Marine thraustochytrid: exploration from taxonomic challenges to biotechnological applications

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Thraustochytrids, as a distinct group of heterotrophic protists, have garnered considerable attention owing to their remarkable adaptability in extreme marine environments, pronounced capacity for metabolic regulation and prolific production of high-value metabolites. The taxonomic classification of these microorganisms presents a substantial challenge due to the variability in morphological characteristics under different culture conditions. And this undermines the efficacy of traditional classification systems on physiological and biochemical traits. The establishment of a polyphasic taxonomic system integrating genomic characteristics in the future will provide new avenues for more accurate classification and identification. Thraustochytrids can effectively accumulate bioactive substances such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), squalene and carotenoids. Through fermentation optimization and genetic modification, scientists have significantly enhanced the production of these metabolites. Moreover, the application of thraustochytrids in aquaculture, poultry and livestock feed has significantly improved animal growth and physiological indicators meanwhile increasing their DHA content. Natural bioactive substances in thraustochytrids, such as terpenoid compounds with antioxidant properties, have been proposed for application in the cosmetics industry. In the field of pharmacology, thraustochytrids have shown certain anti-inflammatory and anti-cancer activities and provide potential for the development of new oral vaccines. Additionally, they can degrade various industrial and agricultural wastes for growth and fatty acid production, demonstrating their potential in environmental bioremediation. Therefore, thraustochytrids not only exhibit tremendous application potential in the field of biotechnology, but also hold significant value in environmental protection and commercialization.

1 Introduction

Thraustochytrids, belonging to the phylum Labyrinthulomycetes, were ubiquitous marine heterotrophic protists and exert of significance roles in coastal and oceanic ecosystems (Liu et al., 2017; Xie et al., 2022). They were widely considered as significant decomposers of organic matters in humus-rich environment or phytoplankton bloom areas by secreting various extracellular enzymes, e.g. cellulase, amylases and proteases (Liu et al., 2014b). Thraustochytrids participated in the carbon cycle and particle sink from the surface to the deep sea by facilitating the formation of marine aggregates (Damare and Raghukumar, 2012). These were contributed largely to the physiological and morphological features of thraustochytrids such as the attachment of biflagellated zoospores and ectoplasmic net to the substrates, hydrophobic effect of the cell wall as well as the production of extracellular polysaccharides (Damare et al., 2020). The diverse habitats of thraustochytrids have led to the production of complex metabolites, positioning them alongside marine bacteria, fungi and microalgae as crucial sources for the development of natural bioactive substances. Due to containing abundant ω-3 polyunsaturated fatty acids (PUFAs), squalene and carotenoids, thraustochytrids are excellent nutrient providers for marine animals through marine food web. And they exhibit great biotechnological application potentials in food, feed and healthy industries.

Ω-3 PUFAs of thraustochytrids were mainly composed of docosahexaenoic acid (DHA) which was significant for modulating the cardiovascular system and promoting cell growth and development of nervous system for human infants (Guesnet and Alessandri, 2011; Graham et al., 2021). Recently, most of the concerns about thraustochytrids were put on the biosynthesis process optimization of PUFAs using fermentation strategies combined with genetic modification and omics techniques as well as wastes screening for fermentation substrates (Heggeset et al., 2019; Yu et al., 2020; Wang et al., 2021a, b; Watanabe et al., 2022; Li et al., 2023). Although former studies had testified the feasibility of utilizing varied agricultural and industrial wastes for DHA production by thraustochytrids, and exhibited great industrial application potential in sustainable DHA production (Chi et al., 2007; Lee Chang et al., 2013; Ryu et al., 2013; Song et al., 2017; Villarroel Hipp and Silva Rodriguez, 2018). High-value utilization of novel wastes such as tofu whey wastewater (Wang et al., 2020), soybean curd wastewater (Lee et al., 2020), volatile fatty acids (Patel et al., 2020), waste acid oil (Laddha et al., 2021), sugarcane bagasse (Watanabe et al., 2022), dairy and brewery wastes (Russo et al., 2021), digestate from methanization (de la Broise et al., 2022) and food waste from Chinese restaurants (Li et al., 2023) were also proved to be excellent feedstock alternatives for DHA production of thraustochytrids. Future endeavors utilizing simple structural, widely sourced one-carbon (methanol, formic acid) and two-carbon (acetic acid) substrates for thraustochytrid fermentation may yield even more exciting outcomes.

In addition, the downstream process of thraustochytrids based biorefinery including lipid extraction and the refining procedure had been developed increasingly. Several extraction methods of lipids containing high levels of DHA were established including enzymatic ethanolysis (He et al., 2019), high shear-assisted solvent extraction (Kwak et al., 2019), supercritical CO_2 extraction (de Melo et al., 2020) and ultrasound-assisted extraction (Li et al., 2021). Remarkably, plenty of recent researches demonstrated that feeding supplementation of biomass or extracts of thraustochytrids was beneficial for aquatic animals such as Nile tilapia (dos Santos et al., 2019), golden pompano (Xie et al., 2019), Pacific white shrimp (Xu et al., 2022) and Penaeus monodon larvae (Visudtiphole et al., 2021), as well as Japanese quail (Gladkowski et al., 2014), layer hen (Moran et al., 2020) and steers (Carvalho et al., 2018) in improving their growth performance, survival, immunity, anti-inflammatory effect, and regulating the blood cells and gut microbiota (Souza et al., 2020). Presumably, thraustochytrids will become an important source of DHA in animal originated foods in our daily diet, and thus improve human health (Figure 1).

2 Taxonomy challenges of thraustochytrids: advancements and issues in classification methods

Considering the great contribution of thraustochytrids to marine ecosystem and humans, it is necessary to figure out the taxonomy characters of Thraustochytriaceae sp. in order to take better advantage of this kind of protists. In the past decades, increasing novel thraustochytrid strains were progressively obtained along with the development of isolation and culture techniques for marine microorganism. However, due to the morphological characters of thraustochytrids vary with their habitats, the traditional classification system based on morphological data are facing many challenges. Besides, the molecular biological method which was widely applied in microorganism identification, also led to confused taxonomy of thraustochytrids because of these disordered reference sequences in database. Moreover, the biochemical markers such as fatty acid profiles had been used as classification features of thraustochytrids. In the following paragraphs, we summarized the development and current issues in the morphological, biochemical and molecular phylogeny methods for thraustochytrid classification (Table 1). Additionally, we discussed polyphasic taxonomy in order to establish an accurate and scientific taxonomic method.

