

ORIGINAL RESEARCH

Culture-based screening of selected bacterial indicators in three freeze-dried lots of the polychaete *Australonuphis* sp. collected on a single date in southern Brazil

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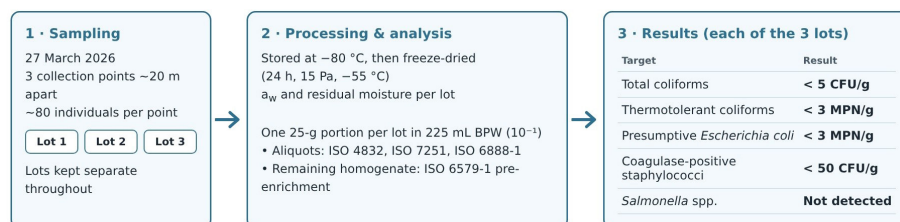
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GRAPHICAL ABSTRACT.

Culture-based screening of freeze-dried *Australonuphis* sp.

Three lots from one sampling date · Laguna, Santa Catarina, southern Brazil · TRL 3



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Interpretation limits

Results apply only to the tested portions. No raw-material, process or storage samples were analysed. Regulatory compliance (MAPA / ANVISA) was not assessed.

Not performed in this study – future steps

Raw-material and multi-season sampling · additional hazards (e.g., *Vibrio* spp., WSSV, EHP) · storage stability · digestibility and feeding trials · feed-specific regulatory classification

ABSTRACT. Polychaetes have been investigated as aquaculture feed ingredients because of their nutritional characteristics, particularly for broodstock diets of commercially important fish and shrimp species. However, the use of polychaetes from Latin America as feed resources for industrial aquaculture remains poorly documented, and microbiological data are an essential requirement for the development of new aquaculture ingredients. Therefore, this study screened three separately freeze-dried lots of *Australonuphis* sp., collected on the same date in southern Brazil, for selected bacterial indicators using culture-based ISO methods. From each lot, one 25-g test portion was homogenized in buffered peptone water; aliquots of this initial suspension were used for the enumeration of total coliforms, thermotolerant coliforms, presumptive *Escherichia coli* and coagulase-positive staphylococci, and the remaining homogenate was pre-enriched for the detection of *Salmonella* spp. No target microorganism was detected above the relevant method-specific reporting or detection limits in the tested portions. In all three lots, total coliforms were below 5 CFU g⁻¹, thermotolerant coliforms and presumptive *E. coli* were below 3 MPN g⁻¹, coagulase-positive staphylococci were below 50 CFU g⁻¹,

and *Salmonella* spp. was not detected. These findings provide preliminary microbiological baseline data for the tested lots of freeze-dried *Australonuphis* sp. only. Results should not be interpreted as evidence of animal-feed regulatory compliance, as the applicable MAPA requirements depend on the regulatory classification and intended use of the material. Further studies including raw-material and multi-season sampling, additional safety parameters, storage stability, digestibility and feeding trials, together with a formal feed-specific regulatory assessment, will be necessary to determine its suitability for future aquaculture applications.

Key words: Marine annelid, feed hygiene, culture-based detection.

Detección de indicadores bacterianos seleccionados a partir del cultivo de tres lotes liofilizados del poliqueto *Australonuphis* sp., recolectados en la misma fecha en el sur de Brasil

