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The effect of different yeasts on fish leucocytes

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Η επίδραση διαφορετικών ζυμομυκήτων στα λευκοκύτταρα των ψαριών

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1. Περίληψη

Όλες οι διεθνείς οργανώσεις τροφίμων συμφωνούν στην επισήμανση της τεράστιας δυνατότητας των ωκεανών να συμβάλλουν σημαντικά στην επισιτιστική ασφάλεια και στην επαρκή διατροφή του παγκόσμιου πληθυσμού. Στο πλαίσιο αυτό, η σημασία της ιχθυοκαλλιέργειας αυξάνεται σε όλο τον κόσμο. Οι θαλάσσιοι μικροοργανισμοί, όπως οι ζύμες, έχουν πολλές πιθανές εφαρμογές στη διατροφή των ψαριών. Οι ζύμες είναι μία πολυφατική ομάδα βασιδιομυκητιακών και ασκομυκητιακών μυκήτων με ένα μοναδικό χαρακτηριστικό της μονοκυτταρικής ανάπτυξης. Έχουν απομονωθεί τέσσερις θαλάσσιες, ακραίοφιλες ζυμομύκητες με δυνητικό ενδιαφέρον για βιομηχανία αγροτικών ειδών όπως το στέλεχος 004 (Dh) του *Debaryomyces hansenii*, το στέλεχος N16 (Sh) *Sterigmatomyces halophilus*, το στέλεχος N6 (YlA) *Yarrowia lipolytica* και το στέλεχος D1 (YIB) του *Y. lipolytica*. Επιπλέον, προσπαθήσαμε να χαρακτηρίσουμε αυτές τις ζύμες μέσω ανοσολογικών δοκιμασιών. Τα δύο κύρια σημεία ήταν η in vitro βακτηριοκτόνος δράση των ζυμομυκήτων έναντι δύο παθογόνων βακτηρίων ψαριών: *Vibrio anguillarum* και *V. Harveyi*, καθώς και η φαγοκυτταρική δραστηριότητα των λευκοκυττάρων των νεφρών της κεφαλής νεογνών τσιπούρων (*Sparus aurata*). Τα αποτελέσματα έδειξαν διακύμανση μεταξύ των στελεχών, με το στέλεχος YIB να είναι εκείνο που κυριαρχούσε τόσο στη βακτηριοκτόνο όσο και στη φαγοκυτταρική δραστηριότητα. Τα παρόντα αποτελέσματα υποδεικνύουν ότι αυτά τα στελέχη ζυμομύκητα θα μπορούσαν να χρησιμοποιηθούν στην υδατοκαλλιέργεια ψαριών, καθώς έδειξαν βακτηριοκτόνες ιδιότητες έναντι δύο παθογόνων βακτηρίων και μπορούν επίσης να εσωτερικοποιηθούν από κύτταρα του ανοσοποιητικού συστήματος, αλλά απαιτούνται περαιτέρω μελέτες για να κατανοήσουμε πλήρως τη φύση αυτών των στελεχών.

1. Abstract

All international food organizations agree on highlighting the enormous potential of the oceans to make a significant contribution to the food security and to the adequate nutrition for the world's population. In this context, the importance of fish farming is increasing all over the world. Marine microorganisms, such as yeasts have many potential applications in fish nutrition. Yeasts are a polyphyletic group of basidiomycetous and ascomycetous fungi with a unique characteristic of unicellular growth. Four marine, extremophile yeasts with potential interest in agrofood industry have been isolated as *Debaryomyces hansenii* strain 004 (Dh), *Sterigmatomyces halophilus* strain N16 (Sh), *Yarrowia lipolytica* strain N6 (YlA) and *Y. lipolytica* strain D1 (YlB). Furthermore, we tried to characterize those yeasts through immunological assays. The two highlights were the *in vitro* bactericidal activity of the yeasts against two fish pathogenic bacteria: *Vibrio anguillarum* and *V. harveyi* as well as the phagocytic activity of gilthead seabream (*Sparus aurata*) head kidney leucocytes. Results showed variation among strains, with YlB strain being the one that dominated both bactericidal and phagocytic activity. Present results indicate that these yeast strains could be used in fish aquaculture, as they showed bactericidal properties against two pathogenic bacteria, and they also can be interiorized by immune cells but further studies are needed before we can completely understand the real potential of these strains.

2. Introduction

2.1 Marine Yeasts

The term "**ecosystem**" refers to the complex of living organisms, their physical environment, and all their interrelationships in a particular unit of space.[1]

Marine ecosystem is the complex of living organisms in the ocean environment. Marine waters cover two-thirds of the surface of the Earth. In some places the ocean is deeper than Mount Everest is high; for example, the Mariana Trench and the Tonga Trench in the western part of the Pacific Ocean reach depths in excess of 10,000 metres (32,800 feet). Within this ocean habitat live a wide variety of organisms that have evolved in response to various features of their environs.[2]

Marine microbes have potential applications in metal detoxification, nutrient cycling, greenhouse gas reduction and form the basis of food webs. Marine microbes, including marine yeasts, live in extreme environments, and this provides a unique potential for the synthesis of functional biomolecules.

Marine yeasts, defined as the yeasts that are isolated from marine environments, are able to grow better on medium prepared using seawater rather than freshwater.

The first marine yeasts were isolated by Bernhard Fischer in 1894 from the Atlantic Ocean, and those were identified as *Torula* sp. and *Mycoderma* sp. Following this discovery, various other marine yeasts have been isolated from around the world from different sources, including seawater, seaweeds, marine fish and mammals as well as seabirds. Among these isolates, some marine yeasts originated from terrestrial habitats (grouped as facultative marine yeast), which were brought to and survived in marine environments. The other marine yeasts were grouped as obligate or indigenous marine yeasts, which confine to marine habitats. However, no sufficient evidence has been found to explain the indispensability of seawater for obligate marine yeasts. It has been reported that

marine yeasts are able to produce many bioactive substances, such as amino acids, glucans, glutathione, toxins, enzymes, phytase and vitamins with potential application in the food, pharmaceutical, cosmetic and chemical industries as well as for marine culture and environmental protection.[3]

Yeasts are a polyphyletic group of basidiomycetous and ascomycetous fungi with a unique characteristic of unicellular growth. The term 'yeast' is derived from the old Dutch word gist and the German word gischt, which refers to fermentation. There are approximately 100 genera and 800 described species of yeasts and estimates suggest that these numbers represent only about 1% of the species that exist in nature, the rest being non-culturable. Yeasts have been used by the food industry principally for the production of ethanol and carbon dioxide, which are important to the brewing, wine distilling and baking industries. Their environmental role is similar to many other fungi, acting as saprophytes by converting plant and animal organics to yeast biomass and by-products, which may have commercial importance. Some yeast are pathogenic to plants and animals. Yeasts are rich with proteins, lipids and vitamins. Biotransformation of raw material into yeast biomass (single-cell protein) is highly significant, due to the nutritional quality of yeast and its possible utilization as animal or aquaculture feed. Yeasts also have immunostimulant properties by virtue of their complex carbohydrate and nucleic acid components. They can be produced very efficiently and economically because of their shorter generation time and use of inexpensive culture media. Lipids, pullulans and enzymes from yeasts are extracellular metabolites of commercial importance. Yeasts are distributed in almost every part of the aquatic environment, i.e. oceans and seas, estuaries, lakes and rivers (Table 1). A truly marine yeast must be able to grow on or in a marine substrate.[4]

Table 1: Details of ecological studies on marine yeasts worldwide. (Sreedevi N. Kutty & Rosamm 2008)[4]

Location/Sample	Generic composition
Sea water	
Central Pacific	*Debaromyces*
Pacific Ocean	*Candida, Torulopsis*
Pacific Ocean	*Metschnikowia*
Loma Trough, off San Diego, California	*Cryptococcus, Rhodotorula*
Pacific Ocean	*Candida*
Pacific Ocean	*Rhodotorula, Cryptococcus*
Info-Pacific and Pacific Ocean	*Leucosporidium, Rhodosporidium, Sympodiomyces*
Rendaji, Shizouka Prefecture	*Torulaspora, Dekkera, Candida*
Atlantic Ocean	*Torula, Mycoderma*
Biscayne Bay	*Candida, Rhodotorula*
Southern Florida	*Candida, Rhodotorula*
Atlantic Ocean	*Klyveromyces*
Gulf Stream, Bahamas	*Candida, Rhodotorula, Cryptococcus, Debaromyces*
Gulf Stream off Florida	*Candida, Rhodotorula*
Atlantic Ocean	*Cryptococcus*
Atlantic Ocean	*Metschnikowia*
Atlantic Ocean	*Sterigmatomyces*
Chesapeake Bay	*Rhodotorula*
Atlantic Ocean	*Leucosporidium, Rhodosporidium, Sympodiomyces*
North Sea	*Debaromyces, Candida*
Southern Sao Paulo, Brazil	*Candida, Cryptococcus, Rhodotorula, Torulopsis, Trichosporon, Debaroryomyces, Hansenula, Pichia, Sporobolomyces*
Olinda Brazil	*Candida*
Off Mumbai, Arabian Sea	*Saccharomyces, Debaryomyces, Pichia, Candida, Torulopsis, Rhodotorula, Cryptococcus*
Indian Ocean	*Rhodotorula, Candida, Sporobolomyces*
Indian Ocean	*Sterigmatomyces*
Off Cochin, Arabian Sea	*Candida, Rhodotorula, Leucosporidium*
Off Cochin, Arabian Sea	*Yarrowia*
Indian EEZ	*Candida, Filobasisium, Leucosporidium, Mastigomyces, Lodderomyces, Debaryomyces, Rhodotorula, Dekkera, Hormoascus, Cryptococcus, Schizosaccharomyces, Kluyveromyces, Williopsis, Aciculoconidia, Pichia, Torulaspora, Saccharomycopsis Lipomyces, Geotrichum, Arxioxyma, Oosporidium, Dipodascus*
Antarctic Sea	*Leucosporidium, Rhodosporidium, Sympodiomyces*

Some of the marine yeasts are extremophiles. Extremophiles are micro-organisms that inhabit some of earth's most hostile environments which produce extremozymes useful in industrial production procedures and research applications because of their ability to remain active under the severe conditions typically employed in these processes.[5] In this study we examined four extremophile marine yeasts *Yarrowia lipolytica* strain *N6* (YlA) and strain *D-1* (YlB), *Sterigmatomyces halophilus* strain *N16* (Sh) and *Debaryomyces hansenii* strain *004* (Dh).

2.1.1 *Yarrowia lipolytica*

The name 'lipolytica' originally comes from the ability of this yeast to hydrolyse lipids. That is possible due to the fact the yeast inherently numerous multi-gene families that play a function within the efficient degradation of these substrates. Its ability is to degrade proteins and lipids and that can be visible by using the manufacturing of extracellular lipolytic and proteolytic activities, proven in Figures 1A and 1B, respectively.[6]

The yeast also utilizes a limited range of sugars, alcohols, sugar alcohols and natural acids. It tolerates bodily parameters including the presence of salt, low temperatures, acidic and alkaline pH. Furthermore, it inherently produces extracellular enzymes such as proteases, lipases, esterases, phosphatases and RNases that aid its growth underneath exclusive situations. The yeast displays wonderful physiological, metabolic and genomic capabilities that differentiate it from other systems together with *Saccharomyces cerevisiae*. These capabilities are summarized in Fig. 2 and they are responsible for the changes that occur during the process of cheese-making.[7-9]

Yarrowia belongs to the family *Hemiascomycetes* and was known as *Candida, Endomycopsis* or *Saccharomycopsis lipolytica*. This species can be found readily in nature, *Y. lipolytica* strains can be isolated from dairy products such as cheeses, yoghurts and sausages. Strains have also been isolated from various environments, such as lipid-rich media (sewage, oilpolluted media) or marine and hypersaline environments.[6]

When plated out on standard lab media, *Yarrowia* usually doesn't make typical smooth and round yeast colonies like most *Saccharomyces* yeast strains. Instead, it makes colonies with ridges, wrinkles, or uneven mounds that tend to be fuzzy in appearance. At a microscopic level, cells of *Yarrowia* are a mix of typical yeast-like cells (small round circles) as well as filamentous cells.
Y. lipolytica can tolerate pretty high salt concentrations (as much as 12% salt), a variety of acidity (from pH 2.5 to pH 8), and really enjoys high fats and excessive protein environments, which includes meats and dairy products.[7-9]

Most strains of *Y. lipolytica* grow most effective up to 34°C, however there are a few traces that are adapted to higher temperatures. The encouraged temperature for growth is 25 to 30°C. Induction of sporulation is highest at 23 °C. Conjugation and sporulation frequencies are highest at 23 to 28°C, but lower strongly above 30°C.[10] Unlike many other types of yeast that can tolerate low-oxygen environments, *Y. lipolytica* needs oxygen so that you can grow. Meaning you'll normally only find this yeast growing on the surface of fermented foods, like the rinds of cheeses, and not within the interior of these foods where oxygen ranges are low.[7-9]

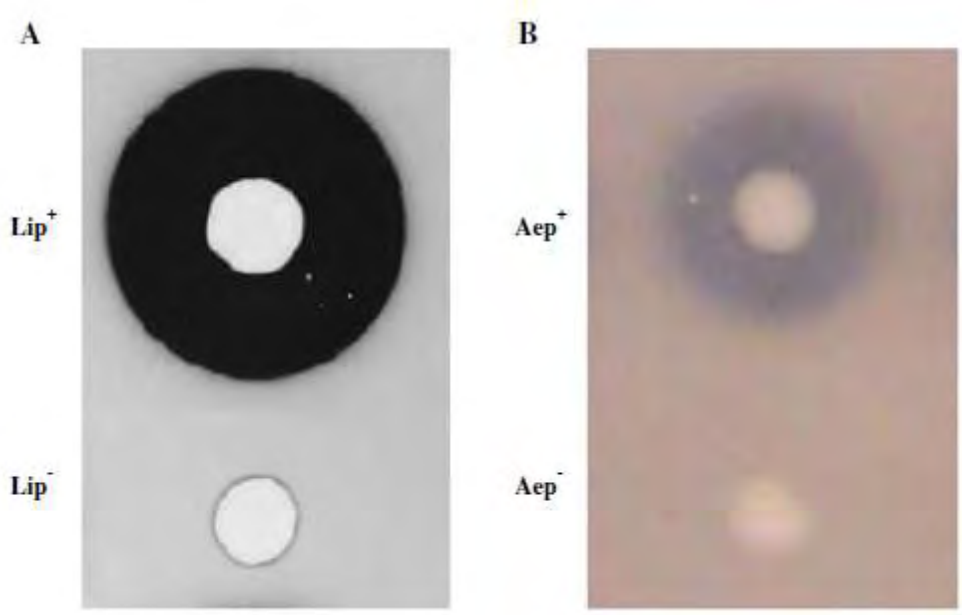

Figure 1: Lipolytic and proteolytic activities produced by *Yarrowia lipolytica* strains. **A:** Lipolytic activity visualized on a tributyrin plate; wild-type strain W29 and a lip2-deleted strain (deletion of the LIP2 gene encoding the secreted extracellular lipase lip2p). **B:** Proteolytic activity visualized on a casein plate; wild-type strain W29 and a xpr2-deleted strain (deletion of the XPR2 gene encoding for the extracellular alkaline protease Aep) (Nicaud 2012)[6]