2.1 Morphological features: challenges and evolution in thraustochytrid identification and classification

At present, the characteristics of life cycle, morphology, ultrastructure, biochemical markers and phylogenetic analysis were typical characteristics for the taxonomy of thraustochytrids. Except for the phylogeny, all the other features are traditional taxonomic features and usually fail to correspond to the phylogenetic relationship of thraustochytrids (Marchan et al., 2017; Dellero et al., 2018a). The life cycle and morphological
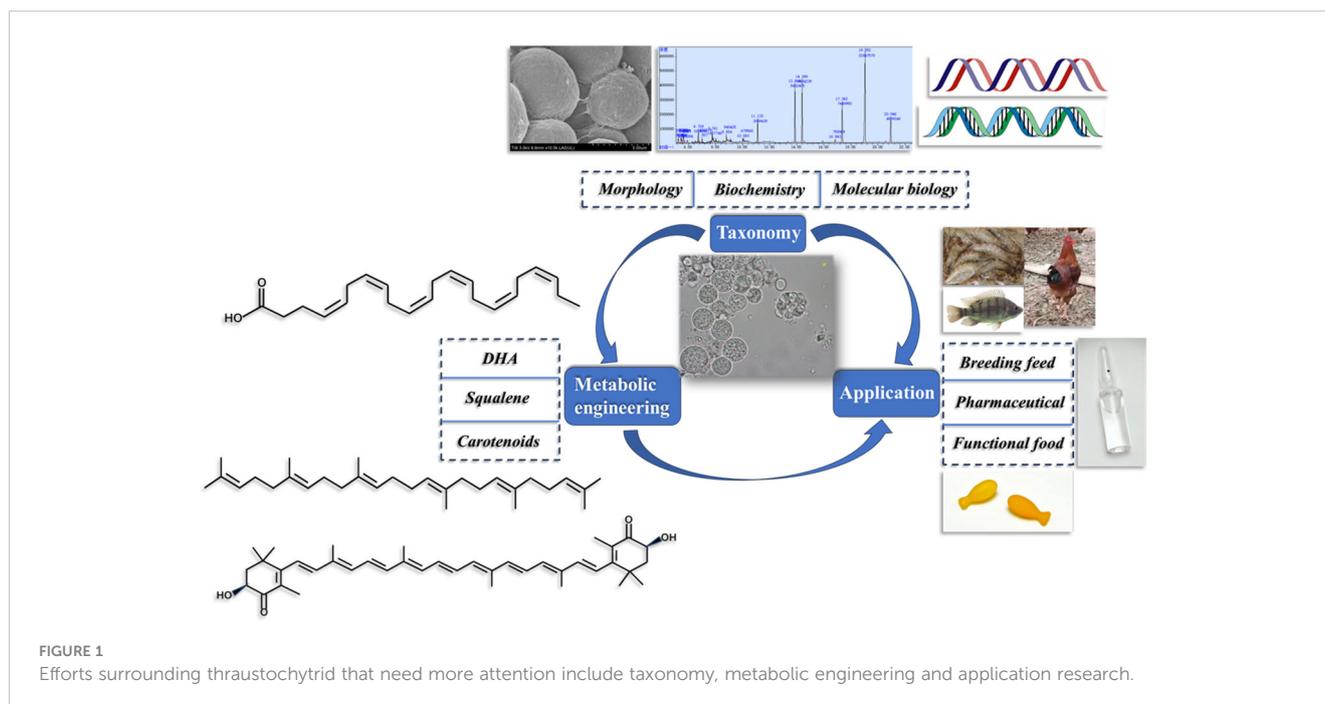
FIGURE 1
Efforts surrounding thraustochytrid that need more attention include taxonomy, metabolic engineering and application research.

development of thraustochytrids are complex. And several attempts have been made to redefine the taxonomic status and phylogenetic relationship of them (Yokoyama and Honda, 2007; Yokoyama et al., 2007; Doi and Honda, 2017; Dellero et al., 2018b). The taxonomic features at the genus and species level were usually dependent on the specific morphological features such as zoospore production (Doi and Honda, 2017), and biochemical characteristics such as the production of specific fatty acids and pigment compounds (Honda et al., 1998). However, differences existing in the life cycle and metabolic profiling with the variation of culture conditions deepened the burden on homogenizing and systematically defining taxa only using phenotypic traits (Raghukumar, 1988). And this thus led to an excessive criteria for identifying and describing specific taxa of thraustochytrids (Raghukumar, 1988). When *Aurantiochytrium limacinum* was grown in artificial medium, the cell body accumulating large amounts of triglycerides at the end of the growth stage produced no zoospores (Dellero et al., 2018a). While large amounts of zoospores produced in oligotrophic medium such as artificial seawater (Dellero et al., 2018a). Besides, the zoospores could swim for at least six days in the absence of organic carbon consuming intracellular triglycerides for energy (Dellero et al., 2018a). While the addition of glucose rapidly triggered the maturation of the zoospores (Dellero et al., 2018a). Therefore, it is necessary to specify the medium for life cycle observation in the identification of thraustochytrid strains (Raghukumar, 1988).

Presently, the most controversial taxonomic species of thraustochytrids exist in the genus *Thraustochytrium*, a typical genus of the family Thraustochytriaceae. Species *Thraustochytrium*. sp. are characterized by the formation of zoospores, including the cell wall shape of sporangium and the presence or absence of proliferates. However, several taxonomic problems remain in this genus,

including the overlap of polyphyletic and interspecific taxonomic features (Gao et al., 2013; Doi and Honda, 2017). For example, the first and second species of this genus, *T. proliferum* and *T. globosum*, were described by observing the morphological characteristics of natural specimens without culture (Doi and Honda, 2017). The cellular morphology of *T. globosum* was first observed on the surface of green algae and was clearly distinguished from the typical species *T. proliferum* by morphological characteristics, namely the proliferates, zoospores and ectoplasms features (Kobayashi and Ookubo, 1953). However, this strain has never been registered in cultured specimens previously, so the taxonomic characteristics cannot be recognized under artificial conditions (Sparrow, 1936). It was believed that the genus *Thraustochytrium* was composed of heterogeneous species (Sparrow, 1969). Later, Doi and Honda (2017) successfully isolated a thraustochytrid strain NBRC 112723, which had typical classification characteristics of *T. globosum* in natural condition (Doi and Honda, 2017). But under culture condition it showed similar taxonomic features with other thraustochytrid species (Doi and Honda, 2017). Finally, combined with its molecular phylogeny and the taxonomic criteria of typical species *T. proliferum*, the genus *Thraustochytrium* was rearranged for *T. globosum*. Thus, a new genus *Monorhizochytrium* was established to clearly distinguish *T. globosum* (Doi and Honda, 2017).

2.2 Biochemical indicators: incorporating biochemical characteristics for enhanced identification

Biochemical characteristics such as lipids and carotenoids, were proposed to be included into the taxonomy of thraustochytrids (Huang et al., 2003; Yokoyama and Honda, 2007; Yokoyama et al., 2007). In the

TABLE 1 Taxonomic summary for each taxonomic criterion of thraustochytrids.

Classification method	Taxonomic criterion	Detailed index	Reference
Morphological features (the life cycle and morphological development)	General morphology	Size, color and shape of the colonies, as well as size and shape of the cells	Bongiorni et al., 2005; Yokoyama and Honda, 2007; Yokoyama et al., 2007; Doi and Honda, 2017; Marchan et al. 2018
	Life cycle	Vegetative reproduction, amoeboid reproduction or sexual reproduction	
	Proliferation	Presence or absence, and the number	
	Sporangium	Size, wall thickness, release mode of spores and persistence of cell wall	
	Amoeboid cells	Presence or absence, and their activity	
	Ectoplasmic nets (EN)	Developed or undeveloped, branched or unbranched, long or not, and the number	
Biochemical indicators	PUFAs profiles	Composition of DHA, DPA, EPA and ARA	Huang et al., 2003; Yokoyama and Honda, 2007; Yokoyama et al., 2007
	Carotenoid profiles	Canthaxanthin, β-carotene, astaxanthin, phenicoxanthin and echinenon, etc	
Molecular Phylogeny	18S rRNA gene	Primers for building tree sequences, i.e. 18S001/18S13, SR01/SR12L1, LabyA/LabyY	Honda et al., 1999; Stokes et al., 2002; Ueda et al., 2015
	Mitochondrial cytochrome C oxidase subunit I (COI) sequence	Primers for building tree sequences: COI-Aur43F/COI-Aur748R	Nishitani and Yoshida, 2018

early stage, thraustochytrids newly isolated from coastal areas of Japan and Fiji had been divided into five main groups (DHA/DPAω6、DHA/DPAω6/EPA, DHA/EPA, DHA/DPAω6/EPA/ARA, DHA/DPAω6/EPA/ARA/DTA) based on PUFA profiles, which were allocated into a single cluster based on the 18S rRNA gene (Huang et al., 2003). However, this method failed to determine the PUFA

profiles of a specific genus, and they were used as biochemical markers for thraustochytrids instead (Huang et al., 2003). Later, two secondary biochemical markers, i.e. the PUFA and carotenoid profiles were combined with the 18S rRNA marker, and were successfully used for the identification of new thraustochytrid genera (Yokoyama and Honda, 2007; Yokoyama et al., 2007). This provided a more robust phylogenetic mechanism for a single phylogenetic branch of Thraustochytriaceae sp. with the exception of genus Thraustochytrium. The genus Thraustochytrium might contain subgenera. Because it did not form a single phylogenetic group based on 18S rRNA and did not exhibit a common PUFA or carotenoid spectrum between species (Huang et al., 2003). Therefore, it is expected that new genera will be proposed in the future.

