UNIVERSIDADE FEDERAL DO RIO GRANDE - FURG

INSTITUTO DE OCEANOGRAFIA

PROGRAMA DE PÓS-GRADUAÇÃO EM AQUICULTURA

Utilização de levedura hidrolisada em dietas paralarvas e juvenis de peixe-rei *Odontesthes argentinensis*

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RIO GRANDE, RS

Fevereiro 2016

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Tese apresentada como parte dos requisitos para a obtenção do grau de doutora em Aquicultura no Programa de Pós-Graduação em Aquicultura da Universidade Federal do Rio Grande - FURG.

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Rio Grande - RS - Brasil
Fevereiro 2016

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“O saber a gente aprende com os mestres e os livros. A sabedoria aprende-se é com a vida e com os humildes.”

Cora Coralina

AGRADECIMENTOS

Ao meu orientador Luis Romano, pelos ensinamentos, paciência e acima de qualquer coisa, tua amizade.

Ao meu co-orientador, Marcelo Tesser, por sempre ser muito disponível e presente, sou muito grata por toda ajuda e todos os ensinamentos...

Ao Ricardo Rodrigues, foste um anjo da guarda durante meu doutorado, me dando as dicas, me ajudando pra caramba durante e após experimentos, tenho muita admiração pelo profissional que és, comprometido com a ciência e com o trabalho, sempre disposto a ajudar à todos.

Aos técnicos Sandro e Diogão (agora professor) pela ajuda durante os experimentos, ao professor João Sarkis que gentilmente liofilizou as amostras, ao motorista Anderson, pelos diversos "leva e trás", aos funcionários da EMA em geral, que sempre com muita presteza, zelam pelo nosso bem estar...

Ao pessoal do Lapem, por ter ajudado muito durante os experimentos, especialmente à Jana, Paola, Oka, Léo e Mario, obrigada mesmo! Ao "povo" da EMA em geral, pela parceria no dia a dia...

Aos velhos amigos do LABIPOA, pela amizade e ajudas: Prof. Joaber, Prof. Rogério, ao Francis, Ana, Amanda, Renatinho.

Àsamigas Marta e Virgínia, pela amizade, pela ajuda imensa que sempre me deram, pelos papos, por me aturar nos dias bons e nos outros, nem tanto... Obrigada pela amizade, acima de tudo!

Aos meus amigos, de perto, de longe, "biológicos" ou não, que apesar da ausência ou distância, sempre estiveram por perto quando precisei!

Ao meu namorado Adriel, obrigada pela ajuda psicológica, racional e até a braçal, durante os finais de semana de experimento. Te agradeço muito pela paciência e amor incondicionais, por se fazer presente (ainda que de longe) em todos momentos.

Aos meus pais Inês e Adelar e irmão Oscar, que sempre foram meu alicerce mais forte, a voz do outro lado da linha me dizendo palavras de apoio e conforto.

À CAPES e ao CNPq pela bolsa e auxílio financeiro concedidos.

A todos que estiveram comigo, me ajudando de qualquer forma durante esse tempo de doutorado, afinal de contas, "ciência não se faz sozinho"

RESUMO GERAL

A levedura é um aditivo nutricional rico em ácidos nucléicos, vitaminas e fatores imunoestimulantes, e a possibilidade de utilização de subprodutos de outras empresas fazem com que seja um produto de valor relativamente baixo. O presente trabalho buscou fazer inicialmente uma compilação de informações a respeito da aplicação de leveduras e seus derivadoscom importância na aquicultura, seus mecanismos de ação e efeitos nas mais diversas espécies aquícolas. Apesar da levedura ser utilizada inteira, ativa (como probiótico) ou inativa, a remoção da parede celular parece ser uma alternativa para o aumento da disponibilidade dos nutrientes intracelulares. De acordo com os fatores que são utilizados para o desencadeamento do processo de lise, podemos diferenciar pelo menos dois produtos: a levedura autolisada e hidrolisada. A levedura ainda pode ser utilizada como veículo para suplementação de nutrientes de interesse, como por exemplo, o selênio, na levedura selenizada. Dentre as principais espécies utilizadas, *Saccharomyces cerevisiae* e *Candida utilis* recebem destaque por serem eficientes imunoestimulantes e também fonte alternativa de proteínas. A levedura hidrolisada foi o produto escolhido para testes em larvas e juvenis de *Odontesthes argentinensis*. Nos dois experimentos realizados foram utilizados dois produtos comerciais, aqui denominados Y1 (constituído de *S. cerevisiae* e *C. utilis*) e Y2 (constituído apenas de *S. cerevisiae*). Náuplios de *Artemia* foram enriquecidos com 1g L^{-1} de cada um dos produtos e oferecidos às larvas durante 20 dias (0 – 20 dias após eclosão). Verificou-se que as larvas que receberam levedura hidrolisada obtiveram maiores taxas de crescimento, mesmo após 10 dias da cessação da suplementação. Ao fim dos 20 dias, com teste de estresse salino verificou-se maior resistência em larvas que foram alimentadas com *Artemia* enriquecida com Y1. Adicionalmente, a levedura hidrolisada teve importante função imune, com aumento da área de órgãos linfóides (timo e rim) e da população linfocitária (T e T helper). Em um segundo experimento, juvenis de peixe-rei receberam a dieta suplementada com 0,5% de levedura hidrolisada (Y1 e Y2) por 50 dias. A concentração utilizada dos respectivos produtos por este período não teve influencia nos parâmetros de crescimento e imunes, tampouco foi efetiva na melhora da morfologia intestinal. A levedura e seus derivados têm um grande potencial para utilização na aquicultura, seja pelos produtos já existentes no mercado,

ou pelo desenvolvimento de novos produtos. A levedura hidrolisada foi efetiva na melhora de parâmetros de crescimento e imunes, além de promover maior resistência ao estresse salino em larvas de peixe-rei, o mesmo não pode ser dito em relação aos juvenis, onde a inclusão de 0,5% de levedura hidrolisada por 50 dias não promoveu crescimento ou resposta imune.

ABSTRACT

Yeast is a nutritional additive rich in nucleic acids, vitamins and immunostimulatory factors, and the possibility of using byproducts from other industries make it a product of relatively low value. The present study aimed initially do a search for information about yeast derived products with importance in aquaculture, their mechanisms of action and effects in various aquaculture species. Although yeast is used whole, active (as probiotics) or inactive, removal of the cell wall appears to be an alternative to increasing the intracellular availability of nutrients. According to the factors that are used to trigger the lysis process, we can distinguish at least two products: yeast autolyzed and hydrolyzed. The yeast may also be used as a vehicle for supplementation of food nutrients, such as selenium in selenized yeast, for example. Among the main species *Saccharomyces cerevisiae* and *Candida utilis* receive highlighted by efficient immunomodulators as well as alternative protein source as well. The hydrolyzed yeast was the product chosen for testing in larvae and juveniles of *Odontesthes argentinensis*. In both experiments two commercial products were used here named Y1 (consisting of *S. cerevisiae* and *C. utilis*) and Y2 (consisting of *S. cerevisiae*). *Artemia* were enriched with 1g L^{-1} of each of the offered products and the larvae for 20 days (0 - 20 days after hatching). It was observed that the larvae that received hydrolyzed yeast had higher growth rates, even after 10 days of cessation of supplementation. At the end of 20 days, with salt stress test, it was seen there was more resistance in larvae that were fed *Artemia* enriched with Y1. Additionally, hydrolyzed yeast plays an important immune function, increasing the area of lymphoid organs (thymus and kidney) and lymphocyte population (T and T helper). In a second experiment, pejerrey juveniles had the diet supplemented with 0.5% hydrolyzed yeast (Y1 and Y2) for 50 days. The concentration used for the product by this time had no effect on the parameters of growth and immune, nor was it effective in improving intestinal morphology. Yeast and derivatives have a great potential for use in aquaculture, either by products already on the market, or the development of new products. The hydrolyzed yeast was effective in improving growth and immune parameters, in addition to promoting greater resistance to salt stress in pejerrey larvae, the same can not be said with regard to juveniles, where the inclusion of 0.5% hydrolyzed yeast for 50 days did not promote growth or immune response.

INTRODUÇÃO GERAL

Peixe-rei

O gênero *Odontesthes*(Teleostei: Atherinopsidae)inclui espécies dulce-aquícolas, estuarinas e marinhas. *Odontesthes argentinensis* (Valenciennes, 1835) está presente em estuários ou regiões marinhas costeiras (Bemvenutti, 1993) edistribui-se no Atlântico sudoeste, desde Santa Catarina, até a Bahia Blanca, na Argentina (figura 1) (Fischer et al., 2004).