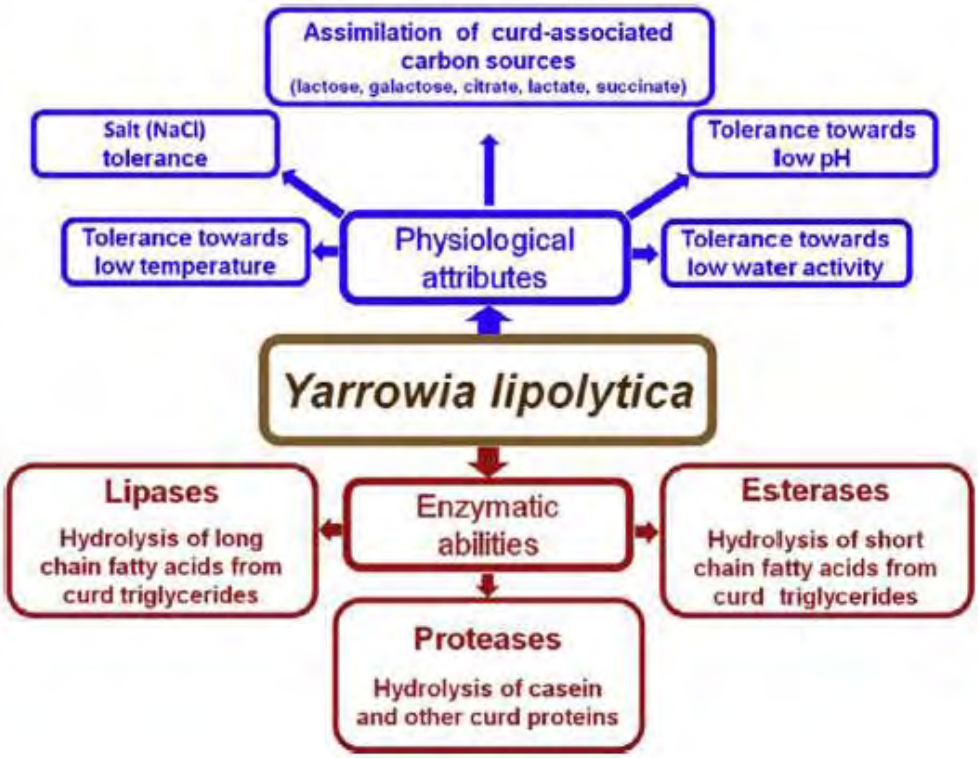

Figure 2: Summary of the attributes of *Yarrowia lipolytica* that are important in its association with cheese varieties. (Zinjarde 2014)[8]

2.1.2 *Sterigmatomyces halophilus*

In 1966, Fell described a new fungal genus, *Sterigmatomyces*, whose members showed a unique type of cell reproduction. The cells developed a long slender projection, the sterigma, producing one asexual spore, the conidium, on its end. The cells produce one or more sterigmata, each giving rise to a single conidium. The conidium is disjointed at a septum in the mid-region of the sterigma. Sexuality was not observed. Potassium nitrate may or may not be assimilated. Distinct carotenoid pigments are lacking. Extra-cellular starch-like compounds are not produced. Cells are round to oval (2.8 - 7) × (3.5 - 8.5) / t. Blastospores and hyphae are not formed, although the sterigma may elongate to give the appearance of hyphae. (Fig. 3).[11,12]

The majority of the observed strains were isolated from or adjacent to marine habitats. The initial isolation was from air in laboratory adjacent to Biscayne Bay, Florida. In addition, the species has been observed in seawater collections in Biscayne Bay and the adjacent Atlantic Ocean. Indian Ocean collections (Fell 1967) were in water depths of 1101977 m with water temperatures as low as 3˚C. The occurrences included several Indian Ocean water masses, specifically, the Indian Ocean Equatorial, Upper Deep, Antarctic Intermediate and Central Water Mass. The species is not endemic to the marine environment as demonstrated by CBS 5449, which is a clinical isolate from Brazil. In addition, W. Gams isolated to strain (CBS 6780) from soil in Sri Lanka, which was identified by phenotypic analysis.

After 7 days at 24˚C, colonies are chalkywhite and pasty, with a dry granular surface and an irregular to lobate margin. A second type of colony may develop that is smooth, glistening or dull and with an entire margin. The cell morphology is similar to that in malt extract. The cells vary in size, 143317 µm, and some cells are thin and long, 127 µm. The stalk-like conidiophores are generally short l330.5 µm, but may elongate to 2630.5 µm.[12-14]

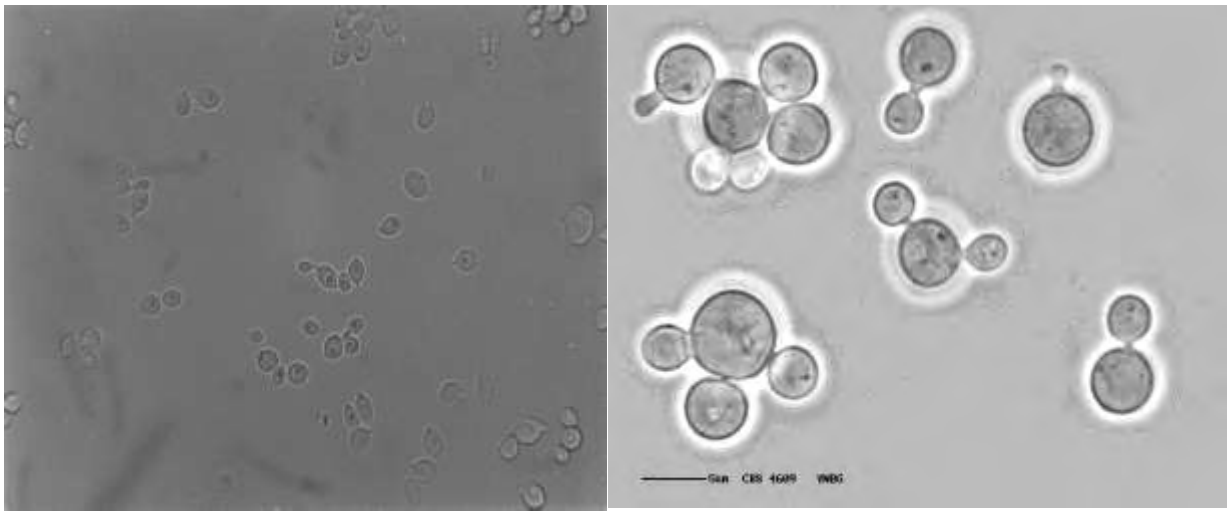

Figure 3: *Sterigmatomyces halophilus* colonies.[12-14]

2.1.3 *Debaryomyces hansenii*

Debaryomyces spp. is a non-pathogenic and extremophilic yeast (salt- and high pH tolerant) found in salty food and seawaters. The halotolerant nature of *Debaryomyces i*s reinforced by the observation that the presence of sodium in the medium efficiently contributes towards protecting *Debaryomyces* cells against additional abiotic stress factors such as extreme pH or temperature.

D. hansenii is a polyamine-producing yeast isolated from the digestive tract of rainbow trout.[15] Normally is classified within the *Ascomycetes*.[16]

D. hansenii occurs in many habitats with low water activity: hypersaline sites, such as seawater, from which it was initially isolated, cheese, meat, wine, beer, soil, fruit as well as in high-sugar products.[16]

Debaryomyces is osmotolerant and can grow in media containing up to 4 M NaCl, whereas growth of *S. cerevisiae* is restricted to medium containing less than 1.7 M NaCl. Its osmotolerance is highly advantageous for some biotechnological applications because it allows quasi-non-sterile production and high product/educt concentrations, conditions which should reduce production costs dramatically, e.g. in the agro-food sector. Unfortunately, *D. hansenii* is also known to be the causal agent of the spoilage of brine-preserved foods, such as gherkins.

D. hansenii is one of the lipid-accumulating, 'Oleaginous' yeasts. Oleaginous yeasts can accumulate lipids to concentrations up to 70% of their dry biomass and their metabolism is clearly dominated by pathways that contribute to lipid

metabolism. The extreme capacity of *D. hansenii* to synthesize, accumulate and store lipids could be advantageous for the biotechnological production of both natural and artificial products. *D. hansenii* is a highly heterogeneous, and thus versatile, species - as shown by the phenotypic.[17]

All *Debaryomyces* species are perfect, haploid yeasts that reproduce vegetatively by multilateral budding (Fig. 4, 5). A pseudomycelium is absent, primitive or occasionally well developed. The sexual reproduction proceeds via heterogamous conjugation, i.e. the conjugation of two cells of different form or size, here a mother cell and a bud. This conjugation generally leads to a short diplophase followed by meiosis and ascospore formation. The asci contain one to four spherical, globular, ovoidal or lenticular smoothy or warty ascospores. Isogamous conjugation also occurs.

D. hansenii is reported to grow optimally at 20–25˚C, but growth between 5˚C and 10˚C and even below 0˚C has also been reported.[17]

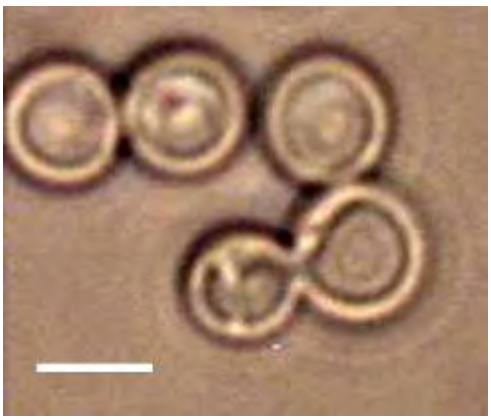

Figure 4: *Debaryomyces hansenii* H158 asci with one ascospore after 6 weeks on YEPD agar at 30˚C. Magnification ×1125. Bar = 2.5 µm. (Breuer & Harms 2006)[17]

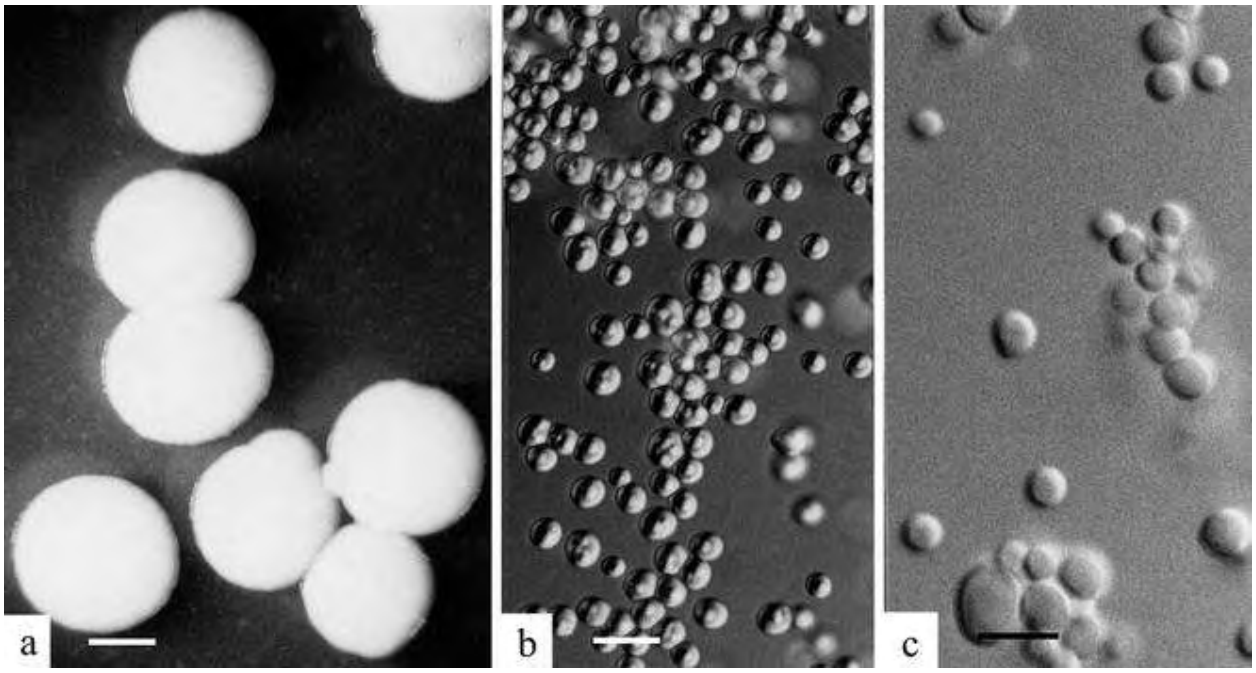

Figure 5: 0.4 *Debaryomyces hansenii* (a) colonies on MEA, 7 d, 25°C, bar = 2 mm; (b,c) vegetative cells (b) bar = 10 µm; (c) bar = 5 µm. (Pitt & Hocking 2009)[18]

2.2 Fish immune system

2.2.1 *Introduction*

Fish, the first vertebrate group appeared in evolution after adaptive radiation during the Devonic period, representing the most successful and diverse group of vertebrates. Fish is a heterogeneous group of organisms that include the agnathans (lampreys and myxines), condryctians (sharks and rays) and teleosteans (bony fish).

This heterogeneous group of organisms occupies an apparent crossroads between the innate immune response and the emergence of the adaptive immune response. Importantly, immune organs homologues to those of the mammalian immune system are found in fish. The immune system of fish is not only conditioned by the particular environment, but also by their poikilothermic nature. Comparative immunology, one of the multidisciplinary approaches of

comparative biology is derived from zoology and immunology which explores the differences as well as similarities among the immune systems of different organisms. Confirming the concept of self/non self, the whole immune system has been emerged as two major components: invertebrates possess natural, non-adaptive, innate, non-clonal responses whereas those of vertebrates are induced, adaptive, acquired, clonal, and anticipatory. So, it yields fascinating perspectives on the evolution and kinship of living beings. The extensive studies on invertebrate immune system show that there are many molecules which share homology with some of those in vertebrates.