RESUMEN. Los poliquetos han sido estudiados como ingredientes para la alimentación en acuicultura debido a sus características nutricionales, especialmente para las dietas de reproductores de especies de peces y camarones de importancia comercial. Sin embargo, el uso de poliquetos provenientes de América Latina como recursos alimenticios para la acuicultura industrial sigue estando poco documentado, y los datos microbiológicos constituyen un requisito esencial para el desarrollo de nuevos ingredientes para este sector. Por ello, en este estudio se analizaron tres lotes de *Australonuphis* sp. liofilizados por separado y recolectados en una misma fecha en el sur de Brasil, en búsqueda de indicadores bacterianos específicos, empleando métodos basados en cultivo conforme a las normas ISO. De cada lote, se tomó una muestra de 25 g, la cual se homogeneizó en agua peptonada tamponada. A partir de esta suspensión inicial, se realizaron recuentos de coliformes totales, coliformes termotolerantes, *Escherichia coli* presuntiva y estafilococos coagulasa-positivos. El resto del homogeneizado se sometió a un pre-enriquecimiento para la detección de *Salmonella* spp. En las muestras analizadas, no se detectaron los microorganismos objetivo por encima de los límites de detección o de informe establecidos por los métodos correspondientes. En los tres lotes, los coliformes totales se situaron por debajo de 5 UFC g⁻¹; los coliformes termotolerantes y *E. coli* presuntiva por debajo de 3 NMP g⁻¹; los estafilococos coagulasa-positivos, por debajo de 50 UFC g⁻¹; y no se detectó *Salmonella* spp. Estos hallazgos proporcionan datos microbiológicos de referencia preliminares exclusivamente para los lotes analizados de *Australonuphis* sp. liofilizado. Los resultados no deben interpretarse como una prueba de cumplimiento de la normativa sobre alimentación animal, ya que los requisitos aplicables, establecidos por el MAPA, dependen de la clasificación reglamentaria y del uso previsto del material. Serán necesarios estudios adicionales que incluyan muestreos de la materia prima en distintas estaciones, parámetros de seguridad complementarios, análisis de estabilidad durante el almacenamiento, estudios de digestibilidad y ensayos de alimentación, junto con una evaluación normativa formal específica para el uso como alimento, a fin de determinar su idoneidad para futuras aplicaciones en acuicultura.

Palabras clave: Anélido marino, higiene de los alimentos, detección mediante cultivo.

INTRODUCTION

Over the last decade, the aquaculture sector has increasingly explored alternative nutritional ingredients to improve feed efficiency and reduce dependence on conventional marine resources (Ahmed and Thompson 2019; Glencross et al. 2020; Ang et al. 2021). Alternative protein sources investigated for aquafeeds include plant meals, fishery by-products, insects, microbial ingredients, animal by-products, and macroalgae (Glencross et al. 2020; Ang et al. 2021; Chen et al. 2021; Aragão et al. 2022; Hussain et al. 2024; Serra et al. 2024;).

Among these alternatives, marine annelids (polychaetes) have been evaluated less frequently, and the published feeding studies have mainly used species from Europe and Asia (Lupatsch 2014; Monteiro et al. 2023; Balqadi et al. 2025; Akbaş et al. 2026). Polychaetes have attracted attention as potential aquaculture-feed ingredients because of their proteins, lipids, long-chain polyunsaturated fatty acids, minerals, vitamins, and pigments (Muli et al. 2016; Martins et al. 2019; Mahulette 2020; Monteiro et al. 2023). Experimental studies have also demonstrated their nutritional value in aquaculture diets. Lupatsch (2014) reported that polychaete meal supported growth performance and efficiency comparable to a standard fishmeal

diet and could replace fishmeal in diets for Pacific white shrimp (*Litopenaeus vannamei*). More recently, Akbaş et al. (2026) reported that polychaete meal could replace up to 50% of dietary fishmeal and partially replace fish oil in European seabass (*Dicentrarchus labrax*) diets without compromising growth, nutrient utilization, or fillet quality. However, nutritional composition alone does not establish the microbiological quality of a potential feed ingredient. Polychaetes collected from marine environments may be exposed to microorganisms associated with water, sediment, other organisms, harvesting, handling, and transportation. Previous reports have described associations between some polychaete genera, including *Dendronereis*, *Marphysa*, and *Nereis*, and biological agents relevant to aquatic animal health, including *Enterocytozoon hepatopenaei* (EHP) and white spot syndrome virus (WSSV) (Herrera-Perez and Ferrando 2026). These reports provide broader biological-risk context for wild-collected polychaetes but do not indicate the presence or absence of these agents in *Australonuphis* sp., which were not investigated in the present study. This context is relevant to *Australonuphis* sp., which has been reported as a live-feed resource transported from northern coastal Peru to Ecuador for use in shrimp farming and their use as bait (Herrera-Perez 2026) (Figure 1).

Australonuphis sp. has also been investigated as a potential source of nutrients for aquaculture applications. Preliminary information on its nutritional and biochemical composition, including protein, lipid, amino acid, fatty-acid and minerals profiles, has been obtained by our group (Herrera-Perez et al. unpublished data). However, these data do not address the microbiological characteristics of the processed biomass. To our knowledge, no culture-based data on bacterial indicators in freeze-dried *Australonuphis* sp. have been published; the new observation reported here is therefore the result of a culture-based screening of five bacterial targets in three freeze-dried lots of this polychaete.

