify the safety of any potentially new species for SCP production [57]. Overall, the potential presence of antinutritional factors in bacterial-based SCP mentioned helps explain why there are constraints on their inclusion in fish diets.

In the present study, increasing the level of dietary SCP caused a significant reduction in the crude fat content of fish muscle tissue. The same significant effects on muscle tissue or whole body have been observed for tilapia and other fish in response to increasing bacterial-based SCP levels in the diet, including rainbow trout (*O. mykiss*), yellowtail (*S. quinqueradiata*), largemouth bass (*M. salmoides*), turbot (*S. maximus*), and large yellow croaker (*Larimichthys crocea*) [17,18,19,21,65,66]. Although not statistically significant, the same trend has also been observed for Atlantic salmon (*S. salar*) [12] and halibut (*Hippoglossus hippoglossus*) [15]. Reduced fat content in muscle tissue or the whole fish body is a consistent response to dietary bacterial SCP. Recent experimental evidence helped elucidate this phenomenon. As fishmeal was gradually replaced from 0 up to 30% by bacterial SCP (*M. capsulatus*) in diets for Pacific white shrimp (*Litopenaeus vannamei*), a significant reduction of the expression of the gene fatty acid synthase (fas), and a significant increase of carnitine palmitoyltransferase (cpt-1) were observed in shrimp hepatopancreas. These enzymes participate in regulating fatty acid synthesis (for fas) and fatty acid catabolism (for cpt-1). In other words, bacterial-based SCP has lipolytic effects and concomitantly reduces lipogenic activity [67]. In the present study, along with the reduced crude fat content, reduced dry matter content (or increased moisture) was observed, a trend that also has been reported in numerous studies of fish fed with bacterial SCP [12,15,17–19,65,66]. Elevating the water content seems to be a recurrent response of the fish body to compensate for the reduced body lipid. Regarding ash and crude protein contents, no differences among treatments were detected in the present study or in studies with *H. hippoglossus* and *L. crocea* fed bacterial SCP [15,66]. However, there is no consistent trend of these parameters in other studies of fish fed with bacterial-based SCP. For example, the content of crude protein increased in *M. salmoides*, and decreased in *S. quinqueradiata*, *O. mykiss*, and *S. maximus*, while ash content increased in *S. quinqueradiata* and *O. mykiss* in response to dietary SCP [17–19,65]. The response may depend on both the species of fish and bacteria under study.

The progressive incorporation of dietary SCP elicited minimal changes in the AA profile of fish muscle. The content of glutamic acid, a non-essential AA, decreased with the dietary levels of SCP, while none of the other AAs were statistically affected. Unfortunately, the amino acid composition of muscle or whole body of tilapia fed bacterial-based SCP in other studies was not reported. In some indirect evidence, employing juvenile tilapia (1.4 g of initial weight), significantly reduced growth was recorded when levels of 15% and 20% of bacterial SCP from *M. glutamicus* were included in the diet [21]. For those diets, the low levels of lysine (0.89-0.95 g/100 diet) and methionine (0.34-0.36 g/100 g) may have caused the depressed growth since they were below the recommended dietary inclusion for juvenile tilapia, i.e., 1.43-2.62 g/100 diet for lysine and 0.75-1.29 g/100 diet for methionine [29,30]. In the present study, the minimum requirements of tilapia for all essential amino acids were met, and all diets were supplemented with methionine and lysine. Therefore, the depressed growth observed at elevated inclusion levels of bacterial SCP was more likely associated with the anti-nutritional characteristics of SCP previously mentioned, e.g., a high nucleic acid content and poor palatability, which may also explain the significantly reduced feed intake. In addition, the digestibility of

bacterial SCP must be considered. At elevated inclusion levels, reduced digestibility for turbot, salmon, rainbow trout, or yellowtail has been evidenced using diets containing SCP from the bacterial species *M. capsulatus* or a bacterial consortium composed of *M. capsulatus, Alcaligenes acidovorans, Bacillus brevis*, and *Bacillus firmus* [12,14,17,19,39]. However, some authors argue that the digestibility of bacterial-based SCP is high [8], especially when compared to fungal and microalgal SCP [11,52]. Diet digestibility was not determined in the present study; therefore, its potential role in the observed growth reduction should be investigated in future research.