An **immune system** "is a system of biological structures and processes within an organism that detect a wide variety of agents, from viruses to parasitic worms, protects against diseases and distinguishes them from the organism's own healthy tissue" (Rauta PR, Nayak B, Das S. 2012 p.148). The immune system present in all the creatures is either a rudimentary immune system (in the form of enzymes which protect against bacteriophage infections present in bacteria) or the more complex immune system (in human). Other basic immune mechanism evolved in ancient eukaryotes and remains in their modern descendants, such as plants and insects, includes phagocytosis, antimicrobial peptides like defensins, and the complement system. All vertebrate gnatostomates share an essential immune structure characterized by: (i) highly conserved innate system, (ii) consistent development of a combined immune system, and (iii) bilateral communication between components of the innate and adaptive immunity. The immune system possesses layered defense mechanisms of increasing specificity to fight against infections. In simple terms, physical barriers prevent pathogens such as bacteria and viruses from entering the organism. If a pathogen breaches these barriers, the innate immune system found in all plants and animals provides an immediate, but non-specific response. If pathogens successfully evade the innate response, vertebrates possess a second type of protection, the adaptive immune system.[19]

Adaptive immune system is activated by the innate response and more adapted by its response during an infection to improve its recognition of the pathogen and retainability of response in the form of an immunological memory. Adaptive

immunity arose early in vertebrate evolution, between the divergence of cyclostomes (lampreys) and cartilaginous fish (sharks) around 450 million years ago in an evolutionary time span estimated to be less than 20 million years. Fish possess both innate immunity as well as adaptive immunity. However, the innate immunity is stronger than adaptive immunity.[19]

2.2.2 *The excised phenotype: fish and its immune system*

Fish is a heterogeneous group of organisms that include the agnathans (lampreys and myxines), condryctians (sharks and rays) and teleosteans (bony fish). As in all vertebrates, fish have cellular and humoral immune responses, and central organs who's the main function is involved in immune defense. Fish and mammals show some similarities and some differences regarding immune function. Taking into account differences due to body compartments and cell organization, most of the generative and secondary lymphoid organs present in mammals are also found in fish, except for the lymphatic nodules and the bone marrow. Instead, the head kidney, aglomerular, assumes hemopoietic functions, and unlike higher vertebrates is the principal immune organ responsible for phagocytosis, antigen processing and formation of IgM and immune memory through melanomacrophagic centers. The kidney in fish is a disperse organ with a Y shape that is placed along the body axis (Fig. 6).[20]

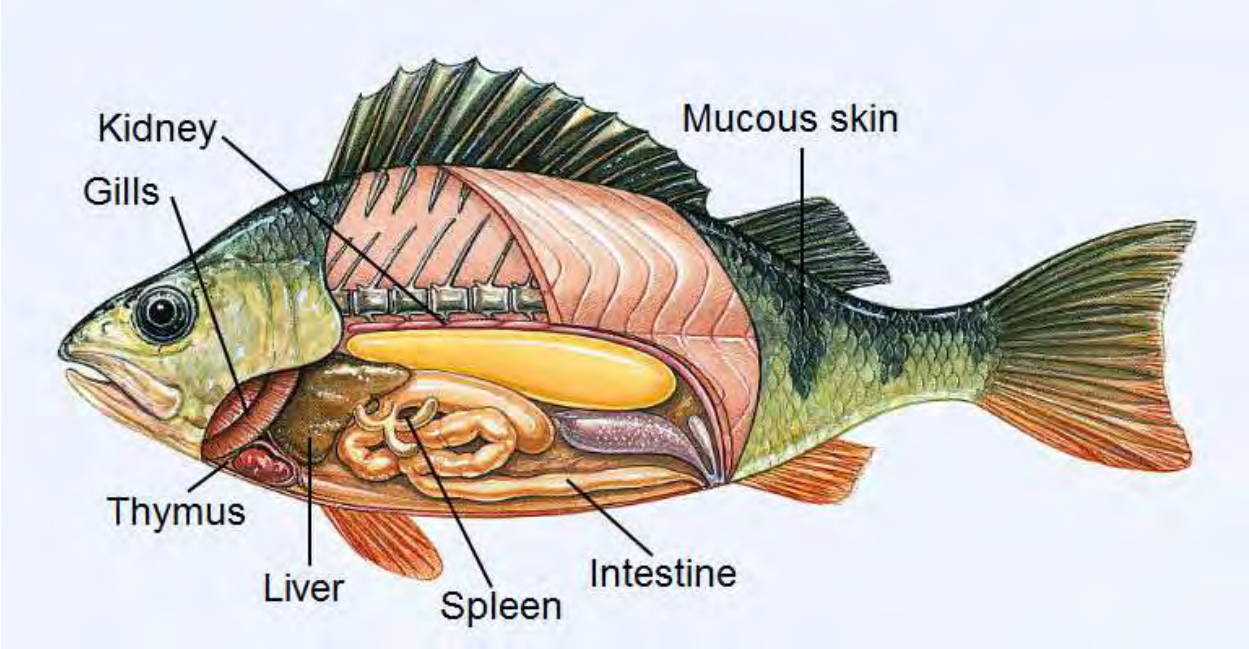

Figure 6: Immune structures in teleost fish. (Kum & Sekkim 2011) [21]

The lower part is a long structure situated parallel to the vertebral column, most of which works as a renal system. The active immune part, the head kidney or pronephros, is formed by two Y arms, which penetrate underneath the gills. In fish, this structure has a unique feature: the head kidney is also an important endocrine organ, homologous to mammalian adrenal glands, releasing corticosteroids and other hormones. In addition, it is a well innervated organ. Thus, the head kidney is an important organ with key regulatory functions and the central organ for immune-endocrine interactions and even neuroimmunoendocrine connections. The thymus, another lymphoid organ situated near the opercular cavity in teleosts (Fig. 7), produces T Lymphocytes involved in allograft rejection, stimulation of phagocytosis and antibody production by B cells. The involution of thymus in fish is more dependent on hormonal cycles and seasonal variations than on the age. Blood filtration and erythrocytic destruction is performed by the melanomacrophagic centres, formed by accumulation of macrophages associated to elipsoid capillaries. These centres may retain antigens as immune complexes for long periods. Although it has been suggested that these centres would assume the functions of germinal centres, no evidence has been presented up to now. Fish immune cells show the same main

features than that of other vertebrates, and lymphoid and myeloid cell families have been determined. The lymphoid system is a relatively recent evolutionary development since most animals prior to vertebrates rely on non-lymphoid cells or serum molecules. The existing functional analysis together with the reactivity of monoclonal antibodies suggests the presence of helper (Th) and cytotoxic (Tc) T lymphocytes and subpopulations of B cells (B1 y B2). The monocyte/macrophage cell lineage is the most studied in fish although no exact cell specific markers are available. Therefore, the term macrophage is often used as a loose definition for phagocytic cells independent of the differentiation status and the anatomical location. The majority of cytokines identified to date and of data concerning cytokine regulation has been obtained in monocyte/macrophage cell cultures.[20]

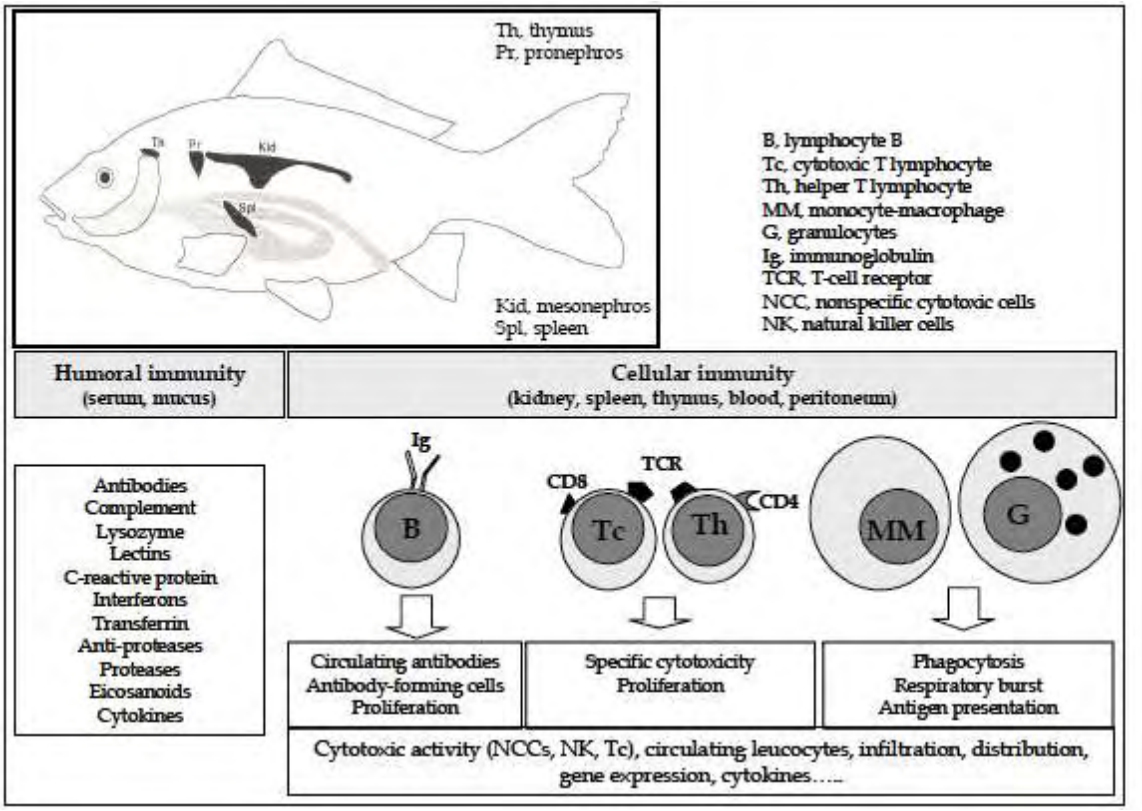

Figure 7: Fish immune system organization and representative humoral and cellular immune responses used in immunotoxicological studies. (Cuesta, Meseguer & Esteban 2011)[22]

2.2.3 *External barriers and integument mechanisms*

In teleosts, the lymphoid tissue associated to teguments is distributed around the skin, gills and intestine, thus complementing the physical and chemical protection provided by the structure. Among the epidermal secretions, complement, antimicrobial proteins, lysozyme, phosphatases and trypsin are often found, although their amount and activity depend on the species. As in other vertebrates the most exposed tissues are the gut, respiratory organs and the skin. This equipment is completed with immunocompetent cells such as leucocytes and intraepithelial plasmatic cells. The gills are a multifunctional wide surface regulating osmotic balance, excretion of part of the waste nitrogen compounds and gas, water and ion exchanges. As suggested by the named filter-feeding species, such as the sardine (*Sardina pilchardus*), the gills may also perform feeding functions. In addition to lymphocytes, the gill lamellae have pillar cells belonging to the reticuloendothelial system. A relevant expression of the β form of the MHC II has been demonstrated in the gills of some salmonids. As an aquatic organism, the fish body is subjected to continuous contact with many different types of microorganims. The first barrier against pathogens, the integumentary surface, is equipped with mechanisms protecting against pathogen entry. Surprisingly, some species are even resistant to immersion in water innoculated with the potent fish pathogen, *Aeromonas salmonicida*. Among the important integumental mechanisms are mucus secretion, and a diverse group of antibacterial molecules. The latter are peptide-based molecules that act both directly and indirectly on components of the bacterial cell wall resulting in lysis. Mucus is an important barrier in fish. Firstly because it provides the substrate in which antibacterial mechanisms may act. Secondly, in most fish species the mucus covers most of the external surfaces, and mainly the skin. Although this is a general trend, it is very clear that most freshwater species have a higher production of mucus compared with marine species. In addition, the production of mucus is significantly increased when subjected to stressing situations, such as chemical aggressions, which induce higher expression and activity of antibacterial agents. In special cases, as in the Myxinoids, mucus production may be as high as 40% of the body weight, using this system for both immune and antipredator

functions. Other surfaces such as gills have mucous cells juxtaposed between respiratory and osmoregulatory cells. Antibacterial peptides have been found in the last years in fish surfaces. However, much work has to be done as important features regarding antibacterial activity and mechanisms of action have to be specifically determined. Similarly, trypsin-like or cathepsin proteases have also been found. Therefore, this is a promising field in fish immune therapy and probably a different trait compared to mammals in terms of the variety and sensitivity against microbial action. Lysozyme is one of the most studied innate responses in fish. Lysozyme can act on the peptidoglycan layer of bacterial cell walls resulting in the lysis of the bacteria. Lysozyme has been found in mucus and ova(38), and serum lysozyme, probably coming from peritoneal macrophages and blood neutrophils, has been used as an indicator of non-specific immune response. The lysozyme response has been found to be variable in its potency depending on the species and the tissue location but is present in all species studied. It appears that the lysozyme response in fish may be induced very rapidly and not only related to bacterial presence but also to other alarm situations such as after stress. Thus lysozyme in fish would be involved in the overall alarm response, acting as an acute-phase protein. Lectins are important immune mediators in lower vertebrates and invertebrates. These proteins have the ability to bind carbohydrates, which are involved in the attachment to cell walls. Therefore lectins can block this attachment and subsequent invasion. Lectins are also involved in the induction of other immune mechanims such as activation of complement.[20]

2.2.4 *Fish immune system organization*

As mentioned before the immune tissues are quite different since fish lack the bone marrow and lymphatic nodules. Thus, pronephros (anterior/head-kidney) is the main lympho-haematopoietic tissue in fish, whilst the posterior part or mesonephros is mainly excretory and the first site for development and B cells production. Thymus is the main tissue for T cells development and maturation whilst spleen is the main secondary lymphoid tissue in fish. Other important site

for the immune response is the mucosal associated-lymphoid tissue (MALT), disperse in the skin, gill and gut.

The leucocyte-types present in fish are quite similar between vertebrates but with some specific differences (Table 2). Thus, fish lymphocytes are responsible for the production of antibodies (B cells) and the specific cellular immune response (T cells). B lymphocytes express and secrete immunoglobulin M (IgM), respond to the mitogen lipopolysaccharide (LPS) and constitute about 30% of the circulating lymphocytes. T lymphocytes are mainly detected in the thymus, express the T cell receptor (TCR) and proliferate with the mitogens concanavalin A and phytohemagglutinin (PHA). They are responsible for the humoral and cellular immune response against T-dependent antigens by the different populations of CD4+ (Th or helper) and CD8+ (Tc or cytotoxic). Moreover, there are also subpopulations of fish lymphocytes lacking proper cell markers, Ig or TCR, and constitute the natural killer (NK) cells. By other side, monocyte macrophages are the leucocytes displaying similar characteristics to both mammalian circulating monocytes and tissular macrophages. Moreover, they are mainly localized in kidney and spleen where they concentrate the ingested particles and aggregate in melano-macrophage (MM) centres. Granulocytes can be divided in neutrophils, eosinophils and basophils according to their staining properties but in the case of fish the distribution and functions do not fit well with their mammalian counterparts. Monocyte-macrophages and some granulocytes form the phagocytic cells involved in phagocytosis of particulated antigens and in production of a machinery of lytic enzymes and the respiratory burst reaction, in which very toxic reactive oxygen species (ROS) and nitrogen intermediates (RNI) are produced. Finally, nonspecific cytotoxic cells (NCCs) are involved in the lysis of tumor cells, virus-infected cells and parasites in a similar fashion than the mammalian NK cells. However, they are a heterogeneous population (lymphocytes, granulocytes and/or monocyte-macrophages) and therefore some authors talk of nonspecific cytotoxic activity more than a cellular type or population.[22]

Table 2: Non-specific immune cells in fish and their functional characteristics and mode of action. (Kum & Sekkim 2011)[21]

Cellular components	**Functional characteristics and mode of action**
Monocytes/Macrophages	Phagocytosis, and phagocyte activation, cytokine production, intracellular killing, antigen processing and presentation, Secretion of growth factors and enzymes to remodel injured tissue, T-lymphocyte stimulation.
Granulocytes (or Neutrophils)	Phagocytosis, secretion and phagocyte activation, cytokine production, extracellular killing, inflammation.
Non-specific cytotoxic cells (or natural killer cells)	Recognition and target cell lysis, induce apoptosis of infected cells, Synthesize and secrete **interferon-gamma (**IFN-γ).

3. Aim of this study

The use of probiotics in aquaculture feeds is in the spotlight. Among the different types of probiotics, yeasts are a good option. For the use of yeasts to be successful, they must be able to survive the passage through the digestive tract. Therefore, the use of extremophile marine yeasts may be of interest.

The overall aim of this work was to characterize the immune properties of four extremophile marine yeast, with the aim of recommending its use as probiotics in aquaculture feeds. The species studied were *D. hansenii* strain *004* (Dh), *Y. lipolytica* strain *N6* (YlA) and *Y. lipolytica* strain *D-1* (YlB) and *S. halophilus* strain *N16* (Sh).