2.3 Molecular phylogeny: advancements and challenges in thraustochytrid identification

Recently, morphological taxonomic features of thraustochytrids have been largely replaced by molecular phylogenetic analysis (Pan et al., 2017). However, the names of many taxa have not been effectively reported because no designated type is specified in the molecule database. Besides, multiple sequences for a single strain will inevitably affect species identification and may lead to an overestimation of genetic diversity. At present, molecular classification based on the 18S rRNA gene has been widely used in different biological systems. Recent studies had shown that no genetic polymorphism existed in the mitochondrial cytochrome C oxidase subunit I (COI) sequence of A. sp., which was conducive to phylogenetic analysis (Nishitani and Yoshida, 2018). Besides, primers designed for this gene can successfully amplify the COI sequences of 16 thraustochytrid strains belonging to five genera, i.e., Thraustochytrium, Aurantiochytrium, Schizochytrium, Botrychytrium and Parietichytrium, with clear single strips (Nishitani and Yoshida, 2018). Moreover, this mitochondrial gene was successfully sequenced without gene cloning, indicating that the mitochondrial region can be used for the identification and genetic diversity analysis of the related genera (Nishitani and Yoshida, 2018). This great facilitated the development of molecular phylogeny of thraustochytrids with multigene locus. Thus, it was beneficial for the clear clarification of natural evolutionary relationship between thraustochytrid species of different genera, enabling their accurate identification.

2.4 Polyphasic taxonomy: integrating phenotype and molecular traits for genus reclassification

The taxonomic characteristics of Labyrinthulomycetes at the genera level are mainly based on the cell morphology at different growth stages. And the influence of culture conditions on morphological characteristics enhances the instability of morphological classification. However, based on 18S rRNA and protein-coding sequence data, species in family Thraustochytriaceae tend to form polyphyletic groups (Wang et al., 2019). The

TABLE 2 Studies on significantly enhancing the production of high-value metabolites in thraustochytrids through fermentation optimization and genetic modification.

Strains	High-value metabolites	Enhancement strategies	Poduction improvement	Reference
Aurantiochytrium sp. SK4	Fatty acids (FA) and astaxanthin	Expression of the emoglobin gene from *Vitreoscilla stercoraria* (VHb)	44% higher total FAs and 9-fold higher astaxanthin contents	Suen et al., 2014
Schizochytrium sp. HX-308	FA profiles and squalene	Introduction of exogenous ω-3 desaturase gene	Improved ω-3/ω-6 ratio, converted 3% docosapentaenoic acid (DPA) to DHA, and reduced squalene in lipid by 37.19	Ren et al., 2015
Aurantiochytrium sp. SD116	PUFA	Overexpression of the G6PDH	enhanced the proportion of PUFA in lipids by 10.6%	Cui et al., 2016
Schizochytrium sp. HX-308	PUFA	Supplementing lipase inhibitor Orlistat into the medium	PUFA yield increased from 39.1 to 48.5 g/L	Ma et al., 2021
Aurantiochytrium sp. SD116	DHA	Heavy-ion irradiation technique was first used, then a two-step adaptive laboratory evolution (ALE), low temperature based ALE and ACCase inhibitor quizalofop-p-ethyl based ALE were employed	DHA content increased by 51%	Wang et al., 2021a
Aurantiochytrium sp. SD116	DHA	Combined metabolic engineering strategy including both disrupting one copy of the fatty acid synthase gene to partial deactivate the competing pathway of DHA biosynthesis, and the overexpression of acetyl-CoA carboxylase and diacylglycerol acyltransferase to strengthen the substrate supply and triacylglycerol synthesis	Increased DHA purity (61% in total FAs) with a content of 331 mg/g dry cell weight	Wang et al., 2021b
Aurantiochytrium sp. PKU#SW8	PUFAs	Nitrogen starvation	A 1.7-fold increase of PUFAs	Chen et al., 2022
Aurantiochytrium sp. SW1	DHA	Strategic plasma mutagenesis coupled with chemical screening to obtain mutant strains with high tolerance toward oxidative stress and high glucose-6 phosphate dehydrogenase (G6PDH) activities	80% higher DHA production	Nazir et al., 2022
Thraustochytrium sp. ATCC 26185	Squalene	Supplementing 5 g/liter NaCl into the medium	The amount of squalene improved 1.9 folds	Zhang et al., 2021
Aurantiochytrium sp. 18W-13a	Squalene and carotenoid	Constitutive expression of the 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR)	The content of squalene and carotenoid were 5.54 (8.3 ± 0.09 mg/L) and 5.11 times (2.76 ± 0.27 mg/g) higher respectively in 400 mL culture.	Yang et al., 2022b
Aurantiochytrium sp. TWZ-97	Squalene	Supplementing 0.7 g/L alpha-tocopherol into the medium	Squalene concentration improved 85% (240.2 mg/L),	Ali et al., 2023
Schizochytrium sp. HX-308	Terpenoid	Overexpression of acetyl-CoA c-acetyltransferase made more acetyl-coA available for the synthesis of terpenes acetyl-CoA	The production of squalene, β-carotene and astaxanthin was increased 5.4, 1.8 and 2.4 times	Huang et al., 2021
Aurantiochytrium sp. O5-1-1	Astaxanthin	Supplementing two-percent ethanol into the medium	Astaxanthin productivity increased 45-fold (2.231 mg/L)	Sakamoto et al., 2023
Aurantiochytrium sp. SZU445.	Carotenoids	Overexpression of KAS III-like enzyme *YxwZ3*	The yield of total carotenoids increased 4.88 folds	Song et al., 2022
Aurantiochytrium limacinum ATCC MYA-1381	Astaxanthin	Overexpression of a β-carotene hydroxylase (CrtZ) candidate gene	A significant increase in astaxanthin production per culture (2.8-fold) and per cell (4.5-fold)	Yoshimi et al., 2023

phylogenetic system constructed using 18S rRNA gene sequences showed that the genera *Schizochytrium* and *Ulkenia* were divided into three and four branches, respectively. Combined with the distribution characteristics of cell morphology, PUFAs and carotenoids, they were individually reclassified into two new genera and three new genera respectively (Yokoyama and Honda, 2007; Yokoyama et al., 2007). In

the constructed molecular phylogenetic system, the genus *Thraustochytrium* is divided into at least seven different branches (Yokoyama and Honda, 2007; Yokoyama et al., 2007). However, this genus has not been reclassified so far (Marchan et al., 2017). In addition, the molecular phylogenetic relationship between two newly isolated thraustochytrid strains were similar, but there were

differences in growth cycle, morphology and biochemical characteristics (Dellero et al., 2018a). And they were finally divided into two different genera, i.e., *Aurantiochytrium* (CCAP 4062/1) and *Hondaea* gen. nov (CCAP 4062/3) (Dellero et al., 2018a). Therefore, phenotypic or molecular traits solely have obvious deficiencies in the classification of *T*. sp. And the polyphase taxonomic method combining phenotypic and molecular traits has great feasibility for the reclassification of this genus.