Processing may further influence the detectability of microorganisms in polychaete biomass. Freeze-drying reduces water availability and can improve the stability of biological materials (Uwin-eza and Zhang 2026); however, it is not inherently a sterilization process, and microorganisms may survive under low-moisture conditions (Lee et al.



Figure 1. Four examples of commercially available dried polychaete products marketed as fishing bait. 1) Dynabait Dry Sandworms: 100% all-natural fishing bait for saltwater and freshwater environments, manufactured in Australia and widely exported and sold across the United States, Canada, the European Union, and South Africa. 2) Dry Sandworms Power Bait, featuring dehydrated lugworms designed for environmentally sustainable sport fishing, manufactured in China and distributed globally via major e-commerce platforms to markets in Brazil, Mexico, and the United States. 3) Dynabait Multi-pack Assortment, available in major outdoor and fishing tackle retail stores throughout Australia, New Zealand, and the United Kingdom. 4) DEGA Blood Worms (Sandwürmer), a product of a prominent German angling brand, distributed and sold across European Union markets, including Germany, Slovenia, Poland, and Croatia.

2021). Microbial survival following drying varies according to the physiological characteristics of the microorganism and the processing conditions, including freezing and drying parameters (Cao et al. 2024). In addition, changes in matrix structure, composition, and water activity may influence microbial culturability and recovery during subsequent analysis (Liu et al. 2022; Rana et al. 2024). Therefore, culture-based screening of freeze-dried biomass can provide preliminary information on the occurrence of selected microorganisms after processing. The intended use of the material determines the regulatory context applicable to its microbiological assessment. In Brazil, materials intended for incorporation into aquaculture feed are subject to the regulatory framework for animal-feeding products administered by the Ministry of Agriculture and Livestock (MAPA), which is distinct from the microbiological standards established by the National Health Surveillance Agency (ANVISA) for human food. Therefore, ANVISA RDC no 724/2022 and IN no 161/2022 should not be used as the regulatory basis for establishing compliance of a polychaete-derived ingredient intended for animal feed. In the present study, neither ANVISA nor MAPA criteria were used to infer regulatory compliance; rather, the results were reported against the method-specific reporting limits described in the Materials and Methods section. The applicable MAPA requirements for this material would need to be established according to its regulatory classification and intended use in animal feed and should therefore be addressed in a subsequent regulatory assessment. Accordingly, this study aimed to perform a culture-based screening of selected bacterial indicators and microorganisms of sanitary relevance in three separately processed freeze-dried lots of *Australonuphis* sp. collected on a single date in southern Brazil. The analyses included total coliforms, thermotolerant coliforms, presumptive *Escherichia coli*, *Salmonella* spp., and coagulase-positive staphylococci, providing preliminary microbiological information on the analyzed freeze-dried biomass.

MATERIALS AND METHODS

Specimen collection and preparation

Wild marine polychaetes were collected on 27 March 2026, between 06:00 and 09:00 h, from a coastal beach in Laguna, Santa Catarina, southern Brazil (28° 25' S, 48° 53' W). The collection site is located in the intertidal zone, and specimens were collected during low tide at an approximate depth of 10-15 cm in the sediment. Three collection points located approximately 20 m apart were sampled, and approximately 80 individuals were collected at each point; the specimens from each point constituted one lot (lots 1, 2, and 3). Collection was conducted with the assistance of local fishermen who routinely collect and sell polychaetes as fishing bait. Authorization for the collection of wild specimens was obtained from the Brazilian Biodiversity Authorization and Information System (SISBIO; authorization no 101907-1). Following collection, the specimens of each lot were placed in separate, labelled containers and were immediately transported to the Food Technology and Bioprocess Research Group at the Federal University of Santa Catarina. Each lot was kept separate and identified by its lot code throughout storage, freeze-drying, subsampling, and microbiological analysis. Because the three lots were collected on the same date and at the same site, they represent separately processed lots from a single sampling occasion rather than temporally or geographically independent batches.