The study was part of my Erasmus+ scholarship and was conducted in the Department of Cell Biology & Histology, Faculty of Biology of the University of Murcia, Spain.

4. Methods

4.1 Yeast culture procedures

Yeasts are eukaryotic microorganisms whose genomes have been comprehensively studied and some have been sequenced. They are relatively easy to grow under laboratory conditions. Moreover, despite their small genome size, they display cellular features and processes that are highly conserved amongst most eukaryotes. For instance, they have membrane-bound organelles, cytoskeleton, nuclear DNA, and transcription mechanisms that are similar to those found in higher eukaryotes. Furthermore, yeasts have many well-characterized secretory proteins and pheromones. Several yeast genes involved in protease processing and secretion have also been identified. Thus, yeasts can be used for several eukaryotic gene and protein studies with the aid of suitable molecular biology tools. Some applications for yeast cultures include synthesis of protein expression systems, the study of specific gene or protein functions, and the analyses of novel protein interactions. They are also used for many industrial applications such as fermentation, baking, and bioremediation.
Yeast culture media play a significant role in supporting growth in both small and large scale purposes. Typically, a yeast culture medium includes peptone, yeast extract, and dextrose or glucose. Even slight differences in media composition can yield yeasts with distinct growth characteristics.[23-25]

During the experiment it was used the YPD medium (yeast extract, peptone and dextrose). For the preparation of the liquid growth medium was mixed 20 gr/L of peptone and 10 gr/l of yeast extract in 500ml of distilled water. 20 gr/L of dextrose was added in 500ml of distilled water. The two liquids were mixed in an 1L bottle with small movements. It has to be sterile so was autoclaved in 100˚C for two hour.

4.2 *Immune parameters*

4.2.1 *Lysozyme assay*

Lysozyme is a mucolytic enzyme with antimicrobial proteins that can be found in serum, mucus and other tissues rich in leukocytes, such as the kidney, spleen and intestine, in both freshwater and seawater. It has the ability to degrade mucopolysaccharides from the cell wall of bacteria, particularly Gram + causing lysis. The lysozyme activity of the yeasts was measured using a method based on the ability of lysozyme to lyse the bacterium *Micrococcus lysodeikticus*. For this determination we proceed with the following:

1. Place in a flat-bottom 96-well plate, 20 µl of the sample (mucus or serum) in triplicate + 180 µl of *M. lysodeikticus* bacterial culture (SIGMA reagent M-3770), which is prepared at a concentration of 0.20 mg/mL in 0.04M sodium phosphate buffer (PBS).
Preparation of 0.04M sodium phosphate buffer in 500 mL of distilled water: Weigh 2.4g of NA_2PO and dissolve in 500 mL of distilled H2O, adjust the pH to 6.2.
Preparation of the bacterial culture *M. lysodeikticus*: to prepare 10 mL of culture in sodium phosphate buffer weigh 0.02 mg and dissolve.
2. Put the standard curve on the plate (Hen Egg White lysozyme); 20 µl of each point of the curve (0,2,4,6,8,10 µg/mL) + 180 µl of the bacterial culture of *M. lysodeikticus*.

Lysozyme concentration(µg/ml)	Mother solution Volume	PBS 0.04M Volume	Total Volume
0	0	100	100
2	2	98	100
4	4	96	100
6	6	94	100
8	8	92	100
10	10	90	100

3. Prepare the reaction's blank: put 20 µl of sample + 180 µl of sodium phosphate buffer.
4. Place a standard curve blank: 200 µl of sodium phosphate buffer.
5. Place on the plate 20 µl of the standard curve + 180 µl of the sodium phosphate buffer.
6. Incubate at 35˚C for 20 min.
7. Read at 450 nm.
To obtain the value of the lysozyme activity, subtract the optical density (OD) of the reaction's blank (20 µl of sample + 180 µl of buffer) to the OD of the reaction with the sample (20 µl of sample + 180 µl of *M. lysodeikticus*).
The points of the standard curve are also subtracted from the blank value; from the new curve the equation of the line is obtained, and interpolating the values of the OD of the plasma (OD reaction's blank – OD sample's blank), the results are obtained.
The values are expressed in µg/mL of equivalent HEWL activity.[26-29]

4.2.2 *Protease assay*

Proteolytic enzymes (also termed peptidases, proteases and proteinases) are capable of hydrolyzing peptide bonds in proteins. They can be found in all living organisms, from viruses to animals and humans. Proteolytic enzymes have great medical and pharmaceutical importance due to their key role in biological processes and in the life-cycle of many pathogens. Proteases are extensively applied enzymes in several sectors of industry and biotechnology, furthermore, numerous research applications require their use, including production of Klenow fragments, peptide synthesis, digestion of unwanted proteins during nucleic acid purification, cell culturing and tissue dissociation, preparation of recombinant antibody fragments for research, diagnostics and therapy, exploration of the structure-function relationships by structural studies, removal of affinity tags from fusion proteins in recombinant protein techniques, peptide sequencing and proteolytic digestion of proteins in proteomics.[30]
Protease activity was quantified using the azocasein hydrolysis.[31]

The procedure was:

1. 10 µl of sample + 100 µl of azocasein 2% in bicarbonate of ammonium was put in a flat-bottom 96-well-plate .

2. The plate was incubated for 24 hours at room temperature in constant agitation and in dark.

3. Then, 250 µl of 10% TCA was added.

4. The plate was centrifuged at 6000g (14000 rpm) for 5 minutes.

5. In a flat-bottom 96-well plate, place 100 µl of the sample and 100 µl of 1N NaOH in triplicate.

6. Measure absorbance at 540 nm.

4.2.3 *Antiprotease assay*

Total antiprotease activity was determined as indicated by the capacity of a sample to inhibit trypsin activity. For this determination we procedure with the following:[31]

1. Put 10 µl of sample + 10 µl of trypsin.

2. Incubate for 2 hours at room temperature in constant agitation and in dark.

3. Add 250 µl of TCA to 4.6%. Let stand for 30 minutes

4. Centrifuge at 6000g (14000 rpm) for 5 minutes.

5. In a flat-bottom 96-well plate, place 100 µl of the sample and 100 µl of 1N NaOH in triplicate.

6. The OD was read at 450 nm using a plate reader.

For a positive (100%) control, buffer replaced the sample, and for a negative control, buffer replaced both sample and trypsin. The inhibitory ability of antiprotease was expressed in terms of percentage trypsin inhibition.

4.2.4 *Phosphatase assay*

Alkaline phosphatases [ALP; orthophosphoric monoester phosphohydrolase (alkaline optimum) EC 3.1.3.1] are plasma membrane-bound glycoproteins (Fig. 8). These enzymes are widely distributed in nature, including prokaryotes and higher eukaryotes, with the exception of some higher plants. Alkaline phosphatase forms a large family of dimeric enzymes, usually confined to the cell surface hydrolyzes various monophosphate esters at a high pH optimum with release of inorganic phosphate.[32]

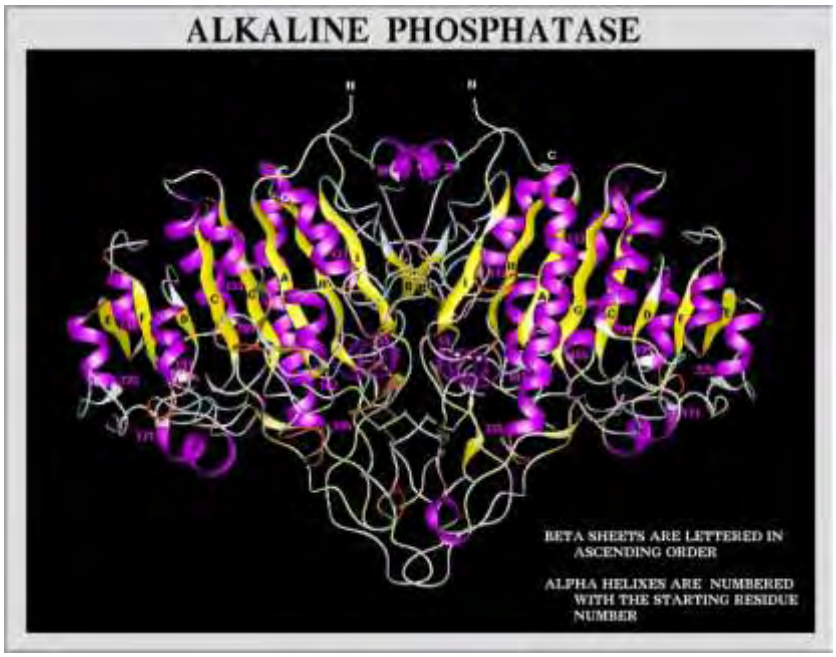

Figure 8: A ribbon diagram of L/B/K ALP protein structure. (Sharma, Pal & Prasad 2014)[32]

The procedure was:

1. In a 96-well plate was added 12 µl of sample (all in triplicate) + 100 µl of the mixture p-nitrophenyl liquid phosphate 4 mM (that it was at -20˚C) + $MgCl_2$ dissolved in 100 mM ammonium bicarbonate with pH 7.8, 30˚C.
For the blank: 100 µl of the ammonium bicarbonate mixture.
For the calculations, the molar extinction coefficient at 405 nm: 18.45 mM is required.
To prepare 100 mL 100 mM ammonium bicarbonate with pH 7.8, 30˚C was weighed 0.002 gr $MgCl_2$ x $6H_2O$ and 0.105 gr of p-nitrophenylliquidphosphate.
Molecular weight of p-nitrophenylliquidphosphate = 263.05 g / mol
Molecular weight of $MgCl_2$ = 203.30 g / mol

2. Once the mixture was made, the optical density of the reaction was measured at continuous intervals of 1-5 min for 3 hours, at a wavelength of 405 nm.

4.2.5 *Esterase assay*

Esterases are subclasses of the hydrolase superfamily of enzymes. Esterases break ester bonds (between a carboxylic acid and an alcohol) in lipids.[33,34]

The procedure was:
1. In a 96-well plate was added 12 of serum (all in triplicate) + 100 µl of the mixture p-nitrophenylmyristate substrate (that it was at -20˚C) + 0.5% of Triton X-100; dissolved in 100 mM ammonium bicarbonate with pH 7.8, 30˚C.
For blank: 100 µl of the ammonium bicarbonate mixture.
For the calculations, the molar extinction coefficient at 405 nm: 16.45 mM is required.To prepare 100 mL 100 mM ammonium bicarbonate with pH 7.8 was weighed 0.0134 g of p-nitrophenylmyristate substrate + 500 µl of Triton X-100.
2. Once the mixture was made, the optical density of the reaction was measured at continuous intervals of 1-5 min for 3 hours, at a wavelength of 405 nm.

4.2.6 *Peroxidase assay*

The peroxidases are enzymes whose primary function is to oxidize hydrogen donors at the expense of peroxides. They are highly specific for hydrogen peroxide, but they accept a wide range of hydrogen donors, including polyphenols. The overall reactions catalyzed are shown in eqns **(a)** and **(b)** :

(a) $AH_2+H_2O_2 \rightarrow A+2H_2O$

(b) $2AH+H_2O_2 \rightarrow AA+2H_2O$

Peroxidases are glycoproteins with a hematin compound as cofactor. Their molecular weights range between 30 and 55 kDa. Depending on the enzyme source, the isoenzyme considered, and the hydrogen donor substrate, the optimum activity is between pH 4 and 7. In many, but not all cases,

the peroxidase isoenzymes are activated by calcium ions. The higher thermostability of some isoenzymes is well known, and the residual peroxidase activity after blanching is often used as an index of thermal treatment.

Although peroxidases are widely distributed, especially in plants, they generally appear to be little involved in enzymatic browning of fruits and vegetables following a mechanical stress. The explanation could be that the peroxidase activity is limited by the internal level of hydrogen peroxide. However, their involvement in slow processes such as internal browning during cold storage of fruits is possible.

Nevertheless, the direct involvement of peroxidase in browning still needs confirmation, just as does that of laccase which may not be present in some fruits and vegetables. Therefore, the following sections will be mainly devoted to PPO activity.[35]

The procedure that was followed is:

1. In a flat-bottom 96-well plate, was placed 5 µl of sample + 45 µl of Hanks buffer without Ca or Mg (all in triplicate).

2. Add 100 µl of the 10MM TMB solution + 0.015% H_2O_2 hydrogen peroxide.

For the preparation of TMB was added 40 mL of distilled water (H_2Od) + 10 µl H_2O_2 + 1 tablet of TMB.

3. Stop the reaction with 50 µl of 2M sulfuric acid.

4. Measure the absorbance at 450 nm.

4.2.7 *Bactericidal assay*

The immune system of teleost fish has mechanisms responsible for the defense against bacteria, through humoral and cell-mediated pathways, which act in a multifactorial approach so as to prevent bacterial colonization. Innate mechanisms against bacterial invasion include the production of antibacterial compounds, such as proteins of the complement activated by an alternative pathway, acute phase proteins, cytokines, and phagocytosis and inflammation.

Proteins of the complement system activated by alternative pathways are considered the most effective antibacterial compounds due to their lytic activity, pro-inflammatory chemotaxis and opsonizing action that influence the defense cell response. Regarding phagocytes, neutrophils and macrophages are very important because of their large quantities of lysosomal enzymes and the production of reactive oxygen species responsible for the destruction of invading bacteria.

Bacteria growth inhibition factors are essential to prevent tissue damage. Among these factors, the transferrin, a soluble blood protein with high iron affinity is present in high concentration mainly in the acute inflammation phase so as to activate macrophages and decrease the available iron, an essential ion for some bacteria in infection establishment. In addition, antiproteases, such as lectins, are blood proteins which act on the proteolytic compound of bacteria responsible for the lysis of fish tissue. Lectins generate pathogen agglutination due to its high affinity to certain carbohydrates on the bacteria wall.

Lysines, found in blood and body tissue, mainly where there are leukocytes (especially monocytes and neutrophils) are antibacterial peptides that attack pathogen membranes. Among lysines, lysozyme lyses peptidoglycan components of Gram-positive and Gramnegative bacteria walls; C-reactive protein binds to phosphorylcholine of the microorganisms walls and may increase its concentration after a heat shock, inflammatory agents, and during warm periods of the year besides promoting the activation of complement and phagocytosis.

The presence of protective proteins in fish blood can be evaluated by serum bactericidal activity and this is an important tool to analyze the innate immune system. This work has standardized the methodology for evaluation of serum

bactericidal activity of the three yeasts, after the *V. angillarum* y *V. harveyi* challenge.[36]

Vibrio harveyi

By definition, *V. harveyi* is a marine Gram-negative luminous organism with a requirement for sodium chloride, in the genus *Vibrio*.[37] *V. harveyi* is rod-shaped, motile (via polar flagella), facultatively anaerobic, halophilic, and competent for both fermentative and respiratory metabolism (Fig. 9). It does not grow below 4 °C or above 35 °C.