2.5 Parasitic thraustochytrids in Labyrinthulomycetes taxonomy: pathogenicity and host interactions

Thraustochytrids coupled with labyrinthulids had been preliminarily reported to be associated with pathological condition in the Lesser Octopus *Eledone cirrhosa*, with symptoms of progressive ulceration of the skin and followed by oedema of body tissues and death (Polglase, 1980). In addition, Quahog Parasite Unknown (QPX), a protistan, had caused high mortalities of wild hard clams *Mercenaria mercenaria* in various regions of Canada and America (Susan et al., 2002). And they were identified to be a member of phylum Labyrinthulomycota based on the morphological and ultramicrostructure features as well as 18S rRNA gene sequences (Maas et al., 1999; Ragan et al., 2000). However, all the QPX protists isolated from infected hard clams from Duxbury, Massachusetts, were proved to be the same species employing phylogenetic analysis (Stokes et al., 2002). Furthermore, possibility existed that the molecular genetic and physiological variation exists among QPX isolates responsible for outbreaks of QPX disease in different areas (Qian et al., 2007). And the variation could most probably be found at the site that directly involved in and potently under selective pressure for the virulence (Qian et al., 2007). Moreover, thraustochytrid had been isolated from the surface mucus of the hermatypic coral *Fungia granulosa*, which established a symbiotic relationship with corals through secreting metabolites such as fatty acids and degradable enzymes (Harel et al., 2008). Future research on the isolation, physiological and biochemical characteristics of parasitic thraustochytrid strains will undoubtedly enrich our understanding of these Labyrinthulomycetes protists.

3 Composition analysis of thraustochytrids: unveiling bioactive components and ecological significance

3.1 Biotechnological exploitation of thraustochytrids: advancements in metabolic product research and enzymatic potential

Marine microorganisms, which have long thrived in extreme environments of low temperature and high pressure, continually adjust their metabolic activities to adapt to external conditions. Marine microorganisms represent a treasure trove for the discovery of natural bioactive metabolic products. And researchers have obtained many potential medicinal active substances from these organisms. Thraustochytrids are most notably recognized for their rich content of natural bioactive components such as PUFAs, squalene and carotenoids, demonstrating immense application potential in industries such as food, pharmaceuticals, feed and cosmetics. Additionally, the presence of substantial amounts of long-chain saturated fatty acids and monounsaturated fatty acids in thraustochytrids makes them a promising candidate for biodiesel production. Furthermore, the lipid and terpenoid components in thraustochytrids can be simultaneously extracted through fractionation, enhancing the efficiency of biomass resource utilization. Research on the metabolic products of thraustochytrids is focused on increasing the yield of these high-value products through biotechnological methods. It also aims at identifying substrates that are efficiently utilized by thraustochytrids, which are widely available and cost-effective, in order to enhance their commercial application potential by reducing production costs. Additionally, employing Raman spectroscopy to characterize the PUFAs and carotenoids in thraustochytrids offers the ability to monitor the composition within individual cells and variations among cells (Sasaki et al., 2023). This method, with its short analysis time and non-destructive nature, holds great potential for application in the production of thraustochytrid metabolic products and molecular domestication breeding (Sasaki et al., 2023).

Due to the high yield and wide application, the intracellular fatty acids and terpenoids in thraustochytrids currently constitute a research hotspot in the biotechnology field. However, the extracellular polysaccharides and enzymes of thraustochytrids, detected in lower quantities, have attracted the attention of only a few researchers. Given their natural habitat and heterotrophic lifestyle, thraustochytrids are capable of degrading extracellular macromolecules like proteins and polysaccharides, thus possessing a variety of extracellular enzyme synthesis and secretion capabilities. The potential and activity of thraustochytrid extracellular enzymes might be underestimated, and related research lacks in-depth study. Limited studies indicate that nitrogen nutrient limitation can induce protease activity in *S. aggregatum* ATCC 28209, suggesting that nutrient limitation strategies could be an effective means to stimulate high-level synthesis of extracellular enzymes (Man et al., 2022).

3.2 Discovery and potential of low-abundance compounds in thraustochytrids: advances in marine biopharmaceuticals

In recent years, components in thraustochytrids present in extremely low quantities have garnered increasing attention from scientists. A new acidic morpholine derivative containing glyceride was isolated and identified from *A*. sp., accounting for about 0.1~0.4% of the freeze-dried cells (Kaya et al., 2021). Its polar part is 2-hydroxy-3-oxomorpholino propionic acid, which may

play a significant role in the lifecycle of thraustochytrids and other organisms (Kaya et al., 2021). Morpholine, a nitrogen and oxygen-containing six-membered heterocyclic compound, is extremely rare in biological compositions (Kaya et al., 2021). Besides, it is an important raw material in many industrial and organic syntheses, often used in the field of pharmaceutical chemistry (Kaya et al., 2021). From 2 kilograms of biomass of *A. mangrovei* BT3, 20 milligrams of (24R)-4α-methyl-5α-stigmasta-7,22-dien-3β-ol (AME1) with 94% purity was obtained with a lipid-lowering effect (Hoang et al., 2022). Besides, a new sterol and 16 known compounds were isolated and identified from the methanol extract of *T. pachydermum* TSL10 (Thuy et al., 2023). Among them, sterols 1-6 showed significant antibacterial activity against Gram-positive bacteria (*Enterococcus faecium* TCC29212, *Staphylococcus aureus* ATCC25923 and *Bacillus cereus* ATCC14579) and yeast (*Candida albicans* ATCC10231), serving as potential antibacterial drugs against Gram-positive bacteria (Thuy et al., 2023). With the saturation of researches on medicinal plant resources, it is promising to explore natural active pharmaceuticals from marine protists like thraustochytrids which has truly begun. However, the extremely low yield of active products is one of the significant challenges especially for some secondary metabolites, with products at microgram or milligram levels. Microbial fermentation is one of the key methods for rapidly obtaining related resources. But there are various limitations in the actual fermentation process. Despite this, microbial fermentation has become one of the important solutions for sourcing marine pharmaceutical development.

3.3 Ecological insights: unraveling the compositional complexity of thraustochytrids

Additionally, analyzing the compositional components of thraustochytrids can provide new perspectives on revealing their ecological roles. Laboratory-cultured marine thraustochytrids including *A.* sp., *Botryochytrium radiatum* and *S.* sp., can produce methyl halides that catalyzing the depletion of stratospheric ozone, potentially serving as sources of methyl halides in marine environments (Sato et al., 2018). Additionally, analyses of the C, N, H and S elemental composition and cell mass quantification of seven thraustochytrid strains isolated from different marine habitats revealed that the proportions of C and H elements were significantly higher in the stationary phase than in the exponential phase (Sen et al., 2021). While the proportions of N and S were the opposite, implying the role of thraustochytrids in the cycling of these elements (Sen et al., 2021). Furthermore, this study also discovered that the carbon density of thraustochytrids cells has been underestimated, suggesting that thraustochytrids contribute more significantly to the oceanic carbon pool than previously thought, surpassing bacteria in this regard (Sen et al., 2021). Consequently, future analyses of the chemical composition of thraustochytrids will provide more insights into their ecological functions and potential biotechnological applications.

4 Metabolic engineering strategies in thraustochytrids: boosting production of lipids, squalene and carotenoids

4.1 Optimizing fatty acid synthesis in thraustochytrids with molecular engineering strategies

Thraustochytrids, which are significant marine oleaginous microorganisms, could accumulate saturated, monounsaturated and PUFAs, constituting over 50% of their dry cell weight. The saturated fatty acids primarily include palmitic acid (C16:0), myristic acid (C14:0) and stearic acid (C18:0). While the monounsaturated ones mainly consist of C16:1 and C18:1. Both saturated and monounsaturated fatty acids are crucial for the synthesis of biodiesel. Polyunsaturated fatty acids, composed of ω-3 fatty acids such as DHA, EPA and arachidonic acid, hold significant economic value and are a key focus in the biotechnological research of thraustochytrids.