Specimens with similar size and morphological characteristics were selected for taxonomic identification. Five individuals from each lot were placed in one Petri dish per lot (three Petri dishes; 15 voucher specimens in total), fixed in 10% formaldehyde-seawater solution, and subsequently preserved in 70% ethanol. Taxonomic identification was performed using stereomicroscopy and compound light microscopy, with genus-level

identification based on morphological characteristics described by Lana (1991) and Carrera-Parra (2021). The remaining specimens of each lot (approximately 75 individuals; 101.25 g wet weight per lot) were stored at -80 °C in separate labelled bags until further processing and analysis.

Freeze-drying process

Frozen samples obtained as described in the last section were subjected to freeze-drying (lyophilization) to obtain the dried polychaete ingredient used in this study. Freeze-drying was selected as the processing method because of its recognized advantages in preserving nutrient quality (Hidir et al. 2026). Each lot was freeze-dried separately. For each lot, a 25-g wet subsample in a sealed bag was used to determine drying yield, moisture content, and water activity, and the remaining wet biomass of the lot was freeze-dried under the same conditions to obtain approximately 57 g of freeze-dried material, from which the 25-g test portion for microbiological analysis was taken. Before freeze-drying, the samples were maintained at -80 °C for 24 h in an ultrafreezer (model CL120-80 V, ColdLab, Piracicaba, Brazil). Samples were subsequently freeze-dried using a benchtop freeze-dryer (model LS, Terroni, São Carlos, Brazil) for a fixed period of 24 h, under a vacuum of 15 Pa and a temperature of -55 °C.

Following freeze-drying, the water activity (a_w) of the lyophilized polychaete samples was determined using a digital hygrometer by measuring the dew point at 25 °C (Aqualab Series 3 TE, Decagon Devices, Pullman, WA, USA). The instrument was calibrated before measurements using certified calibration standards. For each lot, a_w was measured in triplicate, and the results are reported as mean $\pm$ SD (Lot 1: [0.240 $\pm$ 0.005]; Lot 2: [0.250 $\pm$ 0.005]; Lot 3: [0.250 $\pm$ 0.005]). All individual readings were 0.24 when rounded to two decimal places. These measurements were used to verify the final drying state of the freeze-dried material.

Residual moisture content and water activity were determined for each lot on a portion of the

freeze-dried material (5.05 g for Lot 1, 4.98 g for Lot 2, and 5.12 g for Lot 3). The residual moisture contents (wet basis) were 1.07, 1.16, and 0.99 g 100 g⁻¹ for lots 1, 2, and 3, respectively (mean $\pm$ SD: 1.07 $\pm$ 0.09 g 100 g⁻¹). A separate 25-g portion of the freeze-dried material of each lot was used as the test portion for microbiological analysis. Because the freeze-drying period was fixed at 24 h, the process endpoint was defined by the predetermined processing time rather than by reaching a specified a_w value. The measured a_w values were subsequently used as a quality-control indicator of the drying process.

Microbiological analysis

Freeze-dried samples obtained as described in the last section were analyzed for total coliforms, thermotolerant coliforms, presumptive *E. coli*, *Salmonella* spp., and coagulase-positive staphylococci. One 25-g test portion was aseptically taken from each of the three freeze-dried lots (three test portions in total) and homogenized in 225 ml of buffered peptone water (BPW), corresponding to the initial 10⁻¹ suspension. Aliquots of this initial suspension, and of the decimal dilutions prepared from it, were used for the enumeration of total coliforms, thermotolerant coliforms, presumptive *E. coli*, and coagulase-positive staphylococci. Immediately after these aliquots had been withdrawn, the remaining homogenate (corresponding to approximately 24 g of the test portion) was incubated as the non-selective pre-enrichment for *Salmonella* spp. Thus, for each lot, all enumeration and detection assays were performed on the same 25-g test portion rather than on separate 25-g portions for each method; this deviation from the separate test portion specified in ISO 6579-1 is acknowledged as a limitation of the study.

Total coliforms were enumerated using the colony-count technique on violet red bile lactose agar (VRBL), according to ISO 4832:2006a (Table 1). From the initial suspension and the appropriate decimal dilutions, 1 ml of each dilution was inoculated

Table 1. Summary of microbiological analysis protocols.