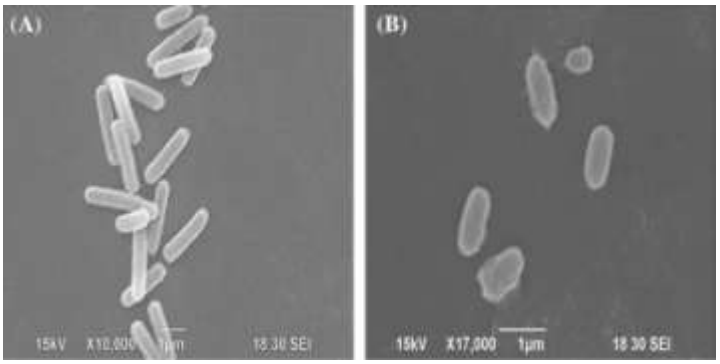

***Figure 9*:** Scanning electronic microscopy of *V. harveyi* cells. **A:** non-starved cells **B:** four-weeks starved cells.

V. harveyi can be found free-swimming in tropical marine waters, commensally in the gut microflora of marine animals, and as both a primary and opportunistic pathogen of marine animals, including Gorgonian corals, oysters, prawns, lobsters, the common snook, barramundi, turbot, milkfish, and seahorses.[38] It is responsible for luminous vibriosis, a disease that affects commercially farmed prawns. *Vibrio* affects both marine fish and invertebrates. In fish, the diseases include vasculitis, gastro-enteritis and eye lesions. The infected fish with pathogenic V. harveyi often exhibit a variety of non-specific symptoms ranging from lethargic swimming, anorexia, a flaccid body, multifocal reddish discoloration spots on the abdominal cuticle, sometimes with melanized erosions around the spots, and white opacity of the abdominal muscle.[37]

Vibrio angillarum

V. anguillarum is a Gram-negative, commashaped rod bacterium, belonging to the family Vibrionaceae. It is polarly flagellated, non-sporeforming, halophilic and facultatively anaerobic (Fig. 10). The bacterium grows rapidly at temperatures between 25 and 30˚C on rich media containing 1.5–2% sodium chloride (NaCl), forming cream-coloured and round-shaped colonies.[39]

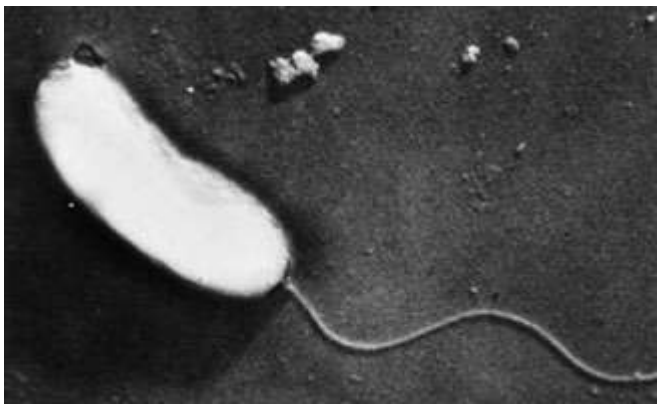

Figure 10: Electron micrograph of Vibrio anguillarum 775 showing the single polar flagellum. Shadow preparation, x10 000. (Frans, Michiels, Bossier, Willems, Lievens & Rediers 2011)[39]

The skin mucus antimicrobial activity was determined by evaluating their effects on the bacterial growth curves using the method of Sunyer and Tort with some modifications:

Preparation of the bacteria solutions:

Next steps have been succesfully carried out for *V. angillarum* and *V. harveyi.*

1. First the bacteria were cultivated in Petri dish. The medium that was used for the two bacteria was TSB (tryptic soy broth, DifcoLaboratories) at a concentration of 30g/l in distilled water, with 1.5% NaCl and 1.5% agar, and was sterilized. The Petri dishes with the bacteria were kept at 25ºC for 24 hours.

2. To grow bacteria, we prepared TSB medium without agar. For example, 50 ml of the medium with a small portion of an individual colony. For a correct growth of the bacteria, 90% of the flask should be air, and 10% the medium with the bacteria. The flask was closed using a tissue as a lid, and cultured at 25ºC with continuous shaking (100 rpm) till next day.

3. The solution was measured in a spectrophotometer using an absorbance of 620nm. A value of 0.275 is equivalent to 10^6 cfu/ml. For blank, medium was used.

Preparation of MTT:

MTT was prepared at 1mg/ml. It was dissolved in sterile PBS, pre heated in a 37ºC bath, and filtered through a 0.22 µm filter.

Procedure:

1. In a round-bottom 96-well plate, in triplicates, 20 µl of sample and 20 µl of *bacteria* were incubated for 5h at 25°C. Hank´s balanced salt solution or sterile PBS instead of sample was used for positive control. It was recommendable to prepare a negative control, adding 20 µl of some of the serum or mucus samples and 20 µl of Hank´s balanced salt solution or sterile PBS instead of bacteria.

2. To each well, was added 25 µl of MTT and incubated for 10 min. at 25°C.

3. Plates were centrifuged at 2000 *x g* for 10 min.

4. The supernatant was removed using multichannel micropipette and inclining the plate in order to avoid removing the bacteria.

5. The precipitate was dissolved in 200 µl of DMSO, well mixed with the micropipette and 100 µl were transferred to a flat-botom 96-well plate.

6. The absorbance was recorded at 570 and 690 nm (final absorbance = Abs. 570 – Abs. 690).

7. The percentage bactericidal capacity is calculated by comparison with the reference sample. Samples without bacteria were used as blanks (negative control). Samples without mucus were used as positive controls (100% growth or 0% bactericidal activity).

% viable bacteria = sample Abs. x 100/ Abs. of the reference sample

% no viable bacteria (bactericidal activity) = 100 - % viable bacteria

4.2.8 *ph Resistance*

For this procedure YPD medium was prepared at different pH (6.5, 5.5, 4.5, 3.5 and 2.5) and the yeasts were inoculated (10%, 600µl in 6ml of YPD medium). Subsequently, the culture was incubated at 30°C with constant agitation (120rpm) for 30 minutes. Once the time had elapsed, serial dilutions (10-1, 10-2 and 10-3) was made, of which 100µl was taken to inoculate plates with YPD Agar medium. The plates were incubated at 30°C and growth was recorded at 24, 48 and 72 hours.

4.2.9 *Resistance to bile*

The bacteria were examined for their ability to grow in the presence of gilthead sea bream bile extract. YPD medium was prepared with bile of gilthead sea bream (2.5 parts YPD medium, 2 parts PBS, 0.5 parts bile). Yeasts (10%, 50µl in 500µl of medium) was inoculated and incubated for one and a half hours at 30°C without agitation (Pedersen et al., 2004). Once the time had elapsed, dilutions were made (10^{-1}, 10^{-2} and 10^{-3}), of which 100µl was taken to inoculate plates with YPD Agar medium. Finally, the plates were incubated at 30°C and growth was measured at 24, 48 and 72 hours.[41]

4.2.10 *Yeast lysis*

Cell lysis or cellular disruption is a method in which the outer boundary or cell membrane is broken down or destroyed in order to release inter-cellular materials such as DNA, RNA, protein or organelles from a cell. Cell lysis is an important unit operation for molecular diagnostics of pathogens, immunoassays for point of care diagnostics, down streaming processes such as protein purification for studying protein function and structure, cancer diagnostics, drug screening, mRNA transcriptome determination and analysis of the composition of specific proteins, lipids, and nucleic acids individually or as complexes.[44]

A large array of enzymatic, chemical and physical based lysis methods have been reported. Enzymatic and chemical methods could be miniaturized, but these methods are associated with high costs, assay inhibition, storage issues and/or pathogen specificity. Therefore the more generic physical based lysis methods are preferred. Methods reported include electrical, mechanical, optical, thermal and ultrasound based methods. In common lab-practice mechanical methods and esp. bead beating is recognized as the golden standard method, especially for rather difficult to lyse microorganisms. Due to the abundant dynamical mechanical characteristics of bead beating, it is one of the most challenging methods to integrate into a small size sample-in result-out system using disposable cartridges.[45]

The specific cell wall composition and growth mode of the yeast cells have been the major limitations for utilization of common procedures.[46] Consequently, in this work we used the beat beating method in order to break the cell wall and release the intracellular components. We examined both the entire cell and the cell after the lysis to see the differences between the components in the supernatant and the components inside the cell.

Bead Mill

Bead mill, also known as bead beating method, is a widely used laboratory scale mechanical cell lysis method. The cells are disrupted by agitating tiny beads made of glass, steel or ceramic which are mixed along with the cell suspension at high speeds. The beads collide with the cells breaking open the cell membrane and releasing the intracellular components by shear force. This process is influenced by many parameters such as bead diameter and density, cell concentration and speed of agitator. In general smaller beads with a range of 0.25–0.5 mm are more effective and preferred for lysis. Using this technique, several kinds of cells can be lysed for example yeast and bacteria. Cell membrane can become totally disintegrated by this method confirming that the intracellular molecules are released. Thus, the efficiency of this method of lysing cells is very high. However, complete disintegration produces small cell debris and thereby separation and purification of sample becomes harder. In addition, heat generation occurs in this process due to the collision between beads and cells. This elevated heat may degrade proteins and RNA.[44,47]

Cell rupture takes place, when the kinetic energy of the colliding beads exceeds the elastic energy stored in the cell. Inter-bead collision lysis requires relative speeds of ~ 0.3 m/s, which could only be achieved at large volumetric chamber vibrations. Inter-bead shear flow to achieve lysis for a typical (Gramm positive) bacterium should be in the range of 5-10 m/s. Therefore inter-beads collisions is the primary mechanism of cell rupture during lysis.[45]

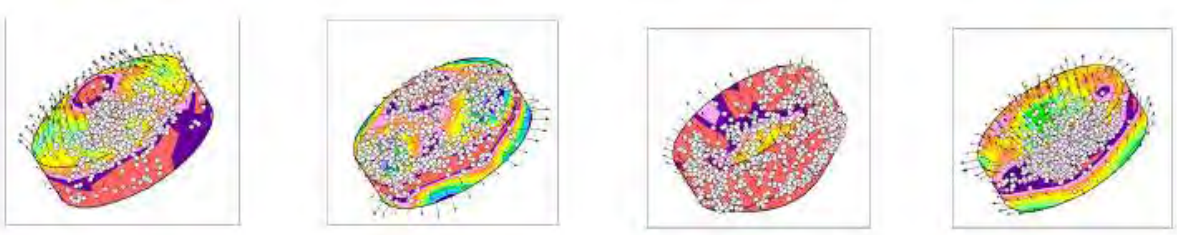

Figure 12: Velocity contours of particles in a vibrating bead-bed (50 Hz frequency and amplitude 80% of the chamber height, a chamber diameter of 10mm, a height of 3mm, a bead filling of 40%, and beads with a diameter between 250 to 500µm and a specific density of = 2.5 – 3 g/cm3) (Beckers, Baragona, Shulepov, Vliegenthart & Doorn 2010).[45]

For the yeast lysis, what we did was to put 1ml of the yeast at the concentration of $3x10^6$ cells/ml together with a volume of 500ml of glass beads, with a size of 0.45mm diameter. We put it in a bed beater (Tissuelisser LT, from Quiagen) for 2 minutes and then we transferred it to eppendorfs.

4.2.11 *Hydrophilic activity (AAH)*

The amount of total antioxidant activity (AAT) is subdivided into hydrophilic antioxidant activity (AAH) and lipophilic antioxidant activity (AAL). We focused on AAH. We measured that activity by comparing the amount of ABTS• (radical) reduced by the antioxidants in the sample with the standard curve of ascorbic acid.[48,49]

Method:

We prepared the reaction medium in which the reagents must remain at a concentration of:

- ABTS 2mM
- H_2O_2 35 µM
- HPR 0.25 µM

1. Phosphate buffer was added to this reaction medium until a fine volume of 1 ml is obtained (spectrophotometer cuvette).

2. 15 µM of sample (depending on the type of sample and the amount of antioxidant capacity it has) was added to the reaction medium and the

absorbance at 730 nm was measured at time zero and at 5 minutes. The hydrophilic antioxidants in the sample neutralize the radical ABTS, producing a drop in absorbance. The difference between these two data was used to determine the antioxidant activity through a straight Ac pattern. L-ascorbic was used as reference compounds.

3. We calculated the amount of ABTS consumed by the sample with the molar extinction coefficient (ε) of the ABTS at 730 nm which is equal to 13000 $M^{-1}cm^{-1}$ and knowing that 1 mol of ascorbic acid reduces two moles of ABTS.

4.2.12 *Phagocytic activity*

Marking with FITC

FITC is one of the most popular fluorescent probes ever created. An isothiocyanate derivative of fluorescein is synthesized by modification of its lower ring at the 5- or 6-carbon positions. The two resulting isomers are nearly identical in their reactivity and spectral properties, including excitation and emission wavelengths and intensities. Their chemical differences, however, may affect the separation of modified proteins from excess reagent or the analysis of tagged molecules by electrophoresis. For this reason, most manufacturers purify the carbon-5 derivative as the FITC reagent of choice.[42]

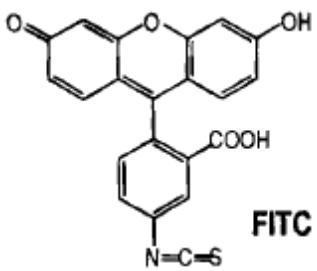

Figure 11: Chemical compounf of FITC. (Brelje, Wessendod & Sorenson 1993)[43]

The protocol was:

1. The yeasts were lyophilized and inactivated by heat (strain 5288C).

2. About 0.05-0.10g were taken and were put in a 15ml tube. The tub was filled up to 15ml with PBS.

3. The tub was shaked strongly with vortex and then with a syringe the lumps were removed.

4. The concentration was adjusted to 108 yeast / ml (10ml).

5. Next, a 15ml tube with PBS with a concentration of 10µg / ml of FITC was prepared (10ml).
6. The two solutions (yeast + FITC) were joined in a 50ml tube and covered with aluminum foil. The final concentration of FITC was 5µg / ml and the yeast concentration was 0.5x108 yeast / ml.
7. It was placed in the agitator at room temperature at 40 cycles per minute for 15 minutes.
8. It was centrifuged at 2500 RPM (858g) 10 minutes.
9. The supernatant was discarded; 40ml of PBS were added, was placed on vortex and was resuspended.
10. Then it was centrifuged, shaked with vortex and resuspended with PBS (for 5 times).
11. In the last wash, after discarding the supernatant, 20ml of RPMI saline was added, shaked with vortex resuspended well. Check the cytometer for marking (using trypan blue to inactivate) with unlabelled yeasts. 400µl PBS + 100µl yeast are used (inactivated with 40µl blue trypan).
12. Last it was aliquoted in 1.5ml eppendorf and was put in the -80ºC freezer.