CONCLUSIONS

At least 50% of the protein provided by fishmeal in a control diet (with a baseline fishmeal content of 8.0% of diet) can be replaced by the bacterial-based SCP MRD-Pro® without significant effects on growth, feed utilization, or survival of tilapia (*O. niloticus*) fry. As fishmeal was substituted on a protein basis, this represents an effective incorporation level of bacterial SCP of 4.25% in the diet. Conversely, fish biological performance declined as dietary SCP levels were increased to 8.50%, 14.50%, or 21.00% of the diet. Increasing the level of dietary SCP caused a significant reduction in crude fat and dry matter content of fish muscle tissue, while the contents of ash and crude protein were unaffected. Minimal changes in the amino acid profile of fish muscle tissue were observed, with glutamic acid content being the only one affected by dietary SCP. Overall, the results of the present study indicate that the bacterial-based SCP MRD-Pro® is a nutritious feed additive that can be effectively incorporated, albeit with certain constraints, into the diet for tilapia fry.

Funding: The authors received no specific funding for this work.

Acknowledgments: The authors would like to thank Jesús Vázquez-Cota at the Kino Bay Experiment Station for his technical assistance during this study. Funding for Ms. Félix-Berumen was partly provided by Consejo Nacional de Humanidades, Ciencias y Tecnología (CONAHCYT, Mexico). The mention of trademarks or proprietary products does not constitute an endorsement of the product by the University of Sonora and does not imply its approval to the exclusion of other products that may also be suitable.

Author contributions: Martin Perez-Velazquez, conceptualization, writing – original draft preparation, supervision; Mayra L. González-Félix, conceptualization, writing – review and editing; Reyna D. Félix-Berumen, methodology, data curation, writing – review and editing. All authors have read and agreed to the published version of the manuscript.

Conflict of interest disclosure: The authors declare that they have no conflict of interest.

Data availability: The data underlying the reported findings have been provided with the submitted article and are available here: https://www.serbiosoc.org.rs/NewUploads/Uploads/Gonz%C3%A1lez-F%C3%A9lix%20et%20al_9719-Raw%20Dataset.xlsx

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SUPPLEMENTARY MATERIAL

Supplementary Table S1. Ingredients (g/100 g of diet) and determined proximate composition (%) of experimental diets for Nile tilapia fry using increasing levels of bacterial-based single-cell protein meal MRD-Pro®.

	Dietary levels of SCP				
Ingredients	**0% (Control) (control)**	**4.25% SCP**	**8.50% SCP**	**14.50% SCP**	**21.00% SCP**
Soy protein isolate[1]	23.96	23.96	23.96	23.96	23.96
Soybean meal extracted[1]	21.35	21.30	21.30	11.06	-
Fishmeal (sardine)[2]	8.00	4.00	-	-	-
MRD-Pro Meridian[3]	-	4.25	8.50	14.50	21.00
Pea Protein[4]	7.60	7.60	7.60	7.60	7.60
Whole wheat flour[5]	12.30	11.715	11.115	15.28	19.735
Wheat starch[6]	4.00	4.00	4.00	4.00	4.00
Soybean oil[7]	9.00	9.06	9.12	9.025	8.875
Fish oil[2]	3.50	3.56	3.62	3.525	3.475
Soy lecithin dry[8]	1.00	1.00	1.00	1.00	1.00
Vitamin/mineral mix[9]	2.00	2.00	2.00	2.00	2.00
CaP dibasic[10]	2.73	3.65	4.54	4.67	4.80
Salt (NaCl)[10]	0.35	0.35	0.35	0.35	0.35
Methionine[11]	0.625	0.665	0.70	0.70	0.695
Lysine[11]	0.015	0.14	0.255	0.35	0.46
Vitamin C[12]	0.10	0.10	0.10	0.10	0.10
Cellulose[13]	0.785	0.795	0.79	1.34	1.95
Diatomaceous earth[10]	2.685	1.855	1.05	0.54	-
Total	100	100	100	100	100
Proximate composition (%)[14]					
Crude protein	45.37	45.17	45.31	44.14	43.82
Crude fat	14.26	14.27	14.24	14.58	14.61
Moisture	5.42	5.41	6.28	7.43	5.57
Ash	12.25	12.08	11.92	11.65	11.66
Crude fiber	2.02	2.52	2.86	2.34	2.68
NFE	20.68	20.55	19.39	19.86	21.66
Gross energy (kJ g^{-1})	19.82	19.80	19.58	19.37	19.50