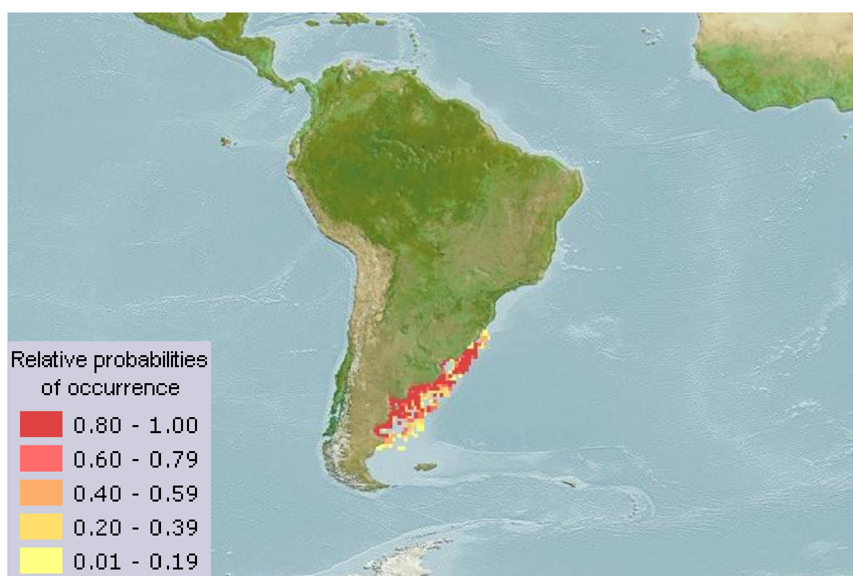

Figura 1. Distribuição geográfica e probabilidade relativa de ocorrência de *Odontesthes argentinensis* (Fish Base).

O período reprodutivo de *O. argentinensis* se estende desde o final do inverno até o início do verão.Seus ovos (2mm de diâmetro) são bentônicos e possuem filamentos coriônicos envolventes que se fixam a substratos disponíveis no fundo do mar. Quando ocorrem tempestades ou ressacas, estes substratos são jogados à praia junto com os ovos. Os embriões conseguem se manter vivos após alguns dias de exposição ao ar (Sampaio e Piedras, 2010).

As larvas de *O. argentinesis* já eclodem com olhos pigmentados e boca funcional (Valadares Costa et al., 2009). Isto significa que já podem se alimentar desde a eclosão com náuplios de *Artemia*, uma vez que seu tamanho avantajado (entre 6 e 7mm), se comparado a outras espécies, dispensa a alimentação com rotíferos

Brachionus plicatilis (120 - 300 μm)(Sampaio e Piedras, 2010), comumente usada com a primeira alimentação em larvas de peixes marinhos.

No ambiente, as larvas apresentam alimentação planctófaga e à medida que crescem, os juvenis passam a se alimentar de animais bentônicos, preservando essa característica até a fase adulta (Bemvenuti, 1990). Em laboratório, Sampaio et al. (1995) estudaram os efeitos da dieta seca, constatando que larvas de peixe-rei se alimentam de ração desde o primeiro dia após a eclosão (dae), porém observaram uma redução do crescimento se comparado a larvas alimentadas exclusivamente com alimento vivo.

Estudos relatam a potencialidade dessa espécie para a aquicultura, devido a características eurialinas, apresentando taxas de sobrevivência e crescimento semelhantes em juvenis criadosem salinidade entre 2 e 35‰ (Sampaio et al., 2000).Existe hoje um considerável número de trabalhos importantes para o desenvolvimento do pacote tecnológico de produção do peixe-rei (Tesser e Sampaio, 2001; Sampaio, 2006; Sampaio et al., 2006; Sampaio et al., 2007; Freitas et al., 2009; Rodrigues et al., 2009). Além disso, o mesmo tem sido utilizado como modelo em estudos toxicológicos (Rodrigues et al., 2010; Gusmão et al., 2012a).

Sistema imune de peixes ósseos

A larvicultura representa um dos gargalos da aquicultura, devido à grande mortalidade (Arg e Sorgeloos, 2005). Estudos demonstram que a maioria dos orgãos linfóides não está totalmente desenvolvida durante a larvicultura (Ellis, 1988), sendo o sistema imune não específico sua maior defesa contra patógenos (ZhouJin, 2003).

O sistema imune é um conjunto de moléculas, células, tecidos e órgãos que têm como função reconhecer o próprio e rejeitar o não próprio (Abbas etal., 2014).O sistema imune dos peixes ósseos é dividido em sistema inespecífico (ou inato) e específico (ou adaptativo), apesar de ambas as defesas trabalharem de maneira colaborativa (Biller-Takahashi e Urbinati, 2014).

Peixes têm como primeira linha de defesa barreiras epiteliais como pele, membranas mucosas (trato gastro intestinal, produção de muco) e barreiras fisiológicas como o pH do estômago, microflora intestinal e mediadores químicos secretados pelo

muco. O muco contem lectinas, lisozima, proteínas do complemento, peptídeos antibacterianos e imunoglobulina M - IgM (Alexander e Ingram, 1992; Nielsen e Esteve-Gassent, 2006).

A lisozima é uma enzima bacteriolítica amplamente distribuída pelo corpo e faz parte da defesa inata da maioria dos organismos. Em salmonídeos, a lisozima foi detectada no soro, secreções, membranas mucosas e tecidos ricos em leucócitos, principalmente rim e intestino (Lie et al., 1989; Saurabh e Sahoo, 2008). A ação bactericida envolve a hidrolização dos peptidoglicanos presentes na parede celular bacteriana, resultando em lise celular (Uribe et al., 2011). Os peptídeos antibacterianos são encontrados, no muco, e também no fígado e no tecido branquial (Birkemo et al., 2003) e têm a habilidade de quebrar as paredes celulares bacterianas (Ellis, 2001). O sistema complemento de teleósteos, assim como dos demais vertebrados, pode ser ativado de três maneiras: via clássica, que é acionado pela ligação do anticorpo à superfície celular; via alternativa,que independe de anticorpos e é acionado diretamente por microorganismos estranhos e; a via da lectina, que é ativada pela ligação de um complexo de proteínas com as células bacterianas(Boshra et al., 2006).

Se o patógeno for capaz de ultrapassar a primeira barreira, ocorre a ação da segunda linha de defesa, representada pelos fagócitos, células natural-citotóxicas (NCC) e resposta inflamatória. As células natural-citotóxicas são linfócitos pequenos e agranulares, comumente encontrados nos tecidos linfóides, como rim cefálico e baço (Shen et al., 2002). Os autores da resposta inflamatória são os interferons (IFN),cuja função é impedir a replicação de ácidos nucléicos de células infectadas por vírus (Robertsen, 2006), interleucinas (ILs),quimiocinas, fatores de necrose tumoral (TNF-α), que causa a ativação dos macrófagos, levando ao aumento do "burst" respiratório, da fagocitose e da produção de ácido nítrico (Yin et al., 1997; Mulero e Meseguer, 1998;).

A ativação do sistema imune inato depende do reconhecimento de estruturas expressas apenas por patógenos, denominadas PAMPs (Pathogens Associated Molecular Patterns).Estas estruturas podem ser lipopolissacarídeos, peptideoglicanos, DNA bacteriano ou RNA/DNA viral, ou outras moléculas encontradas na membrana de organismos multicelulares e que são reconhecidas como não-próprias (Uribe et al., 2011). Os PAMPs são reconhecidos pelos receptores Toll-like (TLR), que constituem a

família de receptores transmembranares que estão relacionados tanto a resposta inata quanto a específica (Takano et al., 2011).

A fagocitose é um importante fator na imunidade inespecífica.As principais células envolvidas são os neutrófilos e macrófagos. Estas células removem bactérias principalmente pela produção de espécies reativas de oxigênio durante o "burst" respiratório, e possuem lisozimas e outras enzimas hidrolíticas no interior de seus lisossomos (Secombes e Fletcher, 1992).Além disso, a fagocitose é um mecanismo fundamental para a apresentação de antígenos ao sistema imune específico, fornecendo desta forma uma ponte entre os dois sistemas (Romano, 2010). As células apresentadoras de antígenos (APC – Antigen Presenting Cells) correspondem aos macrófagos, células dentríticas e linfócitos B.

Se as barreiras do sistema imune inespecífico não forem suficientes para neutralizar o antígeno, a terceira linha de defesa será ativada–a resposta imune específica, com a proliferação de linfócitos, que reconhecem especificamente e respondem aos antígenos. Entre os tipos de linfócitos, os linfócitos B responsáveis pela resposta humoral, que em peixes são produzidos no rim cefálico, são os únicos com capacidade para produzir anticorpos (Abbas et al., 1991). A segunda classe de linfócitos consiste nos linfócitos T, responsáveis pela resposta celular, produzidos no rim,são diferenciados e maturados no timo. Os linfócitos T são subdivididos em subpopulações distintas, a citar: linfócitos T auxiliares (ou Helper) e linfócitos T citotóxicos.