Phagocytosis, is the process whereby cells engulf, kill, and digest different particles (damaged or effete cells, microorganisms, etc.) and plays an important role in the fish nonspecific immune response. For this reason, the study of phagocytic functions shows importance, particularly for those species used in aquaculture. Rapid and precise techniques to estimate leucocyte activities are required, to which end flow cytometry may offer an important contribution.[50]

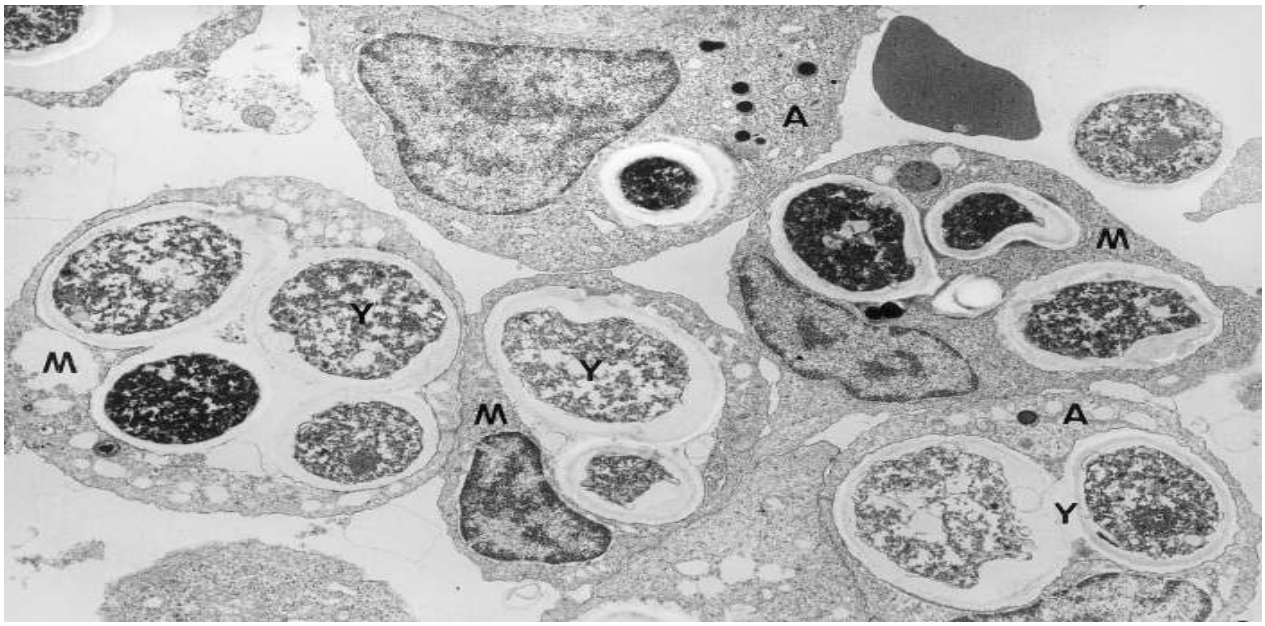

Figure 13: General view of samples containing gilthead seabream head-kidney leucocytes incubated with S. *cerevisiae* (Y), in which are shown two cell types with phagocytic activity. A, acidophilic granulocytes; M, monocyte-macrophages. x 8,000. (Rodriguez, Esteban & Meseguer 2003)[50]

The phagocytosis was studied by flow cytometry (Rodriguez, Esteban & Meseguer 2003).

1. Heat-killed yeast cells were labelled with fluorescein isothiocyanate (FITC, Sigma), washed and adjusted to 5 x 10^7 cells mL^{-1} of sRPMI.[51] Phagocytosis samples consisted of labelled yeast cells and leucocytes.

2. Samples were mixed, centrifuged (5 min, 400 *g*, 22˚C), resuspended in sRPMI and incubated at 22˚C for 30 min.

3. At the end of the incubation period, the samples were placed on ice and 400mL ice-cold PBS was added to each sample to terminate phagocytosis.

4. The fluorescence of the extracellular yeasts was quenched by adding 40mL ice-cold trypan blue (0.4% in PBS). Standard samples of FITC-labelled *S. cerevisiae* or leucocytes were included in each phagocytosis assay as blank. All samples were analysed in a flow cytometer set to analyse the phagocytic cells.

5. Phagocytic ability was defined as the percentage of cells with ingested yeast cells (green-FITC fluorescent cells, $FL1^+$) within the phagocyte cell population. The relative number of ingested yeasts per cell (phagocytic capacity) was assessed in arbitrary units from the mean fluorescence intensity of the phagocytic cells.

4.2.13 *Statistical analysis*

All bioassays and measurements were performed in triplicate and the mean ± standard error, for each yeast strain was calculated. A one way analysis of variance (ANOVA) was performed to determine significant differences of yeast. When there were significant differences among yeast, Tukey was selected as post-hoc analysis (or Games-Howell when results showed lack of homogeneity). Analyses were performed using SPSS v.19.0 software (SPSS, Richmond, VA, USA). Statistical analyses were made with the data obtained for each sample. Differences were considered statistically significant when $P < 0.05$.

5. Results

The results of the experiments, following the protocols mentioned in materials and methods are presented in the following tables. The figures from 14 to 19 show the results from the yeast lysate, means the cells after the lysis and the figures from 20 to 25 show the results from the entire yeast cells. Results from phagocytic activity are presented in the figures 26 and 27.

5.1 *Yeast Lysate*

5.1.1 *Lysozyme assay*

Lysozyme (1,4-β-*N*-acetylmuramidase) levels are widely considered an important index of innate immunity of fish. Lysozyme is a very important enzyme involved in the prevention of bacterial infections, since it attacks the peptidoglycan present in certain bacterial cell walls with lytic action. It is also known to be opsonic in nature and activates the complement system and phagocytes.[52] In the present work, the amount of lysozyme activity was significantly higher in *S. halophilus* strain *N16*, compared to the other yeasts.

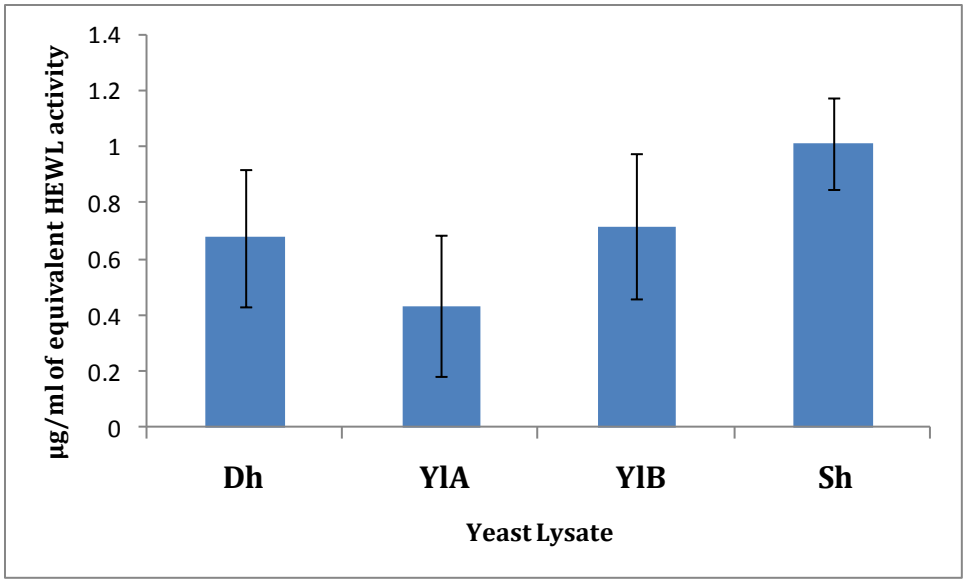

Figure 14 : Lysozyme activity (µg mL^{-1} of equivalent hen egg white lysozyme activity)in yeast lysate. Results are shown as mean ± standard error. There were not detected significant differences among yeast for this activity. Differences were considered significant when $p < 0.05$. *D. hansenii* strain *004* (Dh), *Y. lipolytica* strain *N6* (YlA) and *Y. lipolytica* strain *D-1* (YlB) and *S. halophilus* strain *N16* (Sh).

5.1.2 *Protease assay*

Proteases are proteins charged with the hydrolysis of peptide bonds, while antiproteases have the capacity to maintain the proteases present in tissues. Antiproteases play crucial roles in the inhibition of the action of proteases either by binding to their active sites or by 'trapping' the protease to prevent protein hydrolysis. Furthermore, it is known that a correct balance between protease and antiprotease activities is needed to safeguard the accurate functionality of any tissue or organ. Many microorganisms (mainly bacteria) express a variety of proteases. Some proteases are non-specific and powerful enzymes that degrade many proteins involved in innate immunity, and others are extremely precise and specific in their mode of action.[52] The importance of having antiproteases in serum in order to neutralize these proteases is evident. In the present work, protease in yeast lysate was significantly higher in *Y. lipolytica* strain *D-1*.

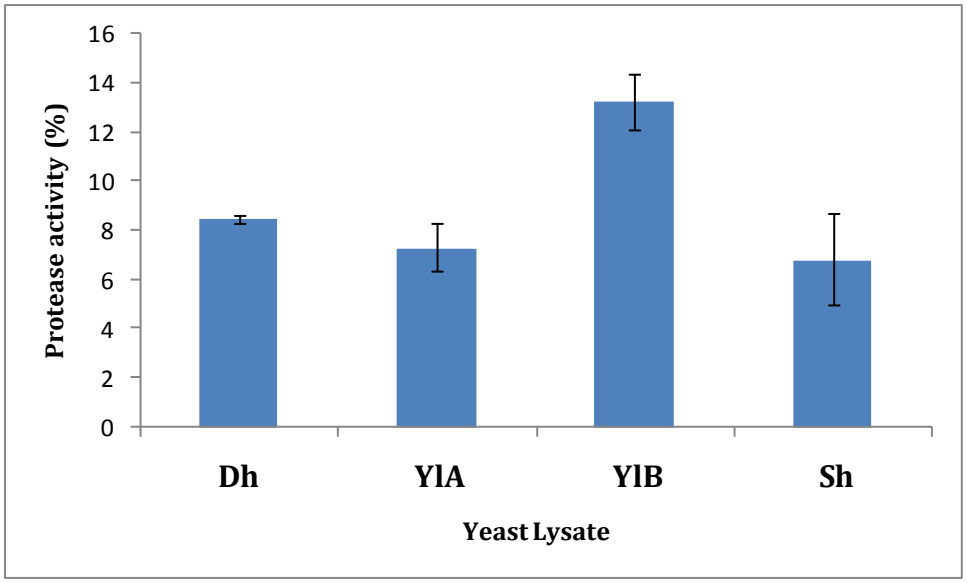

Figure 15 : Protease activity (%) in yeast lysate. Results are shown as mean ± standard error. There were not detected significant differences among yeast for this activity. Differences were considered significant when $p < 0.05$. *D. hansenii* strain *004* (Dh), *Y. lipolytica* strain *N6* (YlA) and *Y. lipolytica* strain *D-1* (YlB) and *S. halophilus* strain *N16* (Sh).

5.1.3 *Esterase assay*

Esterases (EC 3.1.1.X) comprise a diverse group of hydrolases that catalyze the cleavage and formation of ester bonds. They are widely distributed in animals, plants and microorganisms. Many of them show a wide range of possible substrates, leading to the assumption that they evolved to enable access to carbon sources or to be involved in catabolic pathways. These enzymes also display high regio- and stereo-specificity, making them attractive biocatalysts in the production of optically pure compounds in fine chemical synthesis.[53] In the present work, higher esterase activity was found in *D. hanseii*.

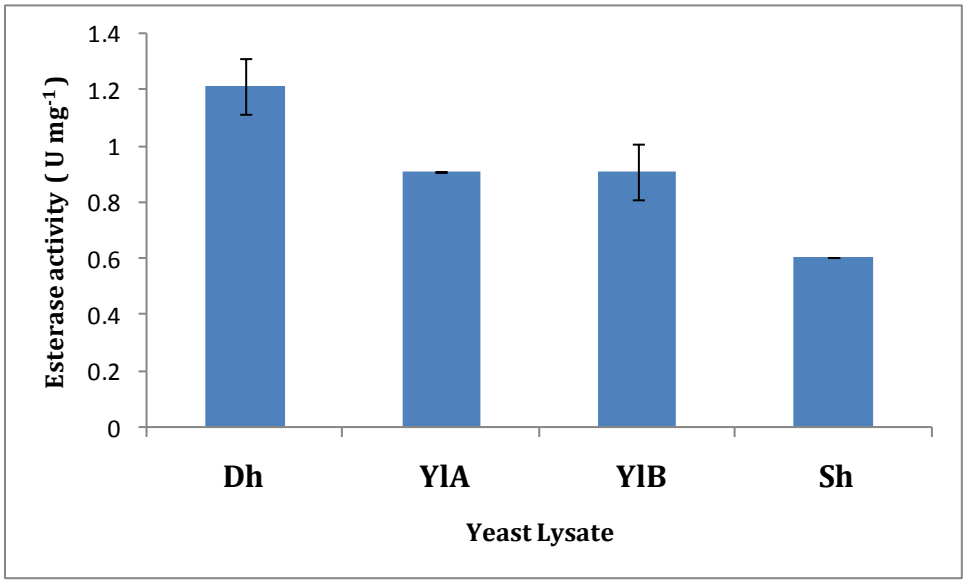

Figure 16 : Esterase activity (U mg^{-1}) in yeast lysate. Results are shown as mean ± standard error. There were not detected significant differences among yeast for this activity. Differences were considered significant when $p < 0.05$. *D. hansenii* strain *004* (Dh), *Y. lipolytica* strain *N6* (YlA) and *Y. lipolytica* strain *D-1* (YlB) and *S. halophilus* strain *N16* (Sh).

5.1.4 *Peroxidase assay*

Peroxidase is an important enzyme that catalyzes the anion superoxide to produce chloride acid. This anion can be produced by other immune defense pathways, such as respiratory burst. Furthermore, peroxidase has microbicidal properties because it uses one of the oxidative radicals (H_2O_2) to produce hypochlorous acid, in a process that is very important as a way of killing foreign microorganisms.[52] In the present work, peroxidase activity was detected in all strains, being the higher values detected in *Y. lipolytica* strain *D-1*.

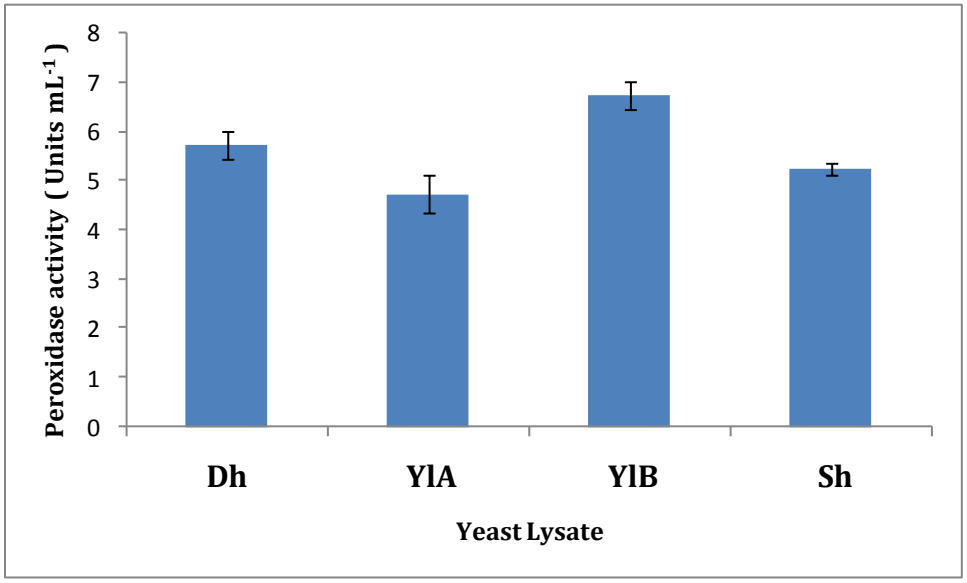

Figure 17 : Peroxidase activity (units mL^{-1}) in yeast lysate. Results are shown as mean ± standard error. There were not detected significant differences among yeast for this activity. Differences were considered significant when $p < 0.05$. *D. hansenii* strain *004* (Dh), *Y. lipolytica* strain *N6* (YlA) and *Y. lipolytica* strain *D-1* (YlB) and *S. halophilus* strain *N16* (Sh).

