Microsporidian heavy infection in a batch of salted and dried cod

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Abstract

The aim of this work is the description and characterization of a severe microsporidian infection in a batch of salted and dried cod. Particularly, the case involves a batch of approximately 800 kg obtained from *Gadus macrocephalus* (Food and Agriculture Organization Zone 61 - Northwest Pacific Ocean), which, after rehydration and sectioning operations, underwent routine company checks before packaging. In about 20% of the samples, the presence of whitish nodules with a diameter ranging from 1 to 2 mm was observed on the surface of the fillets and in cross-section. The lesions ranged from a few units to 10 per cm^2. Some samples were subjected to fresh microscopic observation with the stereomicroscope, confirming the nodular nature of the lesions, which were often confluent, alternating with empty spaces, giving the tissue a honeycombing aspect. The histological examination at low magnification allowed us to observe the heavy vacuolization of nodular lesions irregularly surrounded by a spongy-like wall. The observation at higher magnification of other sections allowed us to identify intra-myofibrillar cists containing presumptive microsporidian elements. The tissue damage derived from the technological processes and gravity of lesions did not allow a morphological characterization of presumptive protozoans. The molecular examination of the nodular lesions and the analysis of the sequence of an 897 bp fragment of the small subunit 16S rRNA revealed 100% identity with *Microsporidium theragrae* (GenBank accession number MT928885-89) first isolated from the skeletal muscles of *Gadus chalcogrammus* specimens from the Sea of Okhotsk. This finding confirms the importance of selecting suppliers and raw materials in the seafood industry, as well as the usefulness of an effective traceability system.

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Introduction

Dried and salted cod is a very well-known and appreciated processed seafood around the world, with an interesting and ancient history since its trade represented an important source of commerce between North and South Europe a lot of centuries ago (EUMOFA, 2021). *Gadus morhua* (Atlantic cod) and *Gadus macrocephalus* (Pacific cod) are the two species that can be used to produce dried and salted cod.

For their production, after catching, bleeding, gutting, beheading, and splitting or filleting, cods are essentially submitted to wet and dry salting processes. Before consumption, products are rehydrated in tap water, with water uptake and salt leaching out of the muscle tissue (Oliveira *et al*., 2012). Several factors affect the quality of products along the production chain. For example, cod freshness has been shown as a conditioning element in the salting, drying, and desalting stages (Barat *et al*., 2006); also, the state of rigor and freezing before salting can have an important influence on the final quality of the products (Lauritzsen *et al*., 2004).

Also, the fishing method (*e.g.*, gillnet or longline) can play a key role in this regard since it can produce several kinds of muscular lesions (*e.g.*, "blood spots") or affect the freshness and microbiological parameters of cods before processing (Esaiassen *et al*., 2004). Finally, the characteristics of the salting and rehydrating processes result in very important differences in the quality and shelf life of the product (Oliveira *et al*., 2012). Besides the above extrinsic factors, cod pathologies concerning parasitosis could be taken into account. Focusing on muscular parasitosis,

Hemmingsen and MacKenzie (2001), in their review of the parasite fauna of Atlantic cod, reported the presence of Anisakids larvae as well as the infection by some protozoans such as *Kudoa thyrsites* (*Myxosporea*) and some microsporidian species (*Pleistophora gadi, Pleistophora* spp. and *Microsporidium* spp.), concluding, regarding these last protozoans, that they are probably the same species. However, the findings of muscular localizations of the *Microsporidium* genus in *G. morhua* are fragmentary, incomplete, and dated (Drew, 1909; Polyansky, 1955; Young, 1969; Karasev, 1984; Waluga *et al*., 1986), as well as those referred only to fresh samples of Atlantic cod. The present paper aims to describe the heavy infection of *Microsporidium* observed in a batch of dried and salted cod obtained from Pacific cod (*Gadus macrocephalus*), observed during processing.