Target	Standard	Portion examined (per lot)	Media and incubation	Confirmation	Controls	Reporting limit
Total coliforms	ISO 4832:2006a (colony count)	1 ml of 10 ⁻¹ (and further dilutions) on 2 plates; 0.2 g at 10 ⁻¹	VRBL, pour plate, 30 °C, 24 ± 2 h	Atypical colonies: BGBL broth, 30 °C, 24 ± 2 h (gas)	<i>S. Typhimurium</i> ATCC 14028 (negative, lactose non-fermenter)	< 5 CFU g ⁻¹
Thermotolerant coliforms	ISO 7251:2005/ Amd 1:2023 (MPN)	3 × 1 ml at 10 ⁻¹ , 10 ⁻² , 10 ⁻³ (3-tube series)	LST, 37 °C, 24 ± 2 h → EC broth, 44.0 ± 0.2 °C, 24 ± 2 h	Gas in EC broth	<i>S. Typhimurium</i> ATCC 14028 (negative, lactose non-fermenter)	< 3 MPN g ⁻¹
Presumptive <i>Escherichia coli</i>	ISO 7251:2005/ Amd 1:2023 (MPN)	Gas-positive EC tubes	Tryptone water, 44 °C	Indole (Kovac's reagent); no species-level confirmation	<i>E. coli</i> ATCC 25922 (positive, indole-positive); <i>S.</i> <i>Typhimurium</i> ATCC 14028 (negative, indole-negative)	< 3 MPN g ⁻¹
<i>Salmonella</i> spp.	ISO 6579- 1:2017/ Amd 1:2020 (detection)	Homogenate remaining after enumeration aliquots (≈ 24 g)	BPW, 37 °C, 18 h → RVS, 41.5 °C, 24 h and MKTTn, 37 °C, 24 h → XLD[+Agar verde brillante (BGA)], 37 °C, 24 h	Biochemical and serological	<i>S. Typhimurium</i> ATCC 14028 (positive); uninoculated BPW (negative)	Detected/not detected in ≈ 24 g
Coagulase- positive staphylococci	ISO 6888- 1:2021	0.1 ml of 10 ⁻¹ (and further dilutions) on 2 plates; 0.02 g at 10 ⁻¹	Baird-Parker agar, spread plate, 37 °C, 48 ± 2 h	Coagulase test (rabbit plasma), read at 4-6 h and 24 h	<i>S. aureus</i> ATCC 25923 (positive); <i>S.</i> <i>epidermidis</i> ATCC 12228 (negative)	< 50 CFU g ⁻¹

One 25-g test portion per lot was homogenized in 225 ml buffered peptone water (BPW; 10⁻¹ initial suspension). Aliquots of this suspension were used for all enumeration assays; the remaining homogenate was pre-enriched for *Salmonella* spp. VRBL, violet red bile lactose agar; BGBL, brilliant green bile lactose broth; LST, lauryl sulfate tryptose broth; EC, *E. coli* broth; RVS, Rappaport-Vassiliadis Soya broth; MKTTn, Muller-Kauffmann tetrathionate-novobiocin broth; XLD, xylose lysine deoxycholate agar.

on two plates per dilution. The plates were incubated at 30 °C for 24 ± 2 h. Typical purplish-red colonies ≥ 0.5 mm in diameter were counted. Atypical colonies were subjected to confirmation in brilliant green bile lactose broth at 30 °C for 24 ± 2 h, with gas production considered a positive reaction. Only plates containing 10 to 150 colonies were used for enumeration. The number of total coliforms was calculated according to the procedure specified in ISO 4832:2006a, considering the dilution factor and the number of countable colonies, and results were expressed as colony-forming units per gram (CFU g⁻¹). Because 2 ml of the 10⁻¹ suspension (0.2 g of sample) was examined in total, the reporting limit was 5 CFU g⁻¹, and the absence of colonies on both plates was reported as < 5 CFU g⁻¹.

Thermotolerant coliforms and presumptive *E. coli* were determined using the most probable number (MPN) technique according to ISO 7251:2005, including the culture media and reagent performance-testing requirements specified in Amendment 1:2023 (Table 1). For each lot, the initial 10⁻¹ suspension described above was used. The decimal dilution series used for MPN determination was 10⁻¹, 10⁻², and 10⁻³. For each dilution, 1 ml was inoculated into three tubes containing Lauryl Sulfate Tryptose (LST) broth, resulting in a three-tube series per dilution. The tubes were incubated at 37 °C for 24 ± 2 h. Tubes showing gas production were considered presumptively positive and were subcultured into *E. coli* (EC) broth, a selective medium containing bile salts to inhibit non-fecal microorganisms, followed by incubation at 44.0 ± 0.2 °C for 24 ± 2 h. Gas production trapped in Durham tubes was interpreted as a positive reaction for thermotolerant coliforms.