Os linfócitos T reconhecem apenas peptídeos antigênicos que estão acoplados a proteínas codificadas pelo complexo de histocompatibilidade (MHC), expresso na membrana das células apresentadoras de antígenos (APC). Como resposta, os linfócitos T respondem a antígenos ligados a superfície celular e não a antígenos solúveis. Após este reconhecimento, os linfócitos T Helper secretam citoquinas, que estimulam a proliferação e diferenciação de linfócitos T, bem como de outras células, tais como linfócitos B, macrófagos e células responsáveis por desencadear resposta inflamatória. Já os linfócitos T citotóxicos têm como função a destruição de células produtoras de antígenos(Abbas et al., 1991).Um dos mais importantes recursos para a identificação destes dois tipos de linfócitos foi a descoberta de proteínas de membrana "CD – cluster of differentiation" diferentes em cada subpopulação. Desta forma os linfócitos T de uma

forma geral, apresentam proteínas $CD3^+$ em sua superfície, já os linfócitos T helper apresentam proteínas superficiais $CD4^+$ e os linfócitos T citotóxicos, proteínas $CD8^+$ (Abbas et al., 1991).

Peixes teleósteos têm um sistema imune parecido com o de mamíferos, distinguindo apenas no órgão hematopoiético, que é o rim cefálico, e não tendo nodos linfóides (Zapata e Amemiya, 2000).Os órgãos linfóides de peixes ósseos são: rim cefálico, timo, baço e tecido associado a mucosas (Press e Evensen, 1999).

O rim é muito importante como órgão hematopoiético e imune. A primeira porção do rim, denominada rim cefálico tem função na produção de células de defesa, diferenciação e maturação de leucócitos, monócitos, macrófagos e granulócitos (Press e Evensen, 1999). As principais células encontradas no rim cefálico são os macrófagos, que se agregam em estruturas denominadas centro de melanomacrófagos (MMC), e células linfóides, que são encontradas em todos os estágios de desenvolvimento e são encontradas principalmente como células B (Press et al., 1994).O timo é um órgão duplo localizado atrás do opérculo, na posição dorsolateral das brânquias (Press e Evensen, 1999). Éconsiderado um importante tecido de desenvolvimento e maturação de linfócitos T (Rombout et al., 2005). A involução do timo em peixes tem maior dependência hormonal e sazonal do que da idade, como é nos mamíferos (Press e Evensen, 1999).

Em relação ao baço, é composto por uma polpa vermelha que promove hematopoiese, com formação de células de defesa, e uma polpa branca, composta por centros de melanomacrófagos, que fagocita células velhas ou defeituosas. Uma vez que o sangue flui através do baço, leva antígenos que são presos a estes centros, a fim de serem processados e apresentados aos linfócitos T (Press e Evensen, 1999).

Em relação à ontogenia dos órgãos linfóides de *O. argentinensis*, foi observado que os primeiros túbulos renais apareceram aos 4 dae, e grupos de células hematopoiéticas adjacentes aos túbulos apareceram aos 7 dae. O timo foi observado no teto da cavidade branquial aos 6 dae e o baço apareceu aos 7 dae próximo ao intestino e ao pâncreas (Gusmão et al., 2012b).

Imunohistoquímica

Nos últimos anos, muitas iniciativas têm sido tomadas no que diz respeito ao controle de patologias de organismos aquáticos. Muitas das técnicas utilizadas para o diagnóstico e controle de enfermidades são adaptadas da medicina humana. A imunohistoquímica é uma técnica histológica, que tem como fim a detecção de antígenos em cortes de tecidos processados e tem enorme utilidade como método diagnóstico. A utilização desta técnica permite a visualização da reação antígeno-anticorpo em células e tecidos tanto em microscopia óptica como eletrônica (Romano, 2008).

A técnica de imunohistoquímica se baseia em aplicar sobre uma amostra de tecido, o anticorpo contra o antígeno que se deseja detectar. Posteriormente este anticorpo específico será utilizado como antígeno para um segundo anticorpo específico, que por sua vez está marcado com um sistema molecular que é detectado por técnicas de coloração, permitindo desta forma, observar a presença ou não do antígeno buscado através de microscopia (Romano, 2008).

A produção comercial de anticorpos consiste na injeção de antígenos de interesse em cobaias (cavalos, coelhos e principalmente ratos). Estas cobaias irão produzir anticorpos contra o antígeno. Os anticorpos policlonais são aqueles que possuem vários epítopos (determinante antigênico) de um mesmo antígeno. A produção de anticorpos monoclonais (MAb) foi cunhada por Köhler e Milstein (1975) e neste processo os anticorpos, produzidos pela cobaia contra o antígeno desejado, são fundidos com células de mieloma formando um hibridoma.Esses hibridomas podem ser cultivados *in vitro*, em grandes quantidades e fornecer anticorpos específicos.Os MAbs conferem maior especificidade, que se dá devido ao reconhecimento de somente um epítopo (Köhler e Milstein, 1975).

Para a realização da técnica de imunohistoquímica são necessários: um MAb específico contra o epítopo que se deseja detectar, um segundo anticorpo relacionado ao MAb, enzimas, substratos e cromógenos.A técnica de imunohistoquimica pode ser utilizada para: estudos de expressão fenotípica de proteínas normais, diagnóstico de enfermidades virais, bacterianas e neuplásicas (Romano, 2008).

Imunoestimulantes

Imunoestimulante é definido como uma substância que aumenta a resposta imune inespecífica através da interação direta com as células do sistema imune, ativando-as (Mastan, 2015). Consistem em aditivos alimentares oriundos de preparados bacterianos, polissacarídeos, extratos animais, vegetais e fúngicos, fatores nutricionais ou citoquinas. Tem como função aumentar os mecanismos de defesa inata (não específica), não havendo desenvolvimento de componentes de memória (Sakai, 1999).

Anderson (1992) propôs que a aplicação de imunoestimulantes deve ser implementada antes de eventos de estresse ou surtos de doenças, para minimizar as perdas. O uso de imunoestimulantes já foi relacionado com aumento da resposta imune em situações de estresse, tais como diminuição da qualidade da água (Yousefi et al., 2012), alterações de salinidade (Salze et al., 2008), altas densidades (Montero et al., 1999) e transferência/ transporte (Zaki, 2004; Abreu et al., 2014).

A dose efetiva e o tempo de exposição são parâmetros muito importantes e ao mesmo tempo muito variáveis entre os diferentes sistemas de criação e regime alimentar (Mastan, 2015).Manoppo et al. (2015) verificou que quando tilápias receberam altas doses de fermento (20 g de levedura kg^{-1} de ração) por período prolongado (4 semanas), o número total de leucócitos e a atividade fagocítica sofreram declínio. Sajeevan et al. (2009) efetuaram estudo para verificar os efeitos da dose e da frequência de administração de β-glucanos em camarão-branco (*Fenneropenaeus indicus*).Concluindo não só que 0,2% de inclusão de β-glucanos foi a mais efetiva entre as doses testadas, como também que camarões que receberam o imunoestimulante em intervalos de sete dias foram mais resistentes quando desafiados ao vírus da mancha branca.

Os mecanismos de ação variam de acordo com a natureza do imunoestimulante (Mastan, 2015). Apesar dos imunoestimulantes não serem propriamente antígenos para os animais, eles funcionam tais como, pois os receptores das células alvo (Toll-like - TLRs) reconhecem-os como moléculas potenciais de alto risco,desencadeando as vias de defesa (Bricknell e Dalmo, 2005).

Uma das primeiras aplicações de imunoestimulantes na aquicultura foi a utilização de β-glucanos na dieta de salmonídeos (Bricknell e Dalmo, 2005). Atualmente existe uma infinidade de produtos cujo potencial imunoestimulante tem sido estudado e aplicado na aquicultura com sucesso, dentre eles a quitina e a quitosana

(Harikrishnan et al., 2012), o levamisol (Kumari e Sahoo, 2006), vitaminas C e E (Ortuño et al., 2001), extratos vegetais (Dügenci et al., 2003; Ardó et al., 2008; Immanuel et al., 2009) e derivados de levedura (Andrews et al., 2009; Gu et al., 2011; Biswas et al., 2012; Kenari et al., 2013).

Os principais efeitos benéficos verificados após imunoestimulação foram: maior proteção contra bactérias (Welker et al., 2011; Selim e Reda, 2015), vírus (Sajeevan et al., 2009) e parasitos (Rio- Zaragoza et al., 2011), e maior eficácia de vacinas (Sudheesh et al., 2016).Em relação àresposta imune inespecífica, foram observados aumento da atividade do complemento e lisozima (Bagni et al., 2005), atividade fagocítica (Jha et al., 2007) e oaumento do burst respiratório (Pal et al., 2007) em peixes. Além disso, também foi verificado aumento no número de linfócitos e imunoglobulinas (IgM) em Kutum *Rutilus frisii*(Rufchaie e Hoseinifar, 2014), bem como aumento na expressão de genes relacionados a resposta imune em pargo europeu*Sparus aurata* (Reyes – Becerril et al., 2008).Para crustáceos foram registrados o aumento do número de hemócitos e da atividade da fenoloxidase (PO) (Sang et al., 2011; Zhang et al., 2012) e aumento da atividade citotóxica (Reyes – Becerril et al., 2008).