5.1.5 *Bactericidal assay*

Serum bactericidal activity is a mechanism known for killing and clearing of pathogenic organisms in fish. Regarding the bactericidal activity measured in yeast lysate, no significant variations were obtained.[52] The bactericidal activity against *V. harveyi* in yeast lysate was high in *D. hansenii*, meanwhile in *Y. lipolytica* strain *D-1* was detected very little bactericidal activity.

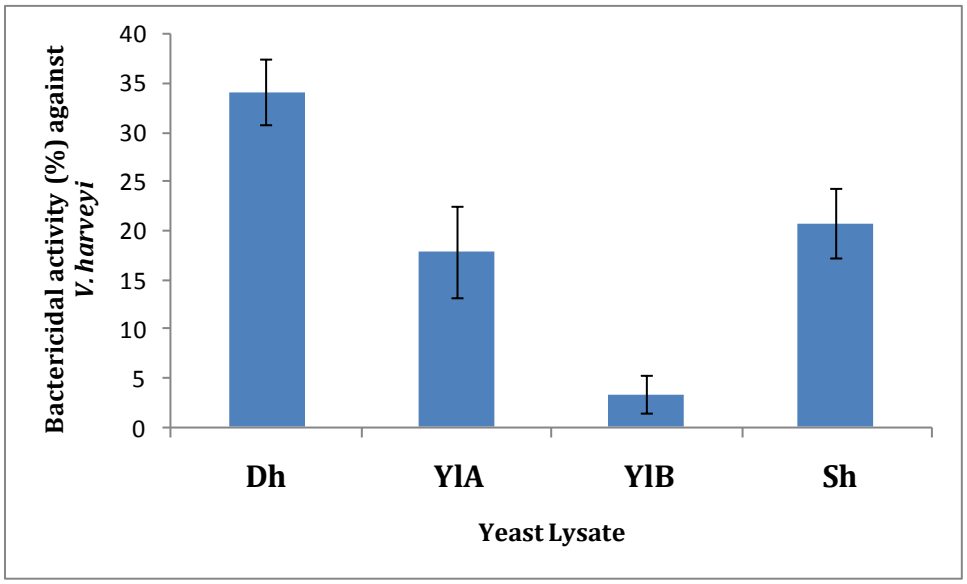

Figure 18 : Bactericidal activity (%) against *V. harveyi* in yeast lysate. Results are shown as mean ± standard error. There were not detected significant differences among yeast for this activity. Differences were considered significant when $p < 0.05$. *D. hansenii* strain *004* (Dh), *Y. lipolytica* strain *N6* (YlA) and *Y. lipolytica* strain *D-1* (YlB) and *S. halophilus* strain *N16* (Sh).

The bactericidal activity against *V. anguilarum* in yeast lysate was high in *Y. lipolytica* strain *D-1*.

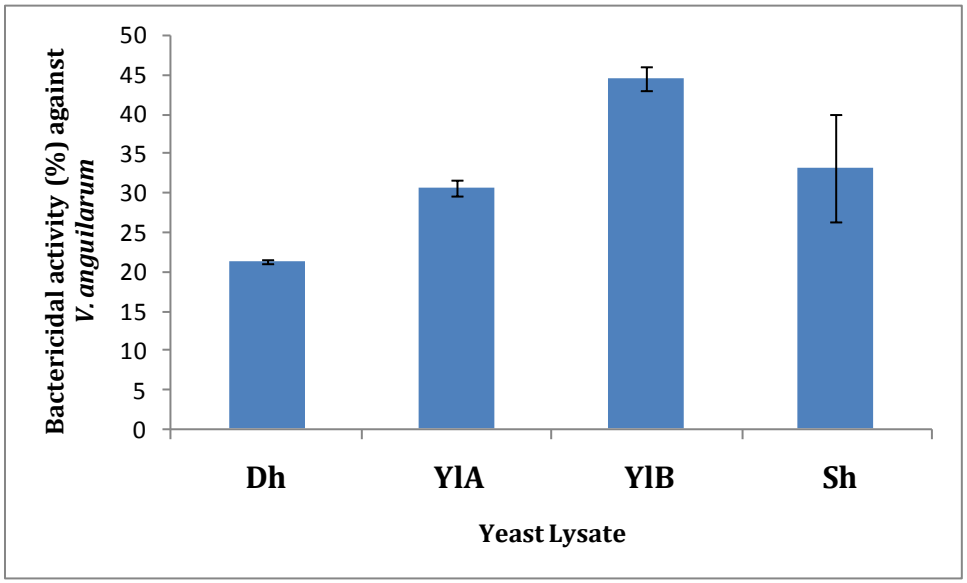

Figure 19 : Bactericidal activity (%) against *V. anguilarum* in yeast lysate. Results are shown as mean ± standard error. There were not detected significant differences among yeast for this activity. Differences were considered significant when $p < 0.05$. *D. hansenii* strain *004* (Dh), *Y. lipolytica* strain *N6* (YlA) and *Y. lipolytica* strain *D-1* (YlB) and *S. halophilus* strain *N16* (Sh).

5.2 *Yeast cells*

5.2.1 *Lysozyme assay*

In the present work, lysozyme activity was significantly higher in *D. hansenii* compared to the other yeasts. Different letters indicate significant differences for each type of yeast.

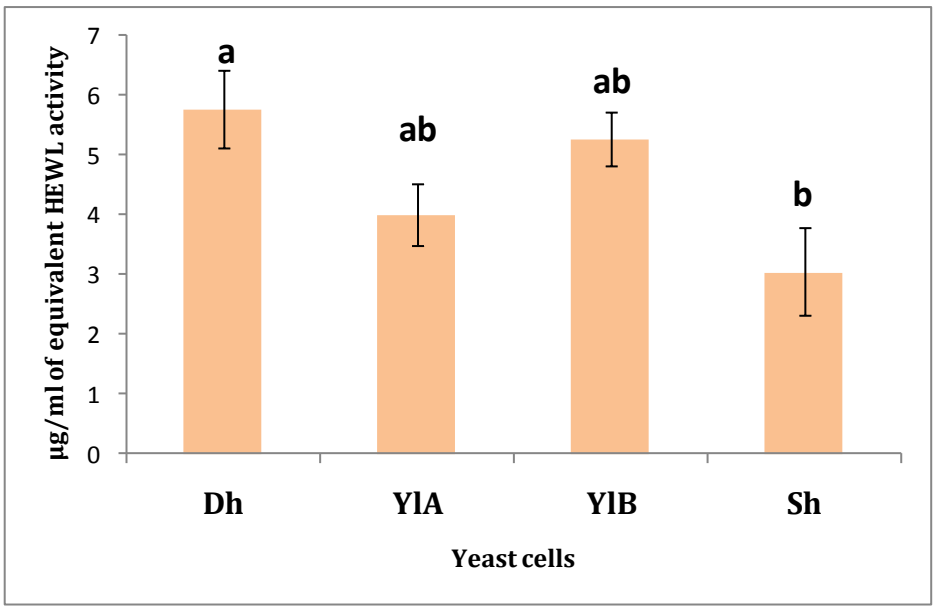

Figure 20 : Lysozyme activity (µg mL^{-1} of equivalent hen egg white lysozyme activity) in yeast cells. Results are shown as mean ± standard error. Different letters indicate significant differences for each type of yeast. Differences were considered significant when $p < 0.05$. *D. hansenii* strain *004* (Dh), *Y. lipolytica* strain *N6* (YlA) and *Y. lipolytica* strain *D-1* (YlB) and *S. halophilus* strain *N16* (Sh).

5.2.2 *Protease assay*

In the present work, protease in yeast cells was significantly higher in *Y. lypolitica* compared to *D. hanseii* and *S. halophilus*.

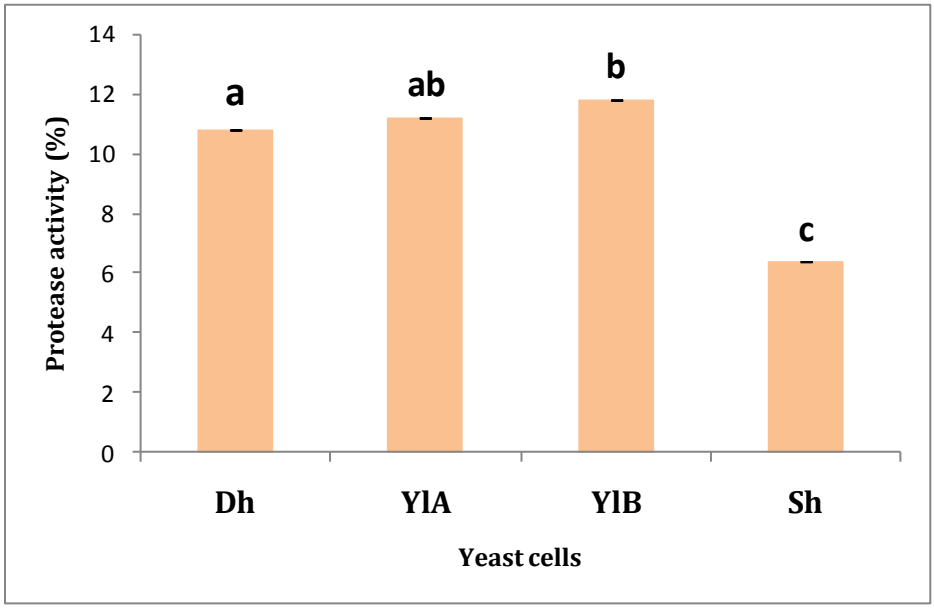

Figure 21 : Protease activity (%) in yeast cells. Results are shown as mean ± standard error. Different letters indicate significant differences for each type of yeast. Differences were considered significant when $p < 0.05$. *D. hansenii* strain *004* (Dh), *Y. lipolytica* strain *N6* (YlA) and *Y. lipolytica* strain *D-1* (YlB) and *S. halophilus* strain *N16* (Sh).

5.2.3 *Esterase assay*

In the present work, *S. halophilus* was the one that presented the most value for this activity.

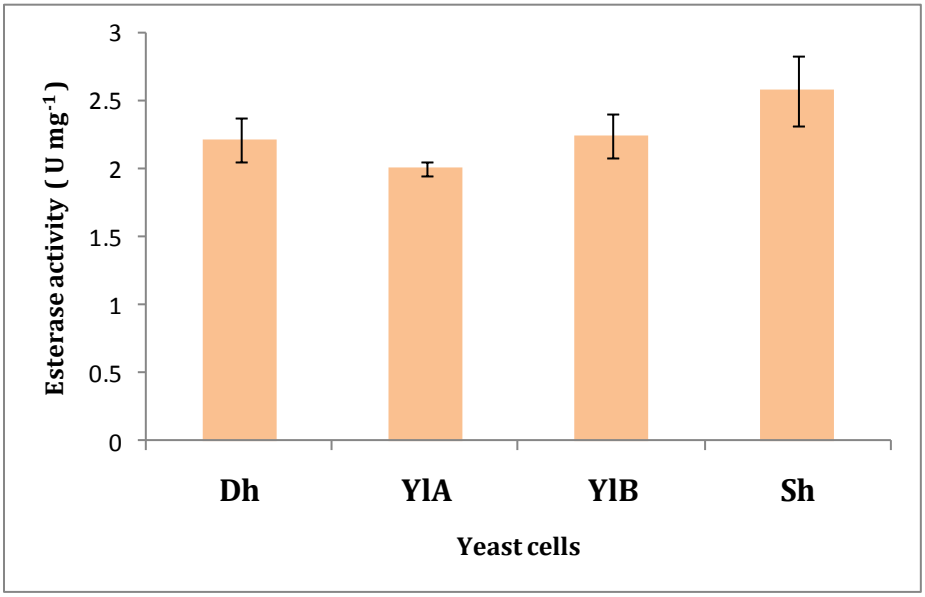

Figure 22 : Esterase activity (U mg^{-1}) in yeast cells. Results are shown as mean ± standard error. There were not detected significant differences among yeast for this activity. Differences were considered significant when $p < 0.05$. *D. hansenii* strain *004* (Dh), *Y. lipolytica* strain *N6* (YlA) and *Y. lipolytica* strain *D-1* (YlB) and *S. halophilus* strain *N16* (Sh).

5.2.4 *Peroxidase assay*

In the present work, *Y. lipolytica* shows tendency to be the yeast with the highest values of this activity of the 4 studied yeasts.

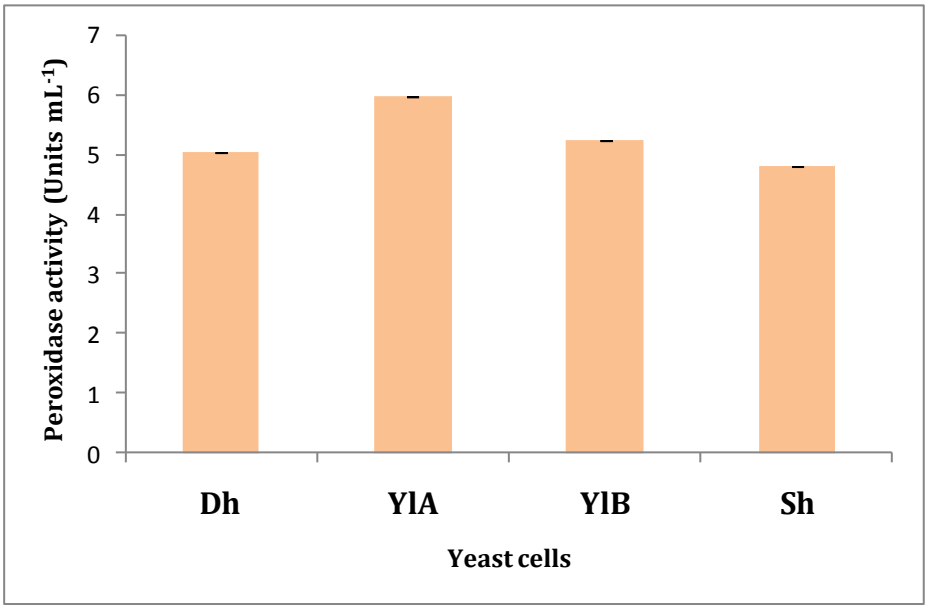

Figure 23 : Peroxidase activity (units mL^{-1}) in yeast cells. Results are shown as mean ± standard error. There were not detected significant differences among yeast for this activity. Differences were considered significant when $p < 0.05$. *D. hansenii* strain *004* (Dh), *Y. lipolytica* strain *N6* (YlA) and *Y. lipolytica* strain *D-1* (YlB) and *S. halophilus* strain *N16* (Sh).

5.2.5 *Bactericidal activity*

In the bactericidal activity against *V. harveyi, S. halophilus* was the one that presented the most value for this activity.