[1]Procesadora de ingredientes, S.A. de C.V., Guadalajara, Jalisco, Mexico.
[2]Productos Pesqueros de Guaymas S.A. de C.V., Guaymas, Sonora, Mexico.
[3]MRD-Pro®, 63.5% crude protein, 3.12% crude fat, Meridian Biotech, LLC, Texas, USA.
[4]HABACUQ Comercializadora Química S.A. de C.V., Guadalajara, Jalisco, Mexico.

[5]Los Gallos, Molino La Fama S.A. de C.V., Hermosillo, Sonora, Mexico.
[6]Gluten y Almidones Industriales, S.A. de C.V., Mexico City, Mexico.
[7]Ragasa Industrias S.A. de C.V., Monterrey, Nuevo León, Mexico.
[8]Golden Harvest, Impulsora Golden, S.A. de C.V., Mexico City, Mexico.
[9]Rovimix, Insumos Nubiot, Obregón City, Sonora, Mexico.
[10]Fagalab, Mocorito, Sinaloa, Mexico.
[11]Alfa Aesar, Ward Hill, Massachusetts, USA.
[12]Stay C® (L-ascorbyl-2-polyphosphate 35% active C), Roche Vitamins Inc., Parsippany, New Jersey, USA.
[13]Sigachi Industries Pvt. Ltd., Madinaguda, Hyderabad, India.
[14]Values are means of triplicate samples, except for gross energy and crude fiber, with two replicate samples.
NFE=Nitrogen Free Extract; SCP=Single-Cell Protein.

Supplementary Table S2. Amino acid composition (g/100 g of dry diet) of experimental diets for Nile tilapia fry using increasing levels of bacterial-based single-cell protein meal MRD-Pro®.

	Dietary levels of SCP				
	0% (Control)	**4.25%**	**8.50%**	**14.50%**	**21.00%**
Essential amino acids					
Arginine	8.87	8.64	7.38	7.84	7.28
Histidine	1.97	2.09	2.69	2.54	2.05
Isoleucine	1.88	2.10	2.25	1.85	2.16
Leucine	3.28	3.19	3.05	3.49	3.77
Lysine	2.02	1.79	1.65	1.59	1.43
Methionine	1.27	1.51	1.68	1.46	1.58
Phenylalanine	1.54	1.70	2.02	1.71	1.92
Threonine	2.56	3.54	3.69	3.64	3.97
Valine	2.42	2.91	3.32	3.27	3.37
Non-essential amino acids					
Alanine	3.09	2.88	2.75	2.95	3.27
Aspartic acid	2.53	2.30	2.05	2.68	2.58
Glutamic acid	5.11	4.94	4.54	4.83	4.60
Glycine	2.69	1.76	1.59	1.17	1.04
Serine	3.13	2.63	2.33	1.86	2.03
Taurine	2.17	1.90	1.75	1.39	1.43
Tyrosine	2.13	2.05	2.00	2.36	2.52

Values are means of triplicate samples. SCP – single-cell protein.