Levedura

Leveduras são organismos unicelulares, que pertencem ao Reino Fungi (Figura 2), sendo a grande maioria das classes Ascomycetes e Basidiomycetes. A reprodução é sexuada ou assexuada, esta última por brotamento ou cissiparidade (Kurtzman, 1994).Variam em tamanho de 5-10μm de comprimento e 5-7μm de largura, variando de acordo com o estágio do ciclo de crescimento, condições de fermentação e idade da célula (células mais velhas são maiores) (Russel, 2006).

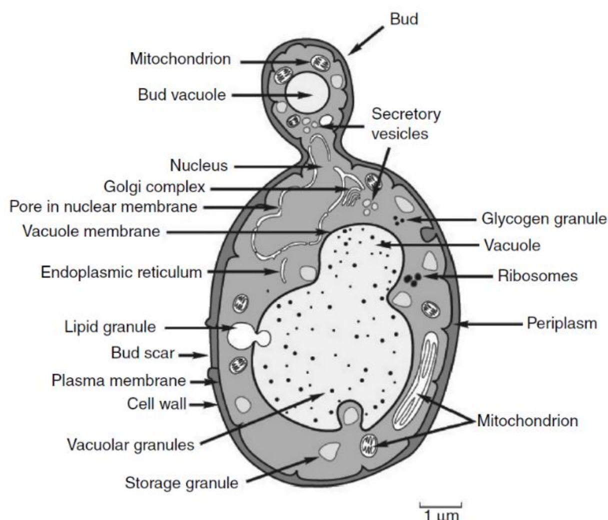

Figura 2: Principais características de uma célula de levedura típica (Russel, 2006).

As leveduras foram identificadas e isoladas por Pasteur no final do século 19 (Geison, 1974) e desde então têm sido utilizadas pelo homem na produção de bebidas alcoólicas e panificação, pela sua capacidade de, sob condições anaeróbias, produzir gás carbônico e etanol.

Saccharromyces cerevisiae é um dos principais subprodutos da indústria de bebidas (cerveja, vinho, cidra e destilados) e de panificação e tem sido amplamente utilizado como fonte de proteínas, enzimas e vitaminas. Seus derivados são utilizados como ingredientes nutritivos e funcionais, em particular o extrato de levedura tem sido largamente utilizado como complemento nutritivo e flavorizante tanto humano, quanto animal. Além de *S. cerevisiae*, *Candida utilis*, comumente conhecida por torula, também recebe destaque como fonte de nutrientes e principalmente como fonte alternativa de proteínas (Bekatorou et al., 2006; Ferreira et al., 2010).

A levedura é uma fonte relativamente barata de nitrogênio já que existe grande disponibilidade de subproduto de cervejarias.Além disso, a levedura pode ser produzida em substratos de carbono alternativos (Lee e Kim, 2001).

De acordo com NRC (2011), a composição proximal da levedura*S. cerevisiae*, é de 42,6% de proteína bruta, 1,0% de lipídios, 3,2% de fibras e 6,6% de cinzas. Em relação à composição de aminoácidos, a levedura se assemelha aofarelo de soja. Leveduras se destacam pelo alto teor de lisina, aminoácido essencial e principal constituiente do tecido muscular dos peixes (Venugopal e Shahidi, 1996), entretanto é deficiente em aminoácidos sulfurados, principalmente metionina (Huige, 2006; Olvera-Novoa et al., 2002).Além de proteínas, as leveduras também são uma excelente fonte de vitaminas do complexo B, ácidos nucléicos e minerais (Ferreira et al., 2010).

A parede celular da levedura representa 10-25% do total da massa celular e é constituída de manoproteínas (31%), glucanos (29%) e pequena quantidade de quitina (2-4%) (Figura 3) (Russel, 2006). Desde a descoberta das propriedades benéficas dos polissacarídeos da parede celular, muitos processos para o isolamento e a purificação desses têm sido desenvolvidos (Peat et al., 1961; Freimund et al., 2003) e utilizadas com êxito na aquicultura.

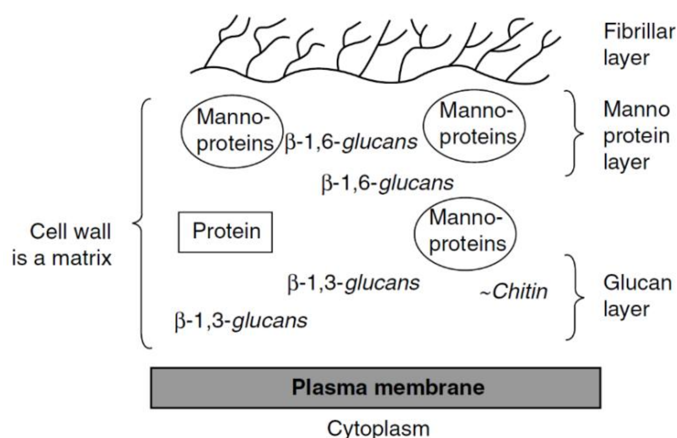

Figura 3: Estrutura simplificada da parede celular de levedura (Russel, 2006).

Os β-glucanos são polissacarídeos de cadeia longa, que podem ser encontrados na parede celular de plantas, algas, bactérias, entre outros. β-glucanos extraídos de levedura, produto conhecido como β-1,3/1,6-glucano, provavelmente é o maior potencializador do sistema imune (Ringo et al., 2012; Barman et al., 2013). Os β-glucanos apresentam PAMPs que são reconhecidos pelos receptores de superfície de células imunes, desencadeando sua resposta. Na aquicultura, a suplementação da dieta com β-glucanos, está vinculada com maior desenvolvimento de mecanismos de defesa celular e humoral, como atividade da lisozima, da fagocitose, do complemento e proliferação de linfócitos (Welker et al., 2007; Siwicki et al., 2010), com aumento de taxas de crescimento e conversão alimentar (Azari et al., 2013). Além disso, os β-glucanos são usados para melhorar a resistência de peixes e crustáceos a infecções virais e bacterianas (Couso et al., 2003; Bai et al., 2014).

Os mananoligossacarídeos (MOS) são carboidratos derivados de levedura e, apesar de não digeríveis, promovem benefícios àsaúde intestinal, aumentando a absorção e imunomodulação (Halas e Nochta, 2012). O receptor de manose, está presente em células dendríticas e macrófagos,e reconhece, além de glicoproteínas patógenas, aqueles presentes nos MOS, desencadeando a cascata do complemento e ativando a resposta imune (Andrews et al., 2009). Os efeitos de MOS sobre o desempenho zootécnico, parâmetros hematológicos e respostas imunes têm sido estudadas em várias espécies aquáticas incluindo a truta arco-íris *Oncorhynchus mykiss* (Staykov et al., 2007; Dimitroglou et al., 2009), o robalo europeu *Dicentrarchus*

labrax(Torrecillas et al., 2007), a tilápia híbrida (*Oreochromis niloticus* × *Oreochromis aureus*) (Genc et al., 2007) e obijupirá*Rachycentron canadum* (Salze et al., 2008). Além disso, MOS possui efeito prebiótico, favorecendoa microbiota benéfica e promovendo melhora na morfologia e fisiologia intestinal (Sang e Fotedar, 2010).

Além dos β-glucanos e MOS, as leveduras também são ricas em nucleotídeos, que constituem o DNA, RNA e ATP (Ringo et al., 2012). O custo energético e a incapacidade de alguns tecidos em produzir nucleotídeos via síntese*de novo*, podem ser minimizados com a suplementação externa de produtos ricos em nucleotídeos como é o caso daqueles derivados de levedura (Quan, 1992; Low et al., 2003). Diversos estudos já relataram os benefícios da suplementação da dieta de organismos aquáticos com nucleotídeos, revisado por Ringo et al. (2012).

As leveduras podem ser usadas na sua forma ativa, como probióticos, ou na sua forma inativa, como ingrediente alimentar rico em nutrientes. Existe no mercado uma série de produtos derivados de levedura inativa, e por vezes a diferenciação entre um e outro é bastante difícil. A levedura encontrada no mercado, na forma inativa, é vendida geralmente na forma seca,obtida por evaporação a vácuo, ou seca pelo método de spray-dry.

Um dos princípios da produção de todos os produtos é a quebra da parede celular.A autólise, ou autodigestão,consiste na quebra de proteínas, glicogênio, ácidos nucleicos, e outros constituintes da célula por ação de enzimas intracelulares. O processo de autólise é um fenômeno que ocorre naturalmente quando as células entram em fase de apoptose. Mas, para interesse comercial, este processo de autólise pode ser induzido, com adição de agentes químicos, físicos ou enzimáticos para aumentar o rendimento, diminuir o tempo de reação, desta forma diminuindo o risco de deterioração e contaminação (Vosti e Joslyn, 1954).