Early researchers improved intracellular lipid production by optimizing the fermentation conditions for thraustochytrids. They found that stress conditions such as low temperature (Ma et al., 2017), low or high dissolved oxygen (Sun et al., 2016; Heggeset et al., 2019) and nitrogen starvation (Chen et al., 2022) could stimulate the accumulation of intracellular lipids. Laboratory domestication and phased cultivation strategies (Wang et al., 2021a; Chen et al., 2022) have been developed. Additionally, the inclusion of lipase inhibitors (Orlistat) (Ma et al., 2021), antioxidants (Zhang et al., 2020) and plant hormones (Yu et al., 2016) in the culture medium can influence PUFAs metabolism and promote lipid accumulation. While fermentation optimization could increase the lipid yields, the targeted modification and alteration of key genes in the fatty acid synthesis pathway represent a rational design approach in molecular breeding with tremendous potential for application (Table 2A). For example, overexpressing glucose-6-phosphate dehydrogenase (Cui et al., 2016) and disrupting the 2,4-dienoyl-CoA reductase (Liang et al., 2022) had both been shown to enhance the accumulation of PUFAs in thraustochytrids. By knocking out a copy of the fatty acid synthase gene to deactivate the competitive pathway of DHA biosynthesis, and overexpressing acetyl-CoA carboxylase and diacylglycerol acyltransferase to enhance substrate supply and triglyceride synthesis, mutant strains of thraustochytrids have been developed (Wang et al., 2021b). The DHA purity in the intracellular lipids of these mutants can account for 61% of the total fatty acids, offering a significant approach for the production of high-purity DHA in thraustochytrids (Wang et al., 2021b). Many scholars have also experimented with non-rational design through mutagenesis and selection, obtaining mutant strains with excellent traits and significant application potential (Qi et al., 2017; Liu et al., 2020b; Nazir et al., 2022). Furthermore, researchers are continually seeking new effective molecular targets to provide novel insights into the rational design of thraustochytrid strains and the construction of thraustochytrid cell factories (Lan et al., 2021; Chi et al., 2022).

4.2 Strategies for enhancing squalene production in thraustochytrids through metabolic engineering

Squalene is a class of terpenoid hydrocarbons, an important precursor in cholesterol biosynthesis, present in many microorganisms, higher plants and animals. In thraustochytrids, the accumulation of squalene can account for about 20% of the cell dry weight (Kaya et al., 2011; Patel et al., 2022). The biosynthetic pathways of squalene in organisms include the mevalonate (MVA) and methyl-D-erythritol 4-phosphate (MEP) pathways. In thraustochytrids, the MVA pathway is predominant and is characterized by low flux. The peak accumulation of squalene usually occurs on the second to third day of cultivation, when most strains are in the logarithmic growth phase. Further extending the cultivation period drastically reduces the squalene yield, to the point where it may not be detectable. Additionally, thraustochytrid strains in squalene synthesis research are mainly focused on the *Aurantiochytrium* and *Schizochytrium* genera. The intracellular accumulation of squalene can be improved by optimizing fermentation culture parameters or using metabolic engineering strategies (Table 2B). The bioactive substance α-tocopherol significantly increases the squalene production in *A. urantiochyum* sp. TWZ-97 by reducing the levels of reactive oxygen species and upregulating key genes in central carbon metabolism pathways (Ali et al., 2023). Compared to sodium-free media, the addition of sodium chloride to the culture medium of *T.* sp. ATCC 26185 doubled the concentration of squalene to 123.6 mg/L (Zhang et al., 2021). This is due to sodium induction shifting energy production from carbohydrate to lipid oxidation and increasing ATP generation. Thereby enhancing squalene synthesis by overcoming thermodynamic constraints in low flux pathways through increasing ATP consumption (Zhang et al., 2021). Moreover, the expression and overexpression of type-2 Acyl-CoA:diacylglycerol acyltransferases (DGAT2) genes, T66ASATb in *A.* sp. T66 and its homologs AlASATb in *A.* limacinum SR21, could improve squalene accumulation by up to 88% in *A.* sp. SR21 (Rau et al., 2022). Additionally, the expression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase 29 (HMGR) is the bottleneck of squalene synthesis, and its constitutive expression could improve squalene production in *A.* sp. 18W-13a (Yang et al., 2022b). Furthermore, the discovery of new molecular targets and the development of efficient gene editing tools will accelerate the construction of thraustochytrid squalene cell factories (Huang et al., 2021).

4.3 Enhancing carotenoids production in thraustochytrids with genetic manipulation strategies

Carotenoids are valuable tetraterpene pigments, and could be synthesized by various organisms such as microorganisms, plants and animals. The majority of organisms capable of synthesizing carotenoids, such as cyanobacteria and microalgae, are phototrophs. However, the non-phototrophic protists like the labyrinthulids can acquire the ability to synthesize carotenoids through horizontal gene transfer from Actinobacteria, Proteobacteria and archaea (Rius et al., 2023). Thraustochytrids can accumulate high levels of astaxanthin and are characterized by short growth cycles and simple astaxanthin extraction processes, making them a promising source of this compound (Table 2C). The synthesis pathway of astaxanthin in thraustochytrids is not fully understood. But overexpression of the β-carotene hydroxylase gene (crtZ) in *A. limacinum* significantly increases astaxanthin content per culture volume (ng/mL) and per cell (ng/mL * OD) by 2.8 and 4.5 times, respectively (Yoshimi et al., 2023). Furthermore, extended light exposure increases astaxanthin yield in wild-type strains and decreases β-carotene content, indicating that light duration is a critical factor influencing astaxanthin synthesis (Yoshimi et al., 2023). Under light conditions, *A. limacinum* reduces ATP consumption to decrease the reactive oxygen species (ROS) occurrence in mitochondria while accumulating astaxanthin to prevent ROS damage (Kubo et al., 2022). The overexpression of the 3-ketoacyl-ACP synthase (KAS) III-like gene YxwZ3 increases carotenoids production in *A.* sp. SZU445, including β-carotene (37.96 μg/g), phytoene, β-cryptoxanthin, antheraxanthin, α-cryptoxanthin and echinenone (Song et al., 2022). This results in the reduced accumulation of fatty acids by increasing the consumption of malonyl-CoA through the MVA pathway of carotenoid synthesis (Song et al., 2022). The geranylgeranyl pyrophosphate synthase encoding gene (AlGGPPS) and isopentenyl pyrophosphate isomerase encoding gene (AlIDI) from *A. limacinum* MYA-1381 were integrated into the *de novo* carotene biosynthetic pathway in *Escherichia coli*, which increased the carotenoid by 2.99-fold compared with the initial strain (Shi et al., 2023). This indicated the participation of the two novel genes functioned coordinately in the carotene biosynthesis and provided novel functional elements for carotenoid engineering improvements (Shi et al., 2023). These studies provide vital references for biosynthesis research of carotenoids and other metabolic products in different thraustochytrid species.

4.4 Challenges in the development of genetic tools for thraustochytrids

Compared to model microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae*, non-model microorganisms like thraustochytrids often exhibit more complex phenotypic characteristics. This offers significant potential advantages for future scale production of biofuels and chemicals (Riley and Guss, 2021). Developing precise genetic editing tools for these non-model microorganisms is crucial. As it allows for rational optimization of their metabolic pathways, and thereby enhances the efficiency of target product synthesis (Riley and Guss, 2021). Metabolic engineering techniques enable microorganisms to meet the titer, rate and yield requirements for industrial applications and to synthesize a diverse array of metabolic products (Riley and Guss, 2021). However, the research and application of non-model microorganisms are limited by the lack of efficient genetic manipulation tools, hindering their widespread use and

development of high-performance strains (Riley and Guss, 2021). Research has been conducted on genetic tools for marine protists across different taxonomic groups, yet no universally applicable solutions have been found (Faktorová et al., 2020). The diversity in genomic sequence features as well as differences in cell structure and morphology lead to significant variations in the strategies for exogenous DNA transformation and selection of DNA elements among different microorganisms. Consequently, the genetic manipulation schemes developed for thraustochytrids have largely been restricted to one or a few strains (Rau and Ertesvåg, 2021). However, advances in sequencing technology and cost reductions, along with the increase of omics analysis data and DNA synthesis technology, have made it more convenient to obtain genomic sequence information and identify gene editing targets. This facilitates the development of genetic tools and the optimization of metabolic engineering strategies (Rau and Ertesvåg, 2021). Furthermore, it is essential to establish an accurate species classification system based on the physiological, biochemical and molecular biological characteristics of thraustochytrids. This will provide a more practical reference for developing genetic tools for new strains and related species within the same genus or species.