Materials and Methods

Sample source

The study concerns an 800 kg batch of dried and salted cod obtained from *G. macrocephalus* caught in the Food and Agriculture Organization Area 61 – Northwest Pacific. After the rehydration and cutting processes, the routinary quality control before packaging pointed out in almost 20% of samples the presence of numerous whitish nodules. These nodules with a diameter ranging from 1 to 2 mm were present on the fillets' surface and on the section. 15 samples were immediately sent, under refrigerated conditions, to the laboratory of Riconnexia SRLS at the Department of Veterinary Sciences of the University of Messina to evaluate the origin of these nodules.

Gross and light microscopy

In the laboratory, all 15 samples were carefully macroscopically observed, recording the shape, distribution, and number of the nodules. After this preliminary evaluation, portions (5×5 cm) of fillets were observed with a stereomicroscope (Leica M205C, Wetzlar, Germany – equipped with Leica Application Suite X vers. 3.0.14.23224).

Smears of nodules were wet mounted with lactophenol solution or fixed and colored with Methylene Blue (Carlo Erba, Milano, Italy) and Giemsa stain (Carlo Erba, Milano, Italy).

Furthermore, samples (10×10×5 mm) of muscle were fixed in 10% neutral buffered formalin, followed by processing using standard histological techniques, and embedded in paraffin. Hematoxylin and Eosin (Carlo Erba, Milan, Italy), Toluidine Blue (Titolchimica spa, Pontecchio Polesina, Italy), and Diff Quik (Titolchimica spa, Pontecchio Polesina, Italy) were used for staining. The upper reported preparations (fresh nodules and histology) were observed under a light microscope Leica DM 2000 (Germany - equipped with Leica Application Suite X vers. 3.0.14.23224).

Molecular and phylogenetic analysis

DNA was extracted from 25 mg of thawed muscle using the PureLink Genomic DNA kits (Invitrogen, Thermo Fisher Scientific, Rome, Italy) according to the manufacturer's instructions. The quality and concentration of DNA extracts were assessed at 260/280 and 260/230 nm using a SmartSpec Plus spectrophotometer (Bio-Rad, Milan, Italy). All purified DNA was stored at -20°C. A fragment of approximately 897 bp of small subunit (SSU) 16S region of rRNA gene was amplified using the microsporidia-specific primers: ss18F (5′-CAC CAG GTT GAT TCT GCC -3′) and ss1492R (5′-GGT TAC CTT GTT ACG ACT T-3′) (Weiss and Vossbrinck, 1998).

Polymerase chain reactions (PCR) were performed in volumes of 50 µL containing PCR buffer, 200 mM dNTP, 2 mM $MgCl_2$, 1.25 units Taq polymerase, 20 pm each primer, and 2 µL DNA template. After an initial denaturing of 3 minutes at 95°C, there were 35 cycles of denaturation at 95°C (30 seconds), annealing at 50°C (30 seconds), and extension at 72°C (2 minutes) with a final extension of 10 minutes at 72°C, using a CFX96 TouchTM (Bio-Rad, Milan, Italy) thermal cycler.

The PCR amplification products were visualized on a 1.5% agarose gel (Biosigma, Venice, Italy), containing GelRed (Biotium, Clinisciences, Rome, Italy) using Gel Doc XR System and the software Quantity One v 4.5.2. (Bio-Rad, Milan, Italy). The resulting products were compared using GeneRuler 100 bp DNA Ladder (Invitrogen, Thermo Fisher Scientific, Monza, Italy). The clearest and brightest products were selected for subsequent Sanger-sequencing by BMR Genomics (Padua, Italy) sequencing service.