Apesar da ação de enzimas durante a autólise ou hidrólise, a parede celular não é totalmente desintegrada, os autólitos/hidrólitos são liberados através de poros na parede celular (Babayan et al., 1981; Kollár et al., 1993). Tecnicamente, o autolisado de levedura compreende o conteúdo inteiro da lise celular, incluindo ácidos nucléicos, aminoácidos, vitaminas, mineirais e componentes da parede celular (β-glucanos, MOS, e quitina) (Huige, 2006).Rumsey et al. (1991), em um estudo comparativo entre levedura íntegra, autolisada e extrato de levedura, verificaram que a ruptura da parede

celular gera um aumento do valor nutricional do produto, da disponibilidade e digestibilidade dos nutrientes.

Watanabe et al. (2010) verificaram que quando a dieta de pacu (*Piaractus mesopotamicus*) foi suplementada com levedura autolisada, a eficácia alimentar e a utilização de proteínas foi maior, sugestivamente devido a uma maior capacidade de utilização dos nutrientes do que quando os peixes recebem levedura inteira. Em um estudo com gastrópodes (*Babylonia areolata*), sugere-se que levedura autolisada é um estimulante de crescimento, além de promover maior resistência a infecções por *Vibrio alginolyticus* (Chaitanawisuti et al., 2011).

Heidarieh et al. (2013), utilizando levedura hidrolisada Hilyses® na dieta de trutas arco-íris, *O. mykiss*, verificaram maior atividade de enzimas digestivas tripsinae amilase e maior quantidade de células caliciformes no intestino. O mesmo produto foi testado também por Sheikhzadeh et al. (2012), verificando maiores taxas de crescimento e melhora na defesa imune inata.

Além das propriedades imunológicas, a levedura também pode ser usada na aquicultura como fonte alternativa de proteínas (Korkmaz e Cakirogullari, 2011; Øverland et al., 2013) ou servir de alimento para rotíferos e artêmias(Robert e Trintignac, 1997; Comabella et al., 2004; Marques et al., 2004; 2006).

Outros produtos à base de leveduras ou de seus derivados já são consolidados no mercado aquícola, como a produção de*Phaffia rhodozyma,* que com sua capacidade de acumular astaxantina (Johnson eAn, 1991) se torna um importante recurso para obtenção da coloração característica de salmonídeos e crustáceos, critério este importantíssimo para o mercado consumidor (Steine et al., 2005).Indo além dos nutrientes importantes já presentes nos produtos e derivados de levedura, o enriquecimento destas com elementos de interesse também é uma alternativa viável e que vem apresentando sucesso, como é o caso da levedura enriquecida com selênio – levedura selenizada, que atua como antioxidante (Rider et al., 2009). Sendo assim, o conhecimento a respeito dos diferentes tipos de leveduras e derivados, bem como sua aplicação na aquicultura, e mais do que isso, o conhecimento da concentração e tempo de administração, são informações importantes.

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OBJETIVOS

Objetivo Geral

O objetivo geral deste estudo foi elucidar os produtos e derivados de levedura com aplicação na aquicultura e verificação dos efeitos da administração de levedura hidrolisada na dietade larvas e juvenis do peixe-rei*O. argentinensis*.

Objetivos Específicos

1. Reunir e elucidar informações a respeito dos produtos e derivados de levedura com utilização na aquicultura, bem como sua aplicação, e estudo de efeitos em espécies de aplicação aquícola;
2. Investigar o efeito do enriquecimento de náuplios de*Artemia* com levedura hidrolisada nos parâmetros zootécnicos e imunes de larvas do peixe-rei, *O. argentinensis*;
3. Investigar o efeito da suplementação da dieta de juvenis do peixe-rei, *O. argentinensis*, com levedura hidrolisada nos parâmetros zootécnicos e imunes;

CAPÍTULO 1

Yeast: Products and derivatives with application in Aquaculture

Artigo nas normas da revista **Reviews in Aquaculture**

Yeast: Products and derivatives with application in Aquaculture

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Yeast: Products and derivatives with application in Aquaculture

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Abstract

Studies around the nutrition of aquatic organisms are increasing together with the demand for development of diets that not only provide essential nutrients but also contribute to disease avoidance and to health maintenance. Yeast is used as alternative source of high nutritional values proteins, vitamins and immunomodulator factors. Many products are being developed with yeast, as probiotic live yeast, autolyzed yeast, yeast fractions and yeast extract. Some of the most prominent yeast with importance in the aquaculture are related in this review, among them *Saccharomyces cerevisiae* (baker's, brewer's yeast), *Candida utilis* (torula) and *Phaffia rhodozyma*. This review commences with a brief synopsis about the yeast composition and its nutritional properties. This is followed by a detailed review about the current products derived from yeast with application on the aquaculture, addressing about the effects, mainly on the immune response, but also on others benefits, providing information about species with importance for aquaculture, which have been studied. In addition, listing the possible mechanisms of action based on current knowledge or hypotheses that can be considered to organize a train of thought. Some future research directions with emphasis on new approaches using enzymes isolated from yeast on aquaculture animal diets and application of genetic knowledge for production of strains that can express important nutrients are also discussed.

Keywords: Immunostimulant, Baker's yeast, Brewer's yeast, *Torula*, *Saccharomyces cerevisiae*, probiotics, single cell protein (SPC), red yeast, selenized yeast

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1. **Introduction**

The global importance of aquaculture is growing and the demand for food and additives with bigger and better quality is increasing year by year. Studies with feed additives, immunostimulants, probiotics and prebiotics in nutrition of aquatic organisms are increasing proportionally by the demand for insurance products produced according sustainable practices (Ringo *et al*. 2012; Barman *et al*. 2013). The challenge in modern nutrition is to develop diets that not only provide essential nutrients but also contribute to disease avoidance and to health maintenance. Feed must be safe and free from pathogens (Gatlin *et al*. 2007).

In order to adapt to market requirements, the recurrent disease and demand for ecofriendly disease-prevention numerous products, natural (bacterial derivatives, yeast derivatives, nutritional factors, hormones, cytokines, polysaccharides, animal and plant extracts, ...) or synthetic (levamisole, FK-565, quaternary ammonium compounds, ...) have been tested and have been proven their potential as immunostimulant (Anderson

&Jeney 1992; Sado & Bicudo 2010; Reverter *et al*. 2014). The concept proposed by Bricknell and Dalmo (2005) for immunostimulants is a naturally occurring compound that modulates the immune system by increasing the host's resistance against diseases that in most circumstances are caused by pathogens.

Yeast is a unicellular organism belonging to the Fungi Kingdom. More than a thousand species were found in the two main classes: Basidiomycota and Ascomycota, which belong species able to replicate by budding or bipartition such as *Saccharomyces cerevisiae* (Kurtzman 1994). Yeasts have been produced and marketed since the late 19th century when they were identified and isolated by Pauster (Geison 1974). Nowadays, knowledge and technology allow for the isolation and production of yeast strains with specific properties to meet the demands of the baking and fermentation industry.

S. cerevisiae, especially,in active form is used in the baking industry, fermentation, and other fermentation processes such brewing. The inactive form, have been widely used in animal feed, as a source of protein and other nutrients, and human consumption, mostly in the form of derivatives, as a nutritional supplement, flavor and flavor enhancer (Ferreira *et al*. 2010).

In aquaculture, the most prominent species are *S. cerevisiae* (baker's, brewer's yeast), *Candida utilis* (torula), *Kluyveromyces marxianus* or *Phaffia rhodozyma* (astaxanthin provider), used primarily as a protein source, but also by their nutritional and immunostimulant properties of some of its components. Its highlight is due to several factors, including the wide availability of yeast biomass from brewery industry (beer, wine, cider, spirits, etc) and excellent nutritional content (Bekatorou *et al*. 2006; Akhtar *et al*. 2008).

Yeast products are getting more and more popular in aquaculture.However, many products (whole or derivatives) (Table 1) are difficult to differentiate. The aim of the present study is to discuss the different types of products on the market today, gather information on the implementation of each of them in the different organisms with importance in aquaculture, and finally assess what still needs and can be accomplished in this field.

2. Chemical composition and nutritional properties of yeast

Yeast protein is known for its easily digestible and nutritious value and has been

widely used as animal and aquaculture feed supplement. Yeast is an inexpensive nitrogen source, generally recognized as safe (GRAS) and has good nutritional characteristics. The proximate composition, according NRC (2011) is 93% of typical dry matter, 42.6% crude protein, 1.0% crude fat, 3.2% crude fiber and 6.6% ash for *Saccharomyces* brewer's yeast. Yeast is considered a cheaper dietary supplement as they are easily produced on an industrial level from a number of carbon-rich substrate by-products (Lee & Kim 2001).

The amino acid composition of yeast is similar to that of soybean meal. Yeast stands out for its high content of theessencial aminoacidlysine, however is deficient in sulfur amino acids, especially methionine. For comparison, methionine content on dry brewer's yeast is 1.6% (Huige 2006), in torula yeast is 3.8% and in fish meal (anchovy meal) is 19.0% (Olvera-Novoa *et al*. 2002).