5 Potential applications in nutritional supplementation and pharmaceutical industry

For the abundant amounts of high-value-added metabolites, especially unsaturated fatty acids (DHA, EPA), squalene and astaxanthin, thraustochytrids had been proposed to be applied in various fields, such as Feed, pharmacological and cosmetics industries. Most of the applicational researches for thraustochytrids were focused on the animal feeds of aquatic animals, poultry and livestock with improved growth and physiological indicators, as well as the higher DHA contents in their bodies or eggs (Yamasaki et al., 2007; Carvalho et al., 2018; dos Santos et al., 2019; Keegan et al., 2019). Besides, some natural products such as terpenoids in thraustochytrids could exert antioxidant effect, thus substitute for the antioxidants and pigments chemicals in cosmetics. In addition, thraustochytrids had exhibited anti-inflammatory and anti-cancer activities (Shakeri et al., 2017; Takahashi et al., 2019), and were proposed to be used to develop oral vaccines for their beneficial characters (Dahmen et al., 2023). The active lipids and defatted by-products of thraustochytrids with important nutritional value, have great potential in the development of functional food (Paulo et al., 2020; Reboleira et al., 2021).

5.1 Thraustochytrid supplementation strategies in enhancing feed nutrition

5.1.1 Enhancing fish nutrition with thraustochytrid-based feeds

Ω-3 long chain polyunsaturated fatty acids (LC-PUFAs) especially DHA and EPA, were proposed to exert vital effects in not only regulating normal physiological activities but the resistance to inflammation related diseases, which were thus suggested to be supplemented into human daily diets (Liu et al., 2014a). As is known, deep-sea fish accumulating large amounts of LC-PUFAs by preying on primary marine microalgae and microorganisms with PUFAs metabolic pathways, was the traditional sources. In the last decades, thraustochytrids have been considered as potential alternatives of DHA to traditional fish oils for several beneficial characters in growth and metabolism. On the other hand, marine animals were supposed to utilize and store LC-PUFAs in their bodies through diet supplementation and simultaneously exhibited excellent growth and physiological indicators. Besides, the feasibility of substitution of thraustochytrid oils for fish oils in full or partially has been demonstrated in several different marine animals.

In the aquatic feed industry, significant efforts had been made on exploring sustainable and environmental alternatives for fishmeal and fish oils (Torrecillas et al., 2017). Terrestrial meals and vegetable oils with high protein and lipid contents were excluded for undesired and adverse effects, for example, absorption suppression for amino acids, vitamins and minerals, as well as the deficiency in LC-PUFAs (Krogdahl et al., 2010; Tocher, 2015). Meanwhile, focus was paid on marine microalgae and microorganism, which was considered as desirable baits with certain physiological active substances. Among them, marine heterotrophic thraustochytrids attracted the most attention for absolute advantages e.g. fast growth, high DHA content and wide-sourced substrates.

Generally, dietary supplementation of thraustochytrids belonging mainly to two genera, namely *Aurantiochytrium* and *Schizochytrium*, could improve growth performance and PUFAs content of fishes. To investigate the feasibility of complete replacement of fish oil with plant oil mixtures (canola oil and palm oil) and *A.* sp. in juvenile yellowtail *Seriola quinqueradiata*, *A.* sp. supplementation at 0% (AM0), 1% (AM1), 2% (AM2), 3% (AM3) and 4% (AM4) into the mixtures of canola oil and palm oil was studied (Fukada et al., 2019). Results showed that the AM2 group exhibited significant higher final body weight than the AM0 group, thus indicated the beneficial effect of *A.* sp. on yellowtail aquaculture (Fukada et al., 2019). In addition, Nile tilapia juveniles fed *A.* sp. meal had significantly greater weight gains when compared to fish fed cod liver oil (CLO) diet, so as the DHA, EPA and LC-PUFAs contents (Nobrega et al., 2019). Similar results were obtained as for shrimp. *A. limacinum* BCC52274 (AL) enriched by instar-II Artemia benefited the growth performance of post-larval (PL) shrimp with an increasing trend of PL biomass gain as percent of the enriched meals increased (Visudtiphole et al., 2018).

Generally, thraustochytrid-based feed has been shown to improve growth, metabolism, digestion, immunity and reproductive indices in farmed fish species such as Nile tilapia (Batista et al., 2021; Nobrega et al., 2022; Luc et al., 2023), European sea bass (Soudant et al., 2022), *Micropterus salmoides* (Habte-Tsion et al., 2020), Salmo salar (Wei et al., 2021), *Cyprinus carpio* (Eljasik et al., 2020), humpback grouper (Cromileptes altivelis) (Sun et al., 2019) and *Oncorhynchus mykiss* (Sevgili et al., 2019). And it is

applicable in the nutrition of larval feeds such as rotifers and *Artemia nauplii* and in DHA enrichment of fish flesh (Yamasaki et al., 2007). Recent transcriptomic approaches have explored the positive impacts of thraustochytrids supplementation on growth, metabolism, immunity and gonadal development in zebrafish, a model organism and provide a new molecular perspective for the application of thraustochytrids feed (Yang et al., 2022a; Huang et al., 2023). Furthermore, compared to other PUFAs sources, such as marine microalgae and oilseed crops, thraustochytrids, with their heterotrophic nature, rapid growth and high-density fermentation capabilities, offer unparalleled advantages. The addition forms of thraustochytrids in feed primarily include lipid extracts and freeze-dried biomass. Lipid extracts in feed are probably more readily absorbed and utilized by animals. While due to the thick cell walls of the organisms, the freeze-dried biomass is not easily digested in the animal gut. Therefore, broken-cell freeze-dried biomass appears more advantageous for simplifying feed preparation processes and improving food utilization rates. In summary, to further explore the potential of thraustochytrids as a sustainable feed resource, future research could focus on the effects of feed supplementation at different aquaculture stages, application in various aquaculture systems, long-term and short-term impacts on farmed animals, as well as its effects on aquaculture water environments. Furthermore, expanding the application of thraustochytrids feed to a broader range of aquatic animals could significantly contribute to the development of the aquaculture industry.

5.1.2 The role of thraustochytrid supplements in growth, health and environmental sustainability in shrimp aquaculture

Long-chain PUFAs play a crucial role in the growth, development and health maintenance of shrimps, which have limited capacity to synthesize these essential fatty acids and rely on dietary intake from feed. Firstly, the incorporation of thraustochytrid biomass has been found to promote the healthy growth of shrimps and PUFAs accumulation in their tissues. Utilizing *A. mangrovei* fiku008 as a juvenile shrimp feed supplement has been shown to enhance growth rate, survival rate and tissue PUFA content in Penaeus semisulcatus (Yilmaz et al., 2023). Additionally, direct supplementation of *A. limacinum* BCC52274 in feed significantly improved growth rate and survival of *Litopenaeus vannamei* juveniles and enhancing their lipid profile meanwhile (Visudtiphole et al., 2021). Furthermore, replacing fish oil entirely with *S.* sp. in shrimp feed did not adversely affect growth performance, survival rate and muscle fatty acid composition (Allen et al., 2019). Secondly, supplementation of thraustochytrid biomass can enhance the antioxidant capacity of shrimps. Low-fishmeal diets supplemented with *S. limacinum* significantly improved the antioxidant capacity of *Litopenaeus vannamei*, altering its transcriptomic expression patterns, particularly genes related to antioxidant and immune functions, thus enhancing the health status of the shrimp (Xu et al., 2022). Moreover, the biofloc technology is an emerging and environmentally friendly aquaculture method. A biofloc system integrated *S.* sp. with *Lactobacillus plantarum* maintained suitable water quality

conditions, promoted shrimp growth and reduced concentrations of common pathogens (e.g., *Vibrio* spp.) (Pacheco-Vega et al., 2018). It is thus demonstrated to have obvious competitive advantage in maintaining water quality and promoting shrimp growth (Pacheco-Vega et al., 2018). Future developments in encapsulation technologies for thraustochytrids, such as microencapsulated bacterial feed (Willer et al., 2020), and the development of thraustochytrid-based probiotic consortia, are significant for developing sustainability aquaculture feeds.