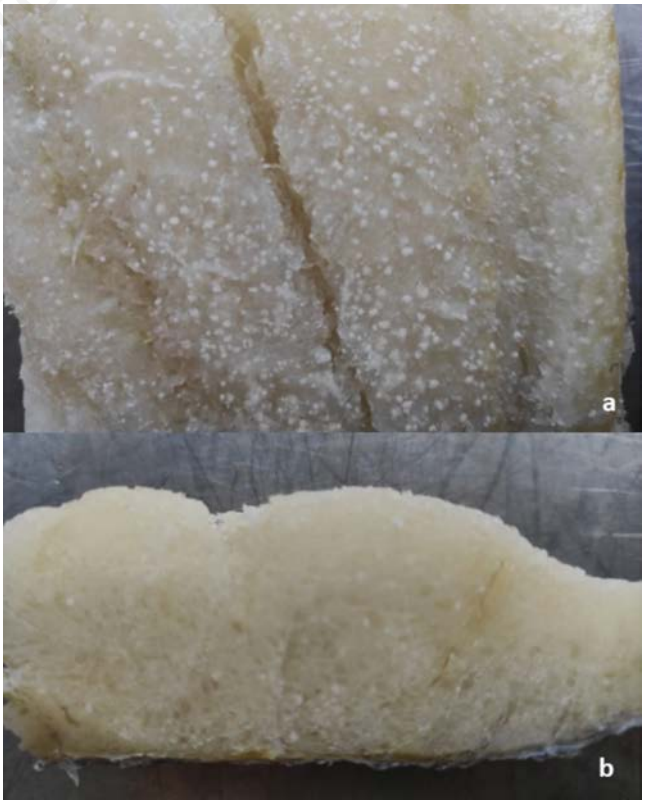

Figure 1. a) Nodular lesions on the surface of salted and dried cod; **b**) section of salted and dried cod in which the honeycombing aspect is evident.

Two partial SSU 16S rDNA sequences of 858-897 bp in length were obtained and blasted against microsporidia sequences from the National Center for Biotechnology Information GenBank database (http://blast.ncbi.nlm.nih.gov/blast, accessed on January 20, 2024). Phylogenetic analyses were conducted using MEGA software, version 11. The sequences were aligned using the CLUSTAL W method (Thompson *et al*., 1994). The phylogenetic tree was constructed using the maximum likelihood method and Kimura 2-parameter model with 1000 bootstrap replicates (Kimura, 1980; Felsenstein, 1985).

Results

Gross and light microscopy

In all 15 samples, nodules were present on the surface and on the section of the fillets, with a number ranging from 1 to 10 per cm^2 (Figure 1). The observation of samples at the stereomicroscope showed the nodular lesions as a conglomerate of smaller nodules as well as the disruption of the surrounding tissue, which takes on a honeycomb appearance (Figure 2a).

Light microscopy of fresh and fixed nodules showed the presence of several basophilic nuclei mixed with amorphous material.

The histological examination at low magnification allowed us to observe the heavy vacuolization of nodular lesions irregularly surrounded by a spongy-like wall (Figure 2b). The observation at higher magnification of other sections (Figure 2 c,d) showed intra-myofibrillar cists containing presumptive microsporidian elements. The tissue damages derived from the technological processes and gravity of lesions did not allow a morphological characterization of presumptive protozoans.

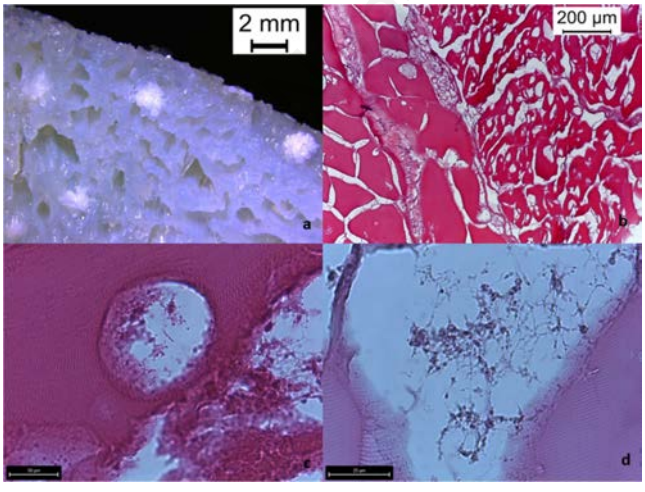

Figure 2. a) Stereomicroscopic observation of the section of products with the honeycombing aspect; **b**) heavy vacuolization of nodular lesions irregularly surrounded by a spongy-like wall (HH stain); **c, d**) intra-myofibrillar cists containing presumptive microsporidian elements.