For presumptive *E. coli*, gas-positive EC broth cultures were further subcultured into tryptone water and incubated as specified in ISO 7251:2005. Following incubation, Kovac's reagent was added, and the development of a distinct red ring at the surface was interpreted as a positive indole reaction. Under ISO 7251:2005, microorganisms that produce gas from lactose in EC broth and indole

from tryptophan at 44 °C are defined as presumptive *E. coli*; the indole step therefore establishes presumptive *E. coli* status only. No further biochemical or molecular confirmation to species level was performed, and the term presumptive *E. coli* is used throughout the manuscript.

The MPN was calculated from the observed pattern of positive and negative tubes across the dilution series using the MPN table specified in ISO 7251:2005. Results were expressed as MPN g⁻¹. The method-specific reporting limit was 3 MPN g⁻¹, corresponding to no positive tubes at any dilution (0-0-0). These analyses were performed as hygiene and fecal-contamination indicators; the results were interpreted only against the method-specific reporting limits and were not evaluated against MAPA or ANVISA criteria.

Salmonella spp. was investigated according to ISO 6579-1:2017, including Amendment 1:2020, comprising non-selective pre-enrichment in buffered peptone water (37 °C, 18 h), selective enrichment in Rappaport-Vassiliadis Soya broth (41.5 °C, 24 h) and Muller-Kauffmann Tetrathionate-Novobiocin broth (37 °C, 24 h), and plating on Xylose Lysine Deoxycholate (XLD) agar and brilliant green agar (BGA) (37 °C, 24 h). Presumptive colonies were confirmed by biochemical and serological testing (Table 1). *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 was processed in parallel as the positive control. The only deviation from ISO 6579-1:2017/Amd 1:2020 was that pre-enrichment was performed on the homogenate remaining after the aliquots for the enumeration assays had been withdrawn (approximately 24 g instead of a separate 25-g test portion).

Coagulase-positive staphylococci were enumerated by the surface-plating technique on Baird-Parker agar according to ISO 6888-1:2021 (Table 1). From the initial suspension and the appropriate decimal dilutions, 0.1 ml of each dilution was surface-plated onto duplicate Baird-Parker agar plates. The plates were incubated at 37 °C for 48 ± 2 h. Plates containing 15 to 150 colonies were selected for enumeration, and the number of coagu-

lase-positive staphylococci was calculated according to the procedure specified in ISO 6888-1:2021, considering the dilution factor and the number of countable colonies. Results were expressed as colony-forming units per gram (CFU g⁻¹). Because a total of 0.2 ml of the 10⁻¹ suspension (0.02 g of sample) was plated, one colony across both plates corresponds to 50 CFU g⁻¹; the reporting limit was therefore 50 CFU g⁻¹, and the absence of confirmed colonies was reported as < 50 CFU g⁻¹.

Typical and atypical colonies were selected for confirmation by the coagulase test using rabbit plasma. The reaction was examined after 4-6 h and, when negative, re-examined after 24 h. *Staphylococcus aureus* ATCC 25923 was used as the positive control for the coagulase test, and *S. epidermidis* ATCC 12228 was used as the negative control. Colonies showing a positive coagulase reaction were considered coagulase-positive staphylococci. The results were based on the confirmed colonies and expressed as CFU g⁻¹.

RESULTS

Microbiological quality of freeze-dried *Australonuphis* sp.

In all three lots analyzed, no confirmed target microorganism was detected above the relevant method-specific reporting or detection limits in the tested portions (Table 2). Total coliforms were < 5 CFU g⁻¹ in each lot. Thermotolerant coliforms and presumptive *E. coli* were < 3 MPN g⁻¹ in each lot, with no positive EC tubes at any dilution (0-0-0). Coagulase-positive staphylococci were < 50 CFU g⁻¹ in each lot. *Salmonella* spp. was not detected in the pre-enriched test portion of any lot: five presumptive colonies isolated on XLD agar (all lots combined) were subjected to biochemical and serological confirmation, and none was confirmed as *Salmonella* (0/5), whereas the positive control

Table 2. Microbiological results for three freeze-dried lots of *Australonuphis* sp. and corresponding method reporting limits.