5.1.3 Enhancing nutritional value of poultry products through thraustochytrid supplementation

Chicken meat and eggs, being among the most consumed protein sources in human diets, can benefit the public health improvement from enhanced nutritional value, particularly in increasing ω-3 fatty acid content. Compared to traditional ω-3 sources such as salmon oil (SO) and flaxseed oil (FO), the supplementation of *Schizochytrium* powder (SP) in chicken feed could significantly increasing DHA content in thigh meat (Jeon et al., 2022). While SO and FO treatments showed notable increases in EPA and linolenic acid (ALA), respectively (Jeon et al., 2022). Furthermore, the tissue DHA concentrations increased by adding *A. limacinum* throughout the whole life and fattening periods of broiler chickens (Keegan et al., 2019). Though DHA enrichment levels supplemented in two different period were similar, the fat deposition was unrelated to increased ω-3 PUFA levels, likely influenced by other nutritional and genetic factors (Keegan et al., 2019). Adding *A. limacinum* as ω-3 fatty acid sources significantly altered certain skeletal characteristics of ISA Brown and Shaver white breeders and progeny, influencing the bone mineralization process in chicken embryos, with varying impacts on the skeletal health of pullet of the two breeds (Kakhki et al., 2020). Therefore, the enrichment of PUFAs in chicken eggs and tissues is related to factors such as the DHA source and dose, feeding duration and chicken breed.

Except for DHA, thraustochytrids also contain antioxidants such as carotenoids and squalene, stabilizing DHA in its non-oxidized state. Supplementation with DHA-rich thraustochytrids significantly increased the DHA content and other bioactive components, such as carotenoids and tocopherols in broilers meat, effectively improving the nutritional value of the meat (Kalogeropoulos et al., 2010). Adding 1.0% *A.* sp. to layer chicken feed significantly increased DHA content in egg yolks within approximately 30 days, reduced the ω-6/ω-3 ratio and improved the anti-atherogenic and anti-thrombotic index of egg yolks (Liu et al., 2020a). Additionally, carotenoid supplementation from thraustochytrids enhanced egg yolk color, helped to slow down lipid oxidation and increased the lipid oxidation stability of DHA-rich egg yolks (Liu et al., 2020b). Future studies are anticipated to further investigate the impact of long-term storage and refrigeration conditions on DHA retention in egg yolks. Additionally, It is expected to explore the mechanisms of absorption and conversion of DHA and carotenoids in egg yolks, which are expected to enhance the nutritional value of eggs.

5.1.4 Omega-3 levels in livestock and insect feeds with thraustochytrid supplements

Meat from the beef, lamb and pork are also staple delicacies in the public daily diet. Although the addition of ω-3 fatty acids to the feed of these large livestock can increase the ω-3 fatty acid content in the meat, the impact on growth, meat color and flavor cannot be overlooked. The supplementation of *S. limacinum* in cattle feed increased the ω-3 fatty acid content in beef, but also increased the degree of meat oxidation, adversely affecting color stability and flavor (Phelps et al., 2016). Adding *S.* sp. to steer feed improved the ω-3 fatty acid content in beef and enhanced insulin sensitivity, but had no significant effect on the growth and carcass characteristics (Carvalho et al., 2018). Feeding pigs with DHA-rich *Schizochytrium* dry biomass improved the growth efficiency, lowered blood triglyceride levels and promoted the expression of fat oxidation genes (Jon Meadus et al., 2011). The longissimus dorsi muscle of pigs, a prime part of pork, showed increased DHA content with the increasing addition of *A. limacinum* in the diet (Moran et al., 2017). And the maximum values were 2.7 and 4 folds higher than the control in pig longissimus dorsi and back fat, respectively, with a greater increase in sows compared to boars (Moran et al., 2017). When fed with *S.* sp., the lambs showed slower growth rates but did not affect carcass weight and GR fat content (Burnett et al., 2016). Overall, research on thraustochytrid supplementation in feed for large livestock is relatively limited. This may be due to the larger size of livestock, requiring a greater amount of thraustochytrids biomass, minor positive effects on growth and DHA enrichment and economic costs, have not shown a clear advantage. Additionally, the black soldier fly, *Hermetia illucens* (HI), due to its high reproduction rate and nutritional value, is a valuable alternative ingredient for feed and food production. A substrate containing 10% biomass of *S.* sp. for insect rearing increased the ω-3 PUFAs content of HI pre-pupae, while higher proportions of *S.* sp. did not provide additional nutritional value (Truzzi et al., 2020). Therefore, future research could focus more on feed addition of thraustochytrid biomass or lipid extracts for insect, to develop novel functional feed and food nutrition components derived from insects.

5.2 Harnessing thraustochytrids for nutraceutical and functional food advancements

Existing research has demonstrated that thraustochytrid PUFAs or intracellular lipid extracts possess anti-inflammatory (Takahashi et al., 2019), antioxidant (Hien et al., 2022; Kalidasan et al., 2022; Kaliyamoorthy et al., 2023), neuroprotective (Hien et al., 2022) and anticancer properties (Shakeri et al., 2017; Kalidasan et al., 2022), highlighting their significant medicinal value (Table 3). Dietary supplementation with DHA has been proven to effectively elevate plasma total DHA levels (reaching 65 μM) (Mayer et al., 2003). Additionally, terpenoids like squalene and carotenoids also exhibit potent antioxidant, anticancer and anti-inflammatory activities. However, when co-produced with PUFAs in thraustochytrids,

they are often emphasized as high-value by-products to reduce production costs. Among all thraustochytrid species, *Schizochytrium* was the only genus approved for commercial production of DHA-rich oils primarily for food and nutraceutical industries with completed feed safety evaluations for animals during 2001-2003 (Hammond et al., 2001a, b, c; Abril et al., 2003). It's worth noting that currently only DHA-enriched oils extracted from *S.* sp. are permitted for use as nutritional additives, but not whole cells. Moreover, oils with high DHA content produced from genetically modified thraustochytrids are not accepted by the food and pharmaceutical industries in many countries (Wang et al., 2021a). Upon the European Commission's request, the European Food Safety Authority (EFSA) Panel on Nutrition, Novel Foods and Food Allergens (NDA) evaluated the safety of *S.* sp. oil, specifically strains WZU477, FCC-3204, TKD-1 and CABIO-A-2, all belonging to the species *S. limacinum*, as novel foods under Regulation (EU) 2015/2283 (Turck et al., 2020; Turck et al., 2021a, b; Turck et al., 2023a, b). These oils, intended for use in infant and follow-on formulae with DHA at a level of 20-50 mg/100 kcal based on Regulation (EU) 2016/127 guidelines (Turck et al., 2020; Turck et al., 2021a; Turck et al., 2023a, b). And strain FCC-3204 was added for food supplements at up to 1 g DHA/day for adults (excluding pregnant and lactating women) (Turck et al., 2021b). These additions were deemed safe despite the absence of strain-specific toxicological studies. This conclusion was drawn from existing toxicological data on *Schizochytrium*-derived oils, their Qualified Presumption of Safety (QPS) status, free from marine biotoxins and viable cells production process. However, the oil from strain *S.* sp. ATCC-20889 was not confirmed safe due to insufficient species-level characterization and a potential unknown toxin risk (Turck et al., 2022). It highlighted the EFSA's cautious approach to ensuring food safety and the necessity for detailed data on specific strains.