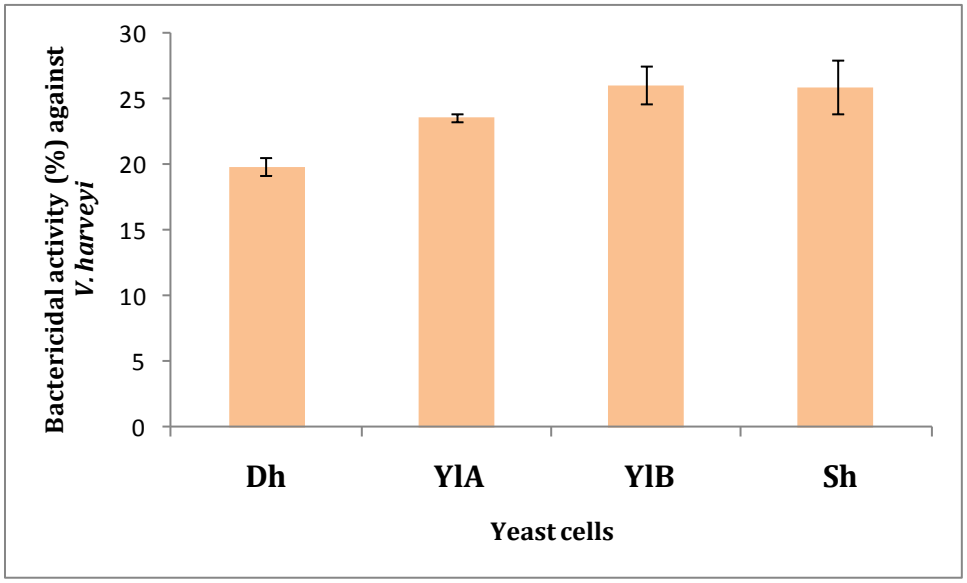

Figure 24 : Bactericidal activity (%) against *V. harveyi* in yeast cells. Results are shown as mean ± standard error. There were not detected significant differences among yeast for this activity. Differences were considered significant when $p < 0.05$. *D. hansenii* strain *004* (Dh), *Y. lipolytica* strain *N6* (YlA) and *Y. lipolytica* strain *D-1* (YlB) and *S. halophilus* strain *N16* (Sh).

In the bactericidal activity against *V. anguilarum* in yeast cells, *D. hansenii* was the one that presented the most value for this activity, meanwhile *Y. lipolytica* strain *N6* presented the lowest.

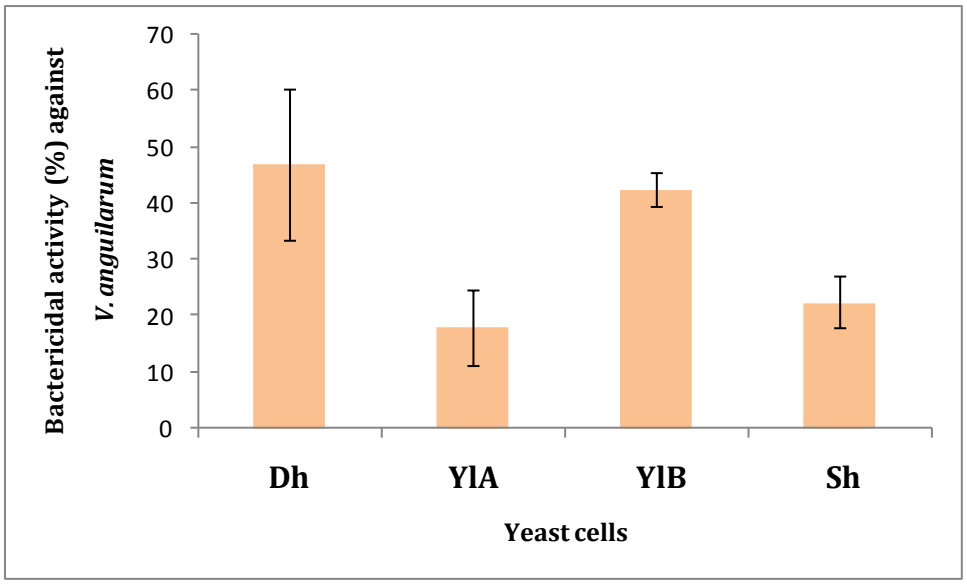

Figure 25 : Bactericidal activity (%) against *V. anguilarum* in yeast cells. Results are shown as mean ± standard error. There were not detected significant differences among yeast for this activity. Differences were considered significant when $p < 0.05$. *D. hansenii* strain *004* (Dh), *Y. lipolytica* strain *N6* (YlA) and *Y. lipolytica* strain *D-1* (YlB) and *S. halophilus* strain *N16* (Sh).

We also performed other assays such as antiprotease activity, phosphatase activity and hydrophilic activity (AAH) that we did not detect activity in the results. That probably means that those strains do not have these activities or that the activity is very little and we cannot detect it with these techniques.

5.3 *pH Resistance*

The yeasts were incubated in YPD medium with different pH(6.5, 5.5, 4.5, 3.5 and 2.5) at 30°C and growth was recorded at 24, 48 and 72 hours. After 3 days in different pH, colonies were grown in all plates but in the plate with *S. halophilus* there were just two colonies.

5.4 *Resistance to bile*

The bacteria were examined for their ability to grow in the presence of gilthead sea bream bile extract. YPD medium was prepared with bile of gilthead sea bream. After 3 days colonies had been grown in all plates but in the plate with *S. halophilus* we did not see any growth.

5.5 *Phagocytic activity*

Before they were incubated with the yeast cells, the gilthead seabream head kidney leucocytes showed no fluorescence. The cells that contained phagocytized yeast cells after challenge with FITC-labeled *Y. lipolytica* strain *N6* and strain *D-1*, *S. halophilus* strain *N16* and *D. hansenii* strain *004* ingested fluorescent microorganisms and then showed green (FITC) fluorescence. Standard samples of FITC-labelled *S. cerevisiae* or leucocytes were included in each phagocytosis assay. All samples were analysed in a flow cytometer set to analyse the phagocytic cells.
Following phagocytosis, the free yeast cells, nonphagocytic leucocytes, and phagocytes were discriminated by the combined measurement of their green (FITC) fluorescence and size (FSC) in dot plots and histograms.
Phagocytic ability was defined as the percentage of cells with ingested yeast cells (green-FITC fluorescent cells, $FL1^{+}$) within the phagocyte cell population. (Fig. 26) The relative number of ingested yeasts per cell (phagocytic capacity) was assessed

in arbitrary units from the mean fluorescence intensity of the phagocytic cells. (Fig. 27)

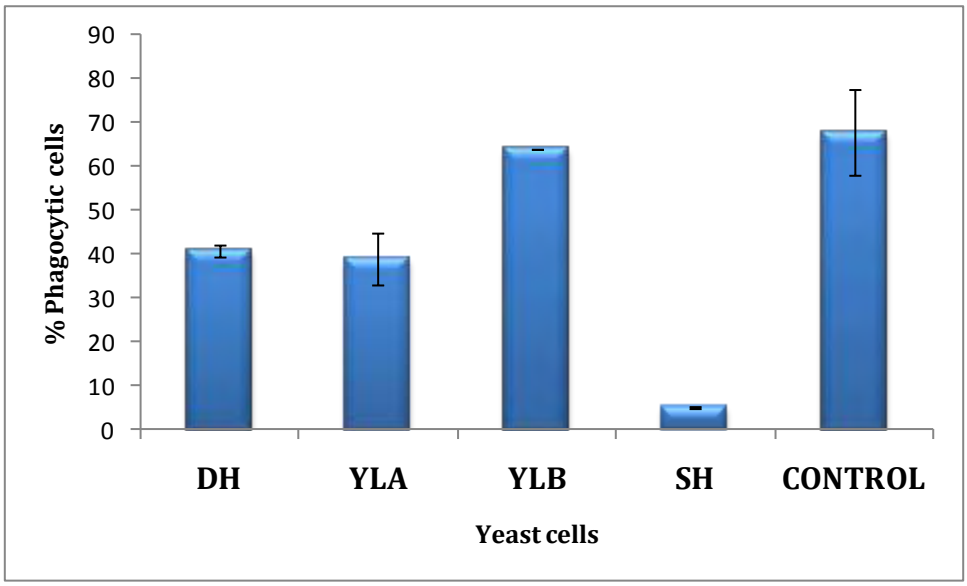

Figure 26 : Phagocytic activity (percentage of cells with ingested yeast cells (green-FITC fluorescent cells, $FL1^{+}$) within the phagocyte cell population) of head-kidney leucocytes from gilthead seabream. were *D. hansenii* strain *004* (Dh), *Y. lipolytica* strain *N6* (YlA) and *Y. lipolytica* strain *D-1* (YlB) and *S. halophilus* strain *N16* (Sh).

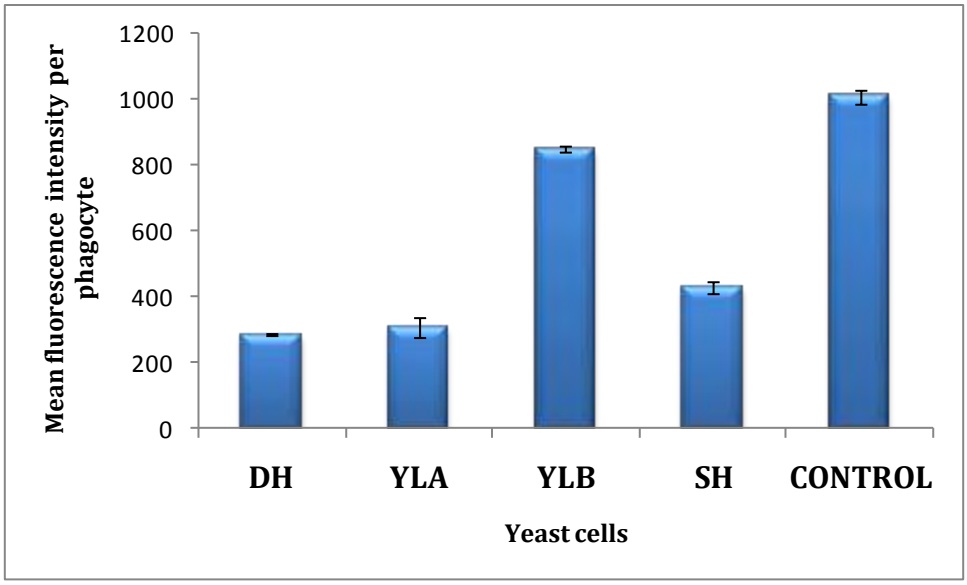

Figure 27 : Phagocytic capacity (mean fluorescence intensity per phagocyte) of head-kidney leucocytes gilthead seabream. *D. hansenii* strain *004* (Dh), *Y. lipolytica* strain *N6* (YlA) and *Y. lipolytica* strain *D-1* (YlB) and *S. halophilus* strain *N16* (Sh).

6. Discussion & future prospective

Marine yeasts live in harsh environments, which provide the potential for several unique desirable properties to be used in various industries. The latest development in the methodology of marine yeast isolation and cultivation offers the opportunity of discovering novel marine yeasts. Various media have been proposed by different research groups in order to suit for the different requirement of marine yeasts. These media are rich in nutrients and it is common to contain antibiotics to reduce the bacterial and mould contamination. Using marine yeasts in bioethanol production shows distinctive advantage on the osmosis tolerance, the possibility of utilisation of seawater instead of fresh water and the potential of advantage in using marine biomass as a substrate. Marine yeasts have already been investigated for the production of pharmaceutical and enzymatic products, such as astaxanthin, siderophore, riboflavin, inulinase and amylases. Yet, the commercial application of marine yeasts is still limited.[3]

The aim of this study was to assess the potential immunological properties of three different extremophile yeasts with future prospective recommending their use as probiotics in aquaculture feeds.

These yeasts live in very harsh environments and may represent a promising source of new bioactive compounds, some of which could perhaps be used as food supplements in aquaculture, one of the most rapidly expanding food production industries in the world.[31]

Yeasts have been used as natural immunostimulants due to their ability to increase growth, improve the digestive function, and stimulate disease resistance by modulating the immune system of fish. The nutritional status of fish is considered one of the important factors for determining the ability of fish to resist disease. The beneficial effects of these probiotics may be attributed to the

improved digestive activity of the host by enhancing the synthesis of vitamins and enzymatic activities, leading an improvement in digestibility, weight gain and immune status of fish, although more studies are necessary to confirm this. Immunological tests (hematological and biochemical) have been adopted in aquaculture as an important tool to assess the health status of fish.[31]

There are many reports that some bacterial compounds act as an immunostimulant in fish and shrimp. Generally, immunity may be improved by the probiotic in three ways:

(1) Increasing macrophage activity, shown by the enhanced ability to phagocytose microorganisms or carbon particles;

(2) Increasing the production of systematic antibodies, usually of immunoglobulin and interferon (a nonspecific antiviral agent);

(3) Increasing local antibodies at mucus surfaces such as the gut wall.[54]

The principal characteristic of a potential probiotic should be its ability to be phagocyted by the fish macrophages. In this study, the phagocytic activity of gilthead seabream (*Sparus aurata*) head kidney leucocytes was studied by flow cytometry using these yeasts as test particles. All the studied yeasts were phagocyted by the seabream leucocytes. The highest and lowest phagocytic activity was obtained when using YlB (64% of leucocytes) and Sh (5% of leucocytes), respectively. The phagocytic capacity was also the highest in YLB strain, while Dh showed the lowest value.

Another characteristic of a fish probiotic is the presence of protective proteins in fish blood, which can be evaluated by serum bactericidal activity. The bactericidal activity is a very important tool to analyze the innate immune system. We studied the *in vitro* bactericidal activity of the yeasts against two fish pathogenic bacteria: *V. anguillarum* and *V. harveyi*. The four yeast strains showed bactericidal activity against the two assayed bacteria, especially against *V. anguillarum*, reaching a 44% of bactericidal activity by the YlB strain. In contrast, YlB strain showed the lowest bactericidal activity against *V. harveyi* (9%), being Dh strain the one which showed the highest bactericidal activity (34%). Nevertheless, all the studied

enzymes were presented in all yeasts in higher or lower concentration. As much as the pH resistance, these yeasts live in extreme environments. Therefore, they can survive under harsh circumstances such as very low or very high pH. The results showed full-grown colonies in all yeasts but in *S. halophilus,* were detected two small colonies. Being able to grow in very low pH means that they could probably survive in stomach's pH. We also examined their ability to grow in the presence of bile extract. Colonies were detected in all plates, except from *S. halophilus,* which did not show any growth. The yeasts are able to grow along bile, results that show that they could probably survive in the intestine.

In conclusion, present results indicate that our results provide new evidence that live yeasts *D. hansenii* strain *004* (Dh), *Y. lipolytica* strain *N6* (YlA) and strain *D-1* (YlB) and *S. halophilus* strain *N16* (Sh) may be considered an interesting probiotic or immunostimulant in fish aquaculture. Not only they do stimulate the seabream innate immune system, but also show bactericidal properties against two pathogenic bacteria. However, further extensive testing is recommended, including oral delivery in fish such as gilthead seabream or in fish farming, to evaluate the actual role played by the probiotic microorganisms on the host under changing environmental conditions and stressful situations. Although, the yeasts have little or no activity, in the studied activities, their ability to be phagocytosed, especially YlB, as well as their bactericidal activity, are sufficient as to make these yeasts interesting candidates for inclusion as probiotics in the feed farmed fish. Though, we would still have to do more studies to complete the information shown here, such as feeding fish with these yeasts at different concentrations and different time and check if the immunological activities of the serum, as well as the phagocytosis of the leucocytes, vary with respect to fish fed with a commercial control feed.

This approach may well provide a safe novel treatment for use as a feed additive for fish farming.

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