Chemical composition of yeast is characterized by vitamins of the B complex, exceeding the levels of the fish meal, except on the B12 vitamins, whose brewer's yeast level is 1.0 mg kg^{-1} while in anchovy fish meal is 352 mg kg^{-1} (NRC 2011). Related to the minerals, brewer's yeast stands out for its high concentration of copper (38.4 mg kg^{-1}, while the level on anchovy fish meal is 9.03 mg kg^{-1}) and low of chlorine (brewer's yeast = 0.07 mg kg^{-1}; anchovy fish meal = 1.00 mg kg^{-1}) and sodium (brewer's yeast = 0.07 mg kg^{-1}; anchovy fish meal = 1.10 mg kg^{-1}) (NRC 2011).

Yeasts contain various immunostimulating compounds such as β-glucans, nucleic acids, and oligosaccharides, and it has the capability to enhance the growth, immune response, stress and disease tolerance of various species (Jha *et al*. 2007; Welker *et al*. 2007; Salze *et al*. 2008). The acid nucleic composition (*C. utilis* 93 g kg^{-1}, *S. cerevisiae* 58 g kg^{-1}) is higher when compared with fish meal (13 g kg^{-1}) (Øverland *et al*. 2013). However, this relatively high non protein nitrogen content, in the form of nucleic acids, mostly RNA (Schulz & Oslage 1976), can lead to elevation of plasma uric acid and produce toxicological effects and disturbance in metabolism.

The nutritive value of yeast products differs according to its specie, harvest time, substrate utilized, temperature, pH and the quality and type of adjuncts added in the brewing process, in the case of brewer's yeast (Mussatto *et al*. 2006).

3. Yeast products and derivatives

3.1 Active yeast

3.1.1 Probiotics

Probiotics, by a wider definition are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002). The natural occurrence of various species of yeast in the gastrointestinal tract of healthy fish are well described and have been implicated as an important constituent of fish gut microbiota (Gatesoupe 2007; Navarrete & Tovar-Ramírez 2014).

The mode of action of yeast as probiotic still unclear, but it could be via the release of polyamine. Polyamines (putrescine, spermine and spermidine) are considered essential growth factors, because they are closely related to cell proliferation, differentiation and maturation (Jänne *et al.* 1978; Dufour *et al.* 1988) by stimulating DNA, RNA and protein synthesis (Tabor & Tabor 1984). The cells have the capacity to produce polyamines, but in some cases, the synthetic capacity of cells or organs is not sufficient to satisfy all requirements. Food from plant and animal origin are the main sources of polyamines supplementation (Eliassen *et al.* 2002; Kalac & Krausová 2005).

The settlement of microorganisms secreting polyamines in the gut constitutes a way to provide a regular supply of polyamine for the host. Yeast could be an appropriate organism because some strains synthesize and secrete different polyamines molecules, and they have strong adhesion potential to intestinal mucus in fish (Vázquez-Juárez *et al.* 1997). Moreover, yeast may improve the health by immunostimulation, whose property is given mainly by glucans, mannan oligosaccharides (MOS)and nucleotides, which are discussed throughout this article.

Tovar *et al.* (2002) revealed maturation of gut and pancreas secretory functions on sea bass larvae related with diet supplementation with *Debaryomyces hansenii* yeast. These authors also found that the main polyamines produced were spermine and spermidine, which are considered the "right polyamines" because putrescine is converted mainly to non-polyamine metabolites, mostly amino acids (Bardócz *et al.* 1995). Once in the intestine, the polyamines readily get into the enterocytes, probably by passive diffusion, and inducing a hormonal cascade that affects organs like the pancreas and liver (Peulen *et al.* 2000). Tovar *et al.* (2004) hypothesized that live yeast induced better performance than inactive yeast, because live yeast provided polyamines

in situ in the intestine.

D. hansenii is commonly found in marine fish intestines and has been isolated from the gut of rainbow trout reared in freshwater for use in many studies to test its probiotic potential. The principal characteristic of a potential probiotic should be its ability to colonize and persist in the host or in its environment (Navarrete & Tovar-Ramirez 2014). Studies done by Tovar *et al*. (2002) and also Sarlin and Philip (2011) compared the probiotic potential of *S. cerevisiae* and *D. hansenii*, found that the second owns greater probiotic potential than the first, to sea bass larvae and Indian white prawn, respectively. Nevertheless, Chiu *et al*. (2010) found that *S. cerevisiae* colonized the intestines of the grouper *Epinephelus coiodes* and induced up regulation of innate cellular and humoral immune responses, besides increasing resistance to *Streptococcus* sp. and grouper iridovirus.

Yeast probiotics are known to have positive effect on growth, survival and increased disease resistance, viral, bacterial or parasitic infections, and resistance to stress events (Lara-Flores *et al*. 2003; Reyes-Becerril *et al*. 2008a; Chiu *et al*. 2010; Sarlin & Philip 2011; Pooramini *et al*. 2014). Furthermore, they can promote the regulation of cellular and humoral innate immune system, with increase of phagocytic, lisozyme, and complement activities, and regulate immune-associated genes, especially in haemotopoietic organs (Reyes-Becerril *et al*. 2008b; Chiu *et al*. 2010). Lara-Flores *et al*. (2003) observed higher feeding efficiency, especially of protein, better conversion and higher digestibility in tilapia that received *S. cerevisiae* as probiotics. Probiotics are also related to an improvement of antioxidant system, with greater respiratory burst activity, and release of Superoxide Dismutase (SOD) and Catalase (CAT) enzymes (Reyes-Becerril *et al*. 2008b; Chiu *et al*. 2010).

The use of probiotics faces the obstacle of manufacturing process because the yeasts generally do not resists the high temperatures, steam, long periods of packaging, etc. Microencapsulation is a process by which very tiny droplets or particles of liquid or solid material are surrounded or coated with a continuous film of polymeric material. It is a technology used to stabilize drugs for the controlled delivery and release of active ingredients and is now being developed in aquaculture nutrition, in order to improve the viability of probiotics in the gastrointestinal tract environment (Bansode *et al*. 2010). There are already reports that encapsulated *S. cerevisiae* can improve intestinal structure

and growth performance in tilapia (Pinpimai *et al*. 2015).

Marine yeasts and yeasts isolated from marine fishes, as *D. hansenii*, seem to be a very logical and more efficient choice.However, there are difficulties in growing these strains in industrial scale and any kind / product has been developed yet. The only products on the market in this sense are the *S. cerevisiae*.

3.1.2 Fermented Yeast

Fermented yeast is usually mentioned in the article as a probiotic. Although not an incorrect approach, this product can be consider as symbiotic, since acting as prebiotic and probiotic, once natural fermented product consist of yeast cell walls (β-glucans and MOS) and cell soluble materials (vitamins, proteins, peptides, amino acids, nucleotides, etc), with a few living cells in the product.

Prebiotics are defined as non-digestible components that are metabolized by specific health-promoting bacteria such as *Lactobacillus* and *Bifidobacterium*. These bacteria are considered beneficial to the health and growth of the host by decreasing the presence of intestinal pathogens and/or changing the production of health related bacterial metabolites (Manning& Gibson 2004).

Fermented yeast is related to an improvement on autochthonous gut bacteria, which by definition are able to colonize epithelial surfaces of the host and be a barrier against colonization of pathogen agents. What in fact it was perceived by He *et al*. (2009), with considerable stimulus of potentially beneficial bacteria and depression of harmful species in hybrid tilapia. Authors suggest that the benefits found for yeast fermented administration may be a sum of factors, namely: production of polyamines, nutritional and immunomodulatory factors (Tabor & Tabor 1984), besides being a protein source rich in essential and nonessential amino acids (Cheng *et al*. 2004).

3.2 Inactive yeast

3.2.1 Autolyzed Yeast

Autolysis is a slow and irreversible process resulting in cellular death. In the course of autolysis, the cytoplasmic material is gradually hydrolyzed and decomposition products are released into the extracellular space (Babayan *et al*. 1981). Hydrolytic enzymes (proteinases, glucanases, nucleases) are responsible for autolytic degradation

of proteins, glycogen, nucleic acids and other cell constituent (Cornett & Shockman1978). While the autolysis occurs naturally when cells complete their growth cycle, when it is applied for industrial purposes, the process has some disadvantages such as low extraction yield, long time of reaction and risk of deterioration due to microbial contamination (Vosti & Joslyn 1954).

The autolytic process can be enhanced with physical, chemical and enzymatic inducers (Chae *et al*. 2001; Tangüler & Erten 2009). These methods allow the release of the autolytic enzymes thus accelerating the process. Induced autolysis process requires application and heat control to kill the cells carefully without inactive the yeast enzymes. This process is usually carried out under stirring and moderate changes in temperature (30-60ºC) for 12-24 h (Huige 2006).

According Babayan *et al*. (1981), the autolysis process can be divided into four stages:

• First, the cell endostructures degrade, releasing vacuolar proteases in the cytoplasm.

• Second, the released proteases are initially inhibited by specific cytoplasmic inhibitors, and are then activated due to degradation of these inhibitors.

• Third, intracellular polymer components hydrolyse, with the hydrolysis products accumulating in the space restricted by the cell wall.