Molecular results

The basic local alignment search tool (BLAST) analyses on the two sequences obtained revealed 100% identity with *Microsporidium theragrae* (GenBank Accession number MT928885-89) (Table 1), first isolated from the skeletal muscles of *Gadus chalcogrammus* specimens from the Sea of Okhotsk (Ovcharenko *et al*., 2022). As described by the authors (Ovcharenko *et al*., 2022), sequence analysis of the SSU rDNA coding region showed that *M. theragrae* is closely related to *M. cypselurus* (Yokoyama *et al*., 2002), infecting flying fish *Cheilopogon japonicus* from the East China Sea. These two species are very close relatives both in geographical distribution (Northwest Pacific region) and molecular and cytological findings. However, even in our case, the percentage of similarity between *M. theragrae* and *M. cypselurus* was much lower (93.37%) than the accepted similarity cut-off (97%) (Aguilar *et al*., 2016; Choi and Park, 2020), confirming that they are different species.

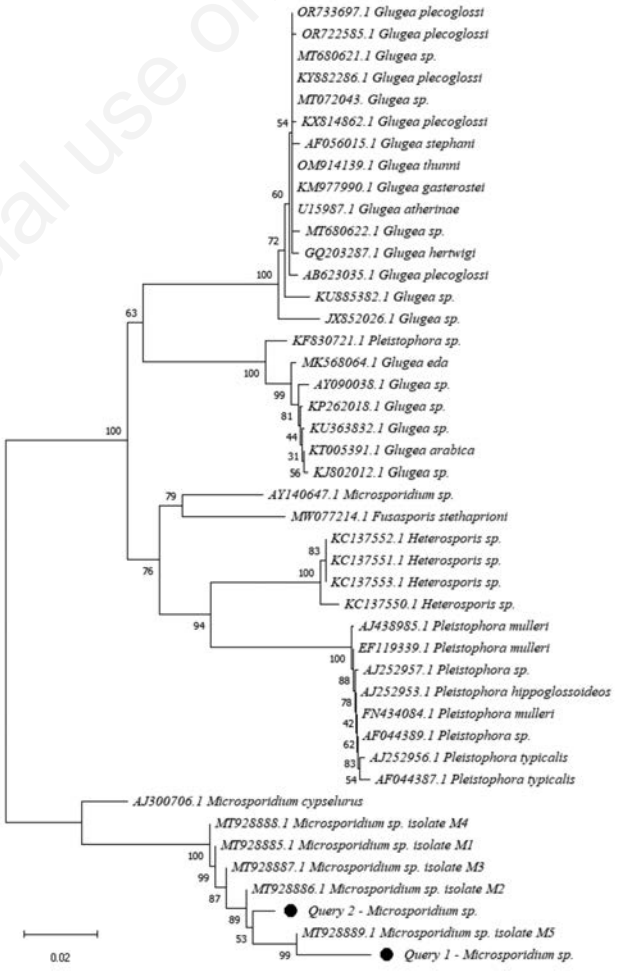

Figure 3. Phylogenetic trees based on SSU 16S rDNA sequences for 41 microsporidian taxa built by using the ML method with 1000 bootstrap replicates. The symbol "●" states for the 2 Microsporidium sp. isolates from Pacific cod skeletal muscle lesions. The percentages of replicate trees in which the associated taxa clustered together are shown next to the branches.

Additionally, BLAST search and phylogenetic analysis support that *M. theragrae* is closely related phylogenetically to *Pleistophora* and *Glugea* but does not form a monophyletic clade with them, indicating the existence of a specific genus (Figure 3).