Parameter	Lot 1	Lot 2	Lot 3	Reporting limit/unit
Total coliforms (CFU g ⁻¹)	< 5	< 5	< 5	5 CFU g ⁻¹
Thermotolerant coliforms (MPN g ⁻¹); EC tube pattern	< 3; 0-0-0	< 3; 0-0-0	< 3; 0-0-0	3 MPN g ⁻¹
Presumptive <i>E. coli</i> (MPN g ⁻¹)	< 3	< 3	< 3	3 MPN g ⁻¹
Coagulase-positive staphylococci (CFU g ⁻¹)	< 50	< 50	< 50	50 CFU g ⁻¹
<i>Salmonella</i> spp. (≈ 24 g)	Not detected	Not detected	Not detected	Detection
Presumptive <i>Salmonella</i> colonies confirmed tested	0/2	0/1	0/2	Total 0/5
Water activity (aw; mean ± SD, n = 3)	[0.240 ± 0.005]	[0.250 ± 0.005]	[0.250 ± 0.005]	-
Residual moisture (g 100 g ⁻¹ , wet basis)	1.07	1.16	0.99	1.07 ± 0.09*
Drying yield (%)	20.20	19.92	20.48	-

“<” Indicates that no target colonies or positive tubes were observed at the lowest dilution examined (results below the method reporting limit). *Mean ± SD.

strain (ATCC 14028) was recovered as expected. These findings indicate only that the target microorganisms evaluated in this study were not detected above the analytical limits of the methods used in the tested portions. However, these results should not be interpreted as establishing legal compliance of the freeze-dried polychaete ingredient as animal feed, because the applicable MAPA regulatory category and corresponding microbiological criteria for this product have not been established in the present study.

DISCUSSION

This study presents a culture-based screening of selected bacterial indicators in three separately processed lots of freeze-dried *Australonuphis* sp. collected on a single date in southern Brazil. Total coliforms, thermotolerant coliforms, presumptive *E. coli*, *Salmonella* spp., and coagulase-positive staphylococci were not detected above the method-specific reporting or detection limits in any of the tested portions. These results were not evaluated against, and do not demonstrate compliance with, ANVISA food standards or MAPA animal-feed requirements. While these negative results for the five evaluated targets provide initial baseline data, they do not establish comprehensive microbiological safety or definitive feed suitability. Nonetheless, this preliminary screening represents an initial step toward evaluating the species as a potential feed ingredient.

Microbiological criteria serve as a standard reference when evaluating novel aquaculture feed ingredients (Milana et al. 2024). In this study, selected hygiene indicators and bacterial pathogens commonly included in microbiological criteria were analyzed to evaluate potential fecal contamination and specific bacterial presence within the tested portions. Because this evaluation lacked a raw-material baseline, environmental sampling, comparators, time series, or storage tests, the re-

sults reflect only the specific status of the analyzed lots. This screening does not demonstrate process hygiene, handling efficiency, or the effects of processing and storage on microbial stability (Ndraha et al. 2024; Snyder et al. 2024; WOA 2024).

Total and thermotolerant (fecal) coliforms are widely used as indicators of the hygienic and sanitary quality of foods and water (Hammad et al. 2022; Martin et al. 2023; Zaffalon et al. 2025). Total coliforms comprise a group of Gram-negative, non-spore-forming, rod-shaped, facultatively anaerobic bacteria defined by their ability to ferment lactose under specified conditions (ISO 4831:2006b; ICMSF 2018). These microorganisms are predominantly associated with the intestinal tract of humans and other warm-blooded vertebrates (Foster-Nyarko and Pallen 2022), although certain members of the coliform group may also persist naturally in soil, surface waters, sediments, and vegetation, particularly under favorable environmental conditions (Bagordo et al. 2024). Thermotolerant coliforms are distinguished by their ability to grow and ferment lactose at elevated incubation temperatures (44–44.5 °C) and are composed predominantly of *E. coli*, although some species of *Klebsiella*, *Enterobacter*, and *Citrobacter* may also exhibit thermotolerance (Hammad et al. 2022; ICMSF 2018). Consequently, results below the reporting limits are consistent with low levels of fecal contamination in the tested portions, but they do not demonstrate hygienic harvesting or processing and do not definitively rule out all foodborne pathogens or low-level fecal contamination (Silva et al. 2021; Rosario et al. 2021).