• Finally, the hydrolytic products are released when their molecular masses are low enough to cross pores in the cell wall.

Although the action of endogenous enzymes during autolysis, there are no break-down of the cell wall. The autolysate is being released by pores in the cell wall and the remainder degradation occurs in the extracellular medium. In the course of autolysis cells get smaller and featuring wrinkles, folds and grooves (Babayan *et al*. 1981; Kollár *et al*. 1993). Autolysate refers to the total content of lysed cell including water-soluble components, solubilized proteins, and cell wall material.

In a comparative study, Rumsey *et al*. (1991a) seen that the disruption of the yeast cell wall (autolyzed yeast or yeast extract) increased the nutritional value of Brewer's dried yeast for rainbow trout (*Oncorhynchus mykiss*).

The inflammatory response and hematological parameters of Nile tilapia fed dry food supplemented with autolyzed *S. cerevisiae* and yeast cell wall were evaluated six and 24 h after inoculation with inactivated *Aeromonas hydrophila* into the swim

bladder. The results suggest a nonspecific increased in the acute inflammatory response, with enhancement of the immune response (greater number of thrombocytes, and lower number of neutrophils, lymphocytes and macrophages) at the focus of the lesion (Reque *et al*. 2010).

Greater feed efficiency and use of proteins was recorded by Watanabe *et al*. (2010), when the diet of Pacu (*Piaractus mesopotamicus*) was supplemented with yeast and derivatives, including autolyzed yeast. These authors suggest that the better capacity in using diet nutrients, which triggered better performance, might be explained by the fact that the diets present higher amounts of insoluble polysaccharides (glucans) and mannan proteins. Also, the microbial production of volatile fatty acids (acetate and propionate) derived from the insoluble fiber fermentation present in yeast diets, which may increase the energy availability.

Li *et al*. (2009) supplement the diet of white shrimp *Litopenaeus vannamei*, with two doses (2 and 5%) of partially autolyzed yeast (Brewtech®) and Grobiotic®-A. Noting, despite the yeast and derivative supplementation not affect growth and immune parameters, a higher cumulative survival when the shrimp were subjected to low salinity (2 ppt).

In a study with gastropods Spotted Babylon*Babylonia areolata*, the autolyzed yeast was suggested as a growth stimulant additive, probably due to increase its digestibility. It was also observed increased resistance to infection with *Vibrio alginolyticus*, suggesting improved non specific immune system (Chaitanawisuti *et al*. 2011). Similar to this study, resistance to pathogen was also seen in rainbow trout when it was challenged with Infectious Hematopoietic Necrosis Virus (IHNV) (Sealey *et al*. 2007) and on hybrid striped bass when infected with *Streptococcus iniae* (Li & Gatlin III 2004).

3.2.2 Hydrolyzed Yeast

The autolysis may be induced by the addition of chemicals or external enzymes that will hydrolyze RNA and proteins – hydrolyzed yeast (Salazar & Asenjo 2007; Oliveira & Oliva-Neto 2011). According to AAFCO (2008), hydrolyzed yeastis a concentrated, non-extracted, partially soluble, yeast digest. Hydrolysis is accomplished by acid or enzymatic methods. Acid hydrolysis is done on a yeast slurry using

hydrochloric acid at elevated temperatures. The product is neutralized by sodium or potassium hydroxide to a pH of 5 or 6, resulting in a product with high salt content. Acid hydrolysis breaks down yeast protein and carbohydrates much more completely than autolysis (Huige 2006). On the enzymatic hydrolysis,proteolytic enzymes hydrolyze proteins more gently than acids, do not require high temperature and usually target specific peptide bonds. The material that results from a proteolytic digestion is a mixture of amino acids and polypeptides of varying lengths. Enzymatic hydrolysis is carried out by proteolytic enzymes or cell wall lysis enzyme (Chae *et al*. 2001; Salazar & Asenjo 2007).

The yeast is consider as a single cell protein (SCP) because it's high protein content and short growth times, leading to rapid biomass production (Bekatorou *et al*. 2006). On the enzymatic hydrolysis, specific enzymes can be used for increase the release of constituents of interest, for example, pancreatin and promease enhance protein hydrolysis (Huige 2006). However, the utilization of yeast as protein source has one obstacle: its relatively high nucleic acid nitrogen content, mostly in the form of RNA (Schulz & Oslage 1976). High levels of dietary nucleic acids, especially purines, elevate plasma uric acid and produce toxicological effects and disturbance in metabolism (Clifford & Story 1976). Moreover, Rumsey *et al*. (1991b) reported that high concentrations of yeast (50 and 75% inclusion) in rainbow trout diet inhibited food intake. Rothenbuehler *et al*. (1982) have reported a non-protein fraction of *S. cerevisiae* that, on hydrolysis, liberated amino acids which are associated with an unpleased taste sensation in humans. In other study, Rumsey *et al*. (1992) verified that the free-based adenine is a potent inhibitor of feed intake and growth in trout.

Two commercial hydrolyzed yeast products (Hilyses®)were used in enrichment of live feed - *Artemia* during the larviculture of pejerrey *Odontesthes argentinensis*, demonstrating increase of growth, improved the immunity, with an increased on CD3 and CD4 labeled cells, and enhanced stress tolerance of larvae (Führ *et al*. 2016). The same commercial product was also studied in rainbown trout, verifying increased activity of digestive enzymes trypsin and amylase and higher density of the goblet cells in the intestine (Heidarieh *et al*. 2013). Sheikhzadeh *et al*. (2012), who also tested Hilyses®, observed better growth performance and skin non-specific immune parameters in rainbow trout.

3.2.3 Selenium Yeast

Selenium (Se) has been known as an essential trace element for normal growth and physiological function of animals (NRC 2011). The main function of Se is the protection of biological components, ie, DNA, proteins and lipids against the attack of free radicals produced during normal metabolism (Watanabe *et al*. 1997). Selenium is incorporated into proteins to make selenoproteins such as glutathione peroxidase (GPx), an important antioxidant enzyme (Rotruck *et al*. 1973).

The yeast *S. cerevisiae* is well known for its ability to accumulate and convert inorganic Se to organic Se (Suhajda *et al*. 2000). Chemical similarities between the sulfur and Se makes the second be absorbed and processed by the same metabolic pathway of the sulfur, in plants and yeast, on the biosynthesis of methionine to produce selenomethionine (SeMet). SeMet can be metabolized to the Se analogue of cysteine, selenocysteine (SeCys), and its metabolites (Birringer *et al*. 2002). This Se is easily available when needed for the synthesis of selenoproteins. This saves valuable time and ensures rapid and efficient reaction in case of stress or illness.

Selenium yeast can be produced by two different techniques. In one, sodium selenite is added to a yeast suspension and the mixture is dried. Selenite can, to some extent, be bound to the cell surface by ionic forces. This product is considered inorganic selenium, and although it is a selenium yeast product, it is not considered a "selenized yeast" product. Despite being economical and widely used, the inorganic Se is a pro-oxidant and may cause oxidative stress (Mézes & Balogh 2009). The second form of selenium yeast is cultivated in a fed-batch fermentation which provides incremental amounts of cane molasses and selenium salts in a manner which minimizes the detrimental effects of selenium salts on the growth rate of the yeast and allows for optimal incorporation of inorganic selenium into cellular organic material. The yeast metabolism reduces selenite to selenide and incorporates it into cellular constituints in place of sulphur. In this selenized yeast (Se-yeast) 50-80% of the selenium is organically bound selenium as SeMet which can replace about 30 percent of the methionine in the yeast proteome SeMet appears to be the predominant selenocompound in selenized yeast (AAFCO 2008; Jacobson 2010). According with Wang and Lovell (1997), organic selenium sources, SeMet and Se-yeast, have higher bioavailability than selenite, in diets for channel catfish (*Ictalurus punctatus*).

Although few studies have been conducted with selenized yeast in fish, Se already had its antioxidant confirmed (Jovanovic *et al*. 1997; Rider *et al*. 2009). Bell *et al*. (1987) related hepatic glutathione S-transferase activity, plasma pyruvate kinase activity, erythrocyte fragility and kidney reduced glutathione were all increased in Se deficiency on Atlantic salmon.

The inclusion of Selplex® (organic form of selenium yeast) in the diet of African catfish could enhance growth, feed utilization and general fish health through increasing their defense mechanism against the environmental Cu toxicity (Abdel- Tawwab *et al*. 2007). When combined with vitamin E, Se was seem to acting synergistically on the reduction of the oxidative stress effects, improvement the immune system and promoting growth in yellowtail kingfish (Le *et al*. 2014).

Moreover, Se-yeast may confer a protective role against pathologies, as verified by Sritunyalucksana *et al*. (2011), whose study found greater resistance of white shrimp to Taura Syndrome Virus (TSV) challenge. Many effects of Se on the immune system have been reviewed, but one of the most widely investigated associations between selenium and the immune system is the effect of the micronutrient on neutrophil function. The antioxidant glutathione peroxidases are likely to protect neutrophils from oxygen-derived radicals that are produced to kill ingested foreign organisms. Dietary Se supplementation reduced oxidative stress and improved the immune response, with increased leukocyte respiratory burst and plasma lysozyme activity and higher plasma immunoglobulin concentration, of grouper after high copper ingestion (Lin & Shiau 2007).