Furthermore, the genus *Aurantiochytrium*, closely related evolutionarily to *Schizochytrium*, exhibits rapid growth, high DHA content and physiological activities such as anti-inflammation, antioxidant and neuroprotection, becoming a significant target in pharmacological research. However, studies on the dietary safety and toxicology of *Aurantiochytrium* for animals are currently scarce. Limited research indicates that unextracted *Aurantiochytrium* biomass, when added to dog food at recommended levels (5 times) and fed for 15 days, showed no adverse effects on the health and safety of adult Beagles (Moran et al., 2018). Studies evaluating the toxicological potential of unextracted *A. limacinum* biomass as a food additive observed no significant mutagenic activity, genotoxic activity, induction of genotoxicity in human lymphocytes and death or acute toxicity (Dillon et al., 2020). Additionally, scientists have already initiated attempts to produce anti-SARS-CoV-2 antibodies related to interventions for Coronavirus disease 2019 (COVID-19) using *A. acetophilum* as a host (Dahmen et al., 2023). Furthermore, the development of novel functional foods enriched with DHA, particularly a yogurt enriched with *A.* sp. biomass (Paulo et al., 2020), appears to underscore the advantages of thraustochytrid-sourced DHA. Unlike DHA derived from fish oil, which has a

TABLE 3 Summaries of studies on the pharmacological activity and functional food application of thraustochytrids.

Strains	Objective	Adding form	Results	Reference
Schizochytrium sp.	Sprague–Dawley-derived rats Crl : CD(SD)BR	*S.* sp. dried microalgae	No effect on estrus cycles or reproductive performance, offspring viability and physical development of F1 animals	Hammond et al., 2001a
Schizochytrium sp.	Sprague–Dawley derived rats and New Zealand White rabbits	Dried orange powder biomass	Neither maternal nor developmental toxicity was observed	Hammond et al., 2001b
Schizochytrium sp.	Sprague–Dawley rats	Dried orange powder biomass	No adverse effects of relevance to humans at dosages up to 4000 mg/kg/day for 13 weeks	Hammond et al., 2001c
Aurantiochytrium sp. qe-4	Gastric cancer AGS cells	Thraustochytrids oil	Exhibited an inhibitory effect on gastric cancer cells	Shakeri et al., 2017
Aurantiochytrium limacinum	Mature Beagle dogs	Unextracted *A. limacinum* biomass	No negative effects on health or safety in mature beagle dogs	Moran et al., 2018
Aurantiochytrium mangrovei 18W-13a	Lipopolysaccharide-induced inflammatory responses in RAW264 murine macrophages	Ethanolic extract	Inhibit the NO production, the secretion of proinflammatory cytokines and exhibited anti-inflammatory effects on murine macrophages	Takahashi et al., 2018
Aurantiochytrium limacinum 4W-1b	Murine Macrophage RAW264 Cells	Ethanol extracts	Anti-inflammatory effect	Takahashi et al., 2019
Aurantiochytrium limacinum	Salmonella typhimurium strains, Sprague Dawley rats, human lymphocytes, rabbits guinea pigs	Unextracted *A. limacinum* biomass (AURA)	No significant mutagenic activity or clastogenic activity, no genotoxic activity in human lymphocytes, non-irritant for the eye and skin of the rabbit and non-sensitizing to guinea pig skin	Dillon et al., 2020
Aurantiochytrium sp. SC145	Mice	PUFAs in the free (FFA) and the alkyl ester (FAAE) forms	Showed antioxidant and AChE inhibitory properties and neuroprotective activities against damage caused by H_2O_2- and amyloid-ß protein fragment 25–35 (A25-35)-induced C6 cells	Hien et al., 2022
Schizochytrium sp. (SMKK1)	Colon cancer cell line (HT29)	Methanolic extract	High total antioxidant activity and the low nitric oxide radical, and could destroy the colon oncoprotein responsible for anti-colon carcinogen	Kalidasan et al., 2022
Aurantiochytrium acetophilum	The ConamaxTM platform based on *A. acetophilum*	Assayed the targets *in vitro* for an activity which confirmed proper structural folding	The produced purified CR3022 antibody could reduce the cytopathic effect of SARS-CoV-2, and exhibited ACE2-specific proteolytic activity	Dahmen et al., 2023
Thraustochytrium sp. and *Aurantiochytrium mangrovei*	Antibacterial activities and antioxidant activities	Biomass extracted in organic solvents	Exhibited antibacterial activity, free radical scavenging and nitric oxide radical scavenging activities	Kaliyamoorthy et al., 2023
Aurantiochytrium sp.	Develop a novel functional lean yogurt supplemented with *A.* sp. biomass rich in bioactive lipids	Freeze-dried microalgal biomass	the fatty acid bioaccessibility was not high (below 60–70%).	Paulo et al., 2020
Aurantiochytrium sp.	To analysis the nutritional characterization and fatty acid composition of defatted biomass	Enzymatically defatted biomass	Rich protein, dietary fiber, glutamic and aspartic acid, a favourable lysine/arginine ratio of 3.73, significant *Weissela cibaria* and *Bifidobacterium bifidum* growth-enhancing potential, and residual ferric reducing antioxidant power (FRAP) activity	Reboleira et al., 2021

pronounced fishy taste, DHA from thraustochytrids is more palatable. Moreover, the presence of potential functional nutritional components in the defatted biomass waste of thraustochytrids, such as abundant protein, dietary fiber, glutamic and aspartic acid, makes the biorefinery process of utilizing these defatted by-products for the development of food supplements more feasible (Reboleira et al., 2021). Overall, future research in the pharmacological study of the genus *Aurantiochytrium*, as well as the development of functional foods, is poised to further enhance the role of thraustochytrids in biotechnological development and functional food and pharmaceutical manufacturing.

6 Conclusions and prospects

As a marine heterotrophic protist, thraustochytrid contribute significantly to marine ecosystems and human welfare. Understanding their taxonomic characteristics is crucial for better utilization of these protists. However, current research on thraustochytrid taxonomy necessitates a more in-depth and systematic approach, incorporating physiological, biochemical and molecular biology methods, particularly genomics, to establish an accurate and scientific polyphasic taxonomy system. The diversity of metabolic products in thraustochytrids renders them a

biotechnological resource with enormous potential. Future use of rapid detection technologies, including Raman spectroscopy, as well as natural product isolation and identification methods, will enrich our understanding of high-value metabolites and low-concentration components in thraustochytrids. Additionally, fermentation optimization and molecular genetic modifications can significantly enhance the accumulation of key bioactive substances including PUFAs, squalene and carotenoids. These will promote the construction of thraustochytrid cell factory.

Currently, studies on the metabolic engineering and application of thraustochytrids primarily utilizes strains from the genera *Schizochytrium* and *Aurantiochytrium*. However, thraustochytrid belonging to other genera and their close relatives *Labyrinthula* sp., could accumulate significant amounts of DHA as well. Future studies with different strains might yield better feed utilization results. Moreover, existing studies on thraustochytrids supplementation to enhance the PUFAs content of meat or egg yolks have overlooked the utilization and transformation of other active components (squalene, carotenoids, proteins and substances in low quantities) in them.

In summary, future challenges and opportunities lie in unlocking the full potential of thraustochytrids through advanced research tools and exploitation of species resources. Innovations in metabolic engineering, cultivation techniques and biorefinery approaches will be critical to increase the efficiency and sustainability of thraustochytrid-based production systems. Furthermore, expanding the understanding of thraustochytrid taxonomy and genetics can facilitate the discovery and optimization of strains with superior production capacities. Besides, regulatory approval and market acceptance will hinge on demonstrating the safety, efficacy and economic viability of thraustochytrid-derived products. As we delve deeper into the capabilities of these protists, we stand on the brink of a new era in biotechnology. Then, thraustochytrids could play a pivotal role in meeting global demands for sustainable and health-enhancing commodities.

Author contributions

QW: Conceptualization, Formal Analysis, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing. YZ: Investigation, Writing – original draft, Writing – review & editing. RH: Investigation, Writing – original draft. YXZ: Investigation, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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