The non-detection of *Salmonella* spp. in the tested portions of freeze-dried *Australonuphis* sp. is particularly relevant because, unlike total coliforms and *E. coli*, *Salmonella* is one of the main foodborne pathogenic bacteria worldwide (ISO 6579-1:2017; Morasi et al. 2022). Non-typhoidal *Salmonella* is estimated to cause approximately 93.8 million cases of acute gastroenteritis and 155,000 deaths globally each year, with the vast majority at-

tributed to contaminated food and water (Majowicz et al. 2010; WHO 2015; Ma et al. 2024).

Coagulase-positive staphylococci were below the reporting limit (< 50 CFU g^{-1}) in each of the three freeze-dried *Australonuphis* sp. lots analyzed. *S. aureus*, the primary coagulase-positive species, is a Gram-positive bacterium arranged in grape-like clusters that colonizes the skin, nasal mucosa, and mucous membranes of humans and warm-blooded animals (González-Machado et al. 2024), as well as food-processing equipment and environments (Léguillier et al. 2024). It is widely monitored as an indicator of personal hygiene and food handling conditions because of its ability to produce heat-stable enterotoxins responsible for staphylococcal food poisoning (Argudín et al. 2010; Li et al. 2024; Xing et al. 2025).

Because no raw (pre-drying) material was analyzed and no inoculated challenge or recovery study was performed, the present data cannot indicate whether freeze-drying reduced, injured, or had no effect on the target bacteria. Freeze-drying is a dehydration process rather than a sterilization treatment, and bacteria may survive in low-moisture matrices, in some cases in a stressed state that may reduce their recovery by culture-based methods (Lee et al. 2021; Liu et al. 2022; Morasi et al. 2022). Non-detection in the freeze-dried product should therefore not be interpreted as evidence that the process inactivates these microorganisms. Accordingly, the results describe only what was observed in the tested post-drying portions, namely that none of the five targets was detected above the method limits. Because no raw material, environmental, or process samples were analyzed, the results cannot be attributed to the initial microbiological quality of the specimens, to the sanitary conditions maintained during harvesting and handling, or to the drying process.

Polychaete meals and freeze-dried polychaete products have been evaluated in shrimp and fish diets, particularly for broodstock maturation and as partial fishmeal replacements (Lupatsch 2014; Monteiro et al. 2023; Balqadi et al. 2025; Akbaş

et al. 2026; Herrera-Perez and Ferrando 2026). However, such feeding-performance evidence does not inform the microbiological quality of a given lot of material. Microbiological data specific to freeze-dried polychaete ingredients are scarce, and the present results add culture-based data for five bacterial targets in freeze-dried *Australonuphis* sp.

CONCLUSIONS

In the three freeze-dried lots of *Australonuphis* sp. tested, total coliforms (< 5 CFU g^{-1}), thermo-tolerant coliforms and presumptive *E. coli* (< 3 MPN g^{-1}), and coagulase-positive staphylococci (< 50 CFU g^{-1}) were below the method reporting limits, and *Salmonella* spp. was not detected in the pre-enriched test portions. These results apply only to the tested portions of three lots collected on a single date at one site and do not establish regulatory compliance or feed suitability. Future work should (i) analyze raw material and process samples alongside the freeze-dried product; (ii) sample several harvest dates, seasons, and sites; (iii) include additional hazards relevant to wild-collected polychaetes, such as *Vibrio* spp. and pathogens of aquatic animals (e.g. white spot syndrome virus and *Enterocytozoon hepatopenaei*); (iv) assess storage stability; and (v) evaluate digestibility and feeding performance, together with a formal feed-specific regulatory classification under MAPA.

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Declaration on the use of AI and automated technologies

During the preparation of this manuscript, the authors used ChatGPT (OpenAI) for language editing and grammatical improvement. The authors reviewed and edited the output and take full responsibility for the content of the manuscript.

Author contributions

Melissa Herrera-Perez: conceptualization; methodology; writing-original draft; writing-review and editing. Helenice Gonzalez de Lima: methodology; writing-review and editing. Natacha Deboni Cereser: methodology. Patricia da Silva Nascente: methodology; writing-review and editing. Camila dos Santos Cardozo: methodology. Ivan De Marco: methodology. Cesar Valmor Rombaldi: conceptualization; writing-review and editing.

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