Discussion

The presence of *Microsporidium* in the muscular tissue of the *Gadus* genus has been reported by some authors (Drew, 1909; Polyansky, 1955; Young, 1969; Karasev, 1984; Waluga *et al*., 1986), but these reports are dated and did not clarify either the involved species (*Pleistophora* or *Microsporidium*) or the morphological diagnostic elements. Concerning the present study, the damages to the examined muscular tissue due to the massive infection and processing (salting and drying) have not allowed a precise morphological characterization of protozoans. However, the molecular analysis allowed us to ensure the presence of *M. theragrae*, yet isolated in *G. chalcogrammus* caught in the Sea of Okhotsk.

Conclusions

Despite the need for further investigation, the present finding would represent the first report of *M. theragrae* in *G. macrocephalus*. The high incidence observed in this batch, as well as the severity of the lesions, leads to the unsuitability for consumption of these products, as they are visibly parasitized and significantly compromised in terms of organoleptic characteristics. Within the framework of fisheries control, this finding confirms the impor-

Table 1. Results of the basic local alignment search tool analysis performed on 16S region of rDNA sequence for microsporidia from the Pacific cod. Best hits (with the highest similarity scores) are shown as retrieved in January 2024 from GenBank.

Species	Coverage (%)	Similarity (%)	Length of sequences in GenBank	GenBank accession number
Microsporidium sp. Isolate M4	82	100.00	897	MT928888.1
Microsporidium sp. Isolate M1	82	100.00	881	MT928885.1
Microsporidium sp. Isolate M3	82	100.00	878	MT928887.1
Microsporidium sp. Isolate M2	82	100.00	898	MT928886.1
Microsporidium sp. Isolate M5	79	100.00	858	MT928889.1
Microsporidium cypselurus	88	93.37	1348	AJ300706.1
Microsporidium sp. STF	98	89.46	1444	AY140647.1
Glugea sp. GC_2015	98	89.03	1820	KU363832.1
Glugea sp. isolate ZH-2023	98	88.94	1328	PP068056.1
Pleistophora sp. LM-2014	98	88.95	1243	KF830721.1
Glugea arabica	98	88.94	1763	KT005391.1
Pleistophora mulleri	98	88.67	1483	AJ438985.1
Glugea sp. LM-2015	98	88.83	1234	KP262018.1
Glugea sp. GC_2014	98	88.83	1782	KJ802012.1
Pleistophora hippoglossoideos	98	88.56	1372	AJ252953.1
Pleistophora mulleri	98	88.44	3698	EF119339.1
Glugea eda	98	88.72	1564	MK568064.1
Glugea sp.	98	88.72	1326	AY090038.1
Pleistophora sp.	98	88.44	1373	AJ252957.1
Glugea sp.	98	88.54	1679	MT072043.1
Glugea plecoglossi	98	88.54	1813	KY882286.1
Glugea gasterostei	98	88.54	1305	KM977990.1
Glugea thunni	98	88.54	1751	OM914139.1
Glugea sp.	98	88.54	1345	MT680622.1
Glugea plecoglossi	98	88.43	1548	KX814862.1
Glugea sp.	98	88.44	1349	MT680621.1
Pleistophora mulleri	98	88.25	1306	FN434084.1
Glugea plecoglossi	97	88.55	1194	OR733697.1
Glugea atherinae	98	88.44	1335	U15987.1
Glugea hertwigi	98	88.31	1856	GQ203287.1
Glugea stephani	98	88.31	1165	AF056015.1
Glugea plecoglossi	97	88.44	1194	OR722585.1
Fusasporis stethaprioni	98	88.34	1286	MW077214.1
Pleistophora typicalis	98	88.05	1373	AJ252956.1

tance of selecting suppliers and raw materials. The implementation of increasingly effective traceability systems is, of course, essential.

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