Several recent studies have demonstrated that rotifers provide insufficient Se to meet the Se requirement of Atlantic cod *Gadus morhua* (Penglase *et al*. 2010). As rotifers can ingest and digest yeast (Rodriguez *et al*. 1996), feeding rotifers Se-yeast appears a logical method of increasing rotifer Se concentration. Penglase *et al*. (2011) investigated the enrichment and retention of Se in rotifers fed Se-yeast. Rotifers enrichment with Se-yeast resulted in reproducible concentrations of Se that were then retained for extended periods of time, allowing that Se-yeast enriched rotifers can be used as a Se delivery method for fish larvae nutritional requirement.

The use of selenium yeast still consists of a field with many gaps. The maximum selenium level into the diet as non-toxic for animals, according EPA (2004) is 8.0mg Se

kg^{-1} (fresh weight). However, the toxic effects related to the concentration of Se appear to vary greatly between species depending on specific factors (Hilton *et al*. 1980; Gatlin & Wilson 1983).Furthermore, suppliers also need to provide more information on the specific type of Se used in supplements, since there are records of the selenite has pro-oxidant effect. The increasing demand from consumers for higher quality foods provides an excellent opportunity to produce functional foods rich in Se.

3.2.4 Yeast Extract

According to AAFCO (2008),yeast extract (YE)is the concentrated soluble of mechanically ruptured cells of a selected strain of the yeast, and can be obtained by methods of autolysis, plasmolysis or hydrolysis (Moresi *et al*. 1995; Chae *et al*. 2001; Eddy & Barnett 2007; Dolinska *et al*. 2012). This derivative are commercially marketed as liquid, paste or powder, and are usually produced from baker's or spent brewer's yeast, *S. cerevisiae*, but other yeast, in particular, *Candida utilis* (Guan *et al*. 2013) and *Kluyveromyces marxianus* (Revillion *et al*. 2003) are also used. YE is rich in peptides, amino acids, vitamins and nucleotides (in the form of nucleic acids), used as supplement in both human and animal foods, in pharmaceuticals, as well as flavor and taste enhancer (Bekatorou *et al*. 2006).

The manufacturing process consists on breaking down the cells using endogenous or exogenous enzymes. Autolysis is an usually disruption method for YE production, but it has some disadvantages, like low yield, difficulty solid-liquid separation due to the higher content of residue in the autolysate, poor taste characteristics as a flavor enhancer and risk of deterioration due to microbial contamination. To obtain YE, the insoluble cell wall material, provenient of the lysis process, is removed by filtration or centrifugation.

Many studies have been conducted in order to find economic and effective methods, as regards higher yield compared to the release of nutrients (Tangüler & Erten 2008; Chae *et al*. 2001; Guan *et al*. 2013). Tangüler and Erten (2009) verified that the optimum temperature and time for the production of yeast extract was 50°C for 24h, based on α-amino nitrogen content, which was 3.7%. Furthermore, under the same conditions, protein content was 52.5% and total solids content was 1.98%. Often hydrolyzed and inactive yeast are sold as yeast extract. This deadlock can be solved by

observing the levels of carbohydrates: autolyzed yeast has about 20-22% (most of the rest of the cell wall) while YE contains only 3-6% carbohydrates (Tacon 2012).

Ghosh *et al*. (2005) verified that rohu *Labeo rohita* fingerlings fed YE had better growth performance and nutrient digestibility, probably due to the production of extracellular enzymes by the gut microflora, which helped in a better utilization of feed. Later, Andrews *et al*. (2011) found that when rohu fingerlings received three concentrations of YE, brewer's yeast and spirulina, fingerling which receiving YE at lower inclusion (1%) showed improvement of the immune system and increased resistance to infection to *A. hydrophila*.

Biswas *et al*. (2012) found a significant improvement in the immune system of common carp which received daily doses of 5mg YE for 3 days, with increase of cytokine gene expression, of superoxide anion production, phagocytic activity and resistance to *A. hydrophila*.

In addition to the effects on the immune system, supplementation of the diet with YE improves liver function with decreased levels of alanine and aspartate aminotransferase, increase of the supranuclear zone in the intestinal epithelial cells, thereby increasing the intestinal absorption surface (Jarmolowicz *et al*. 2012).

Rumsey *et al*. (1991a) found that the yeast-based products, whose cell wall has been degraded have better digestibility. Allied to this, the composition of YE is rich in important factors to growth and immune system improvement. One of the products used in the studies with YE is Nupro® (Alltech Inc.) which is a yeast-based product formed from the collection of the cytoplasmic contents of the yeast (*S. cerevisiae*) cell.

The large concentration of proteins in YE makes it be an alternative to fish and soybean meal. It has been shown that complete replacement of fish and soybean meal with NuPro® is possible in rainbow trout (Hunt *et al*. 2014) and white shrimp (McLean *et al*. 2006), although cobia showed a reduced growth rate at levels of 50% replacement and higher (Lunger *et al.* 2006). Furthermore, yeast extract is rich in glutamic acid which has a dietary flavor-enhancing effect and is a vital energy substrate for cells involved in rapid cell division, including intestinal cells (Lacey & Wilmore 1990).

3.2.5 Yeast cell wall components

According Klis *et al*. (2006), the cell wall has four main functions, namely: (1) stabilization of internal osmotic conditions, avoiding the influx of water; (2) protection

against injury; (3) maintaining the cell format and (4) the cell wall as a scaffold for protein, retaining the periplasmic proteins and limiting the accessibility of foreign enzymes to the cell.

Under normal conditions, the yeast cell wall represents about 10-25% of the total cell mass. The macromolecules that constitute the cell wall are mannoprotein (30-50%), β- 1,6-glucan (5-10%), β- 1,3-glucan (30-45%) and chitin (1.5-6.0%) for *S. cerevisiae*.

The inner layer of the *S. cerevisiae* yeast cell wall consists of β-glucan and chitin. β-1,3-Glucan and chitin are synthesized by plasma membrane bound enzyme complexes while β-1,6-glucan is synthesized in the endoplasmic reticulum and processed outside the cell. The β-1,3-glucan chains have a triple helical structure and are responsible for the mechanical strength of the cell wall (Cabib & Roberts 1982). β-glucans and chitin are responsible for rigidity of the cell wall and mannoproteins are responsible for cell-cell recognition and interactions, interactions with the environment and determine immunological specificity of the yeast (Klis 1994).

Since the finding that the polysaccharides from yeast cell wall have beneficial properties, many processes for the isolation and purification of the polysaccharide have been developed (Peat *et al*. 1961; Thanardkit *et al*. 2002; Freimund *et al*. 2003). Most of them use hot alkali, acids or a combination of both, which solubilize proteins and other polysaccharides (Whistler & BeMiller 1958). The following will detail the components of the yeast cell wall with a description of their chemical and biological nature and its application on aquaculture organisms.

β-glucansare heterogeneous group of glucose polymers, consisting of a backbone of β-1,3- linked β-D-glucopyranosyl units with β-1,6- linked side chains of varying distribution and length. They are widespread in plants, algae, bacteria, yeast and mushrooms. β-glucan from different sources are different in their structure and immunomodulatory properties (Brown & Gordon 2003; Volman *et al*. 2008). Yeast β–glucans, for example, have β-1,6 branches further with addicional β-1,3 regions (reviewed by Meena *et al*. 2013). According Ooi and Liu (2000), insoluble 1,3/ 1,6-β-glucans have greater biological activity than that soluble 1,3/ 1,4-β- glucans. Basically, glucan molecules are of two types according the basis of glycosidic bonds present in them, that is, α-glucan (dextran with 1,6, starch with α-1,4- and α-1,6-glycosidic bonds) and β-glucan (cellulose with β-1,4, zymosan with β-1,3, laminarin with β-1,3- and β-

1,6, lichenin with β-1,3 and β-1,4 glycosidic bond) (Meena *et al*. 2013).

Recognition of pathogens by the innate immune system depends on molecules called "pattern recognition receptors” (PRR). These PRRs exist in different forms and include soluble proteins such as complement, membrane bound receptors such as the scavenger receptors, integrins and Toll-like receptors. The PRRs are expressed on cells of the innate immune system such as macrophages, leukocytes, dendritic cells and Natural Killer (NK) cells. Molecules that bind to those receptors are called "pathogen associated molecular patterns (PAMPs). Examples of PAMPs are lipoteichoic acid on Gram-positive organisms, lipopolysaccharide on Gram-negative organisms and β-glucan on fungi. Recognition of particles derived from yeast was attributed to a variety of receivers, including the Dectin-1, which recognizes β-1,3 and β-1,6-glucans and is present in monocytes / macrophages, neutrophils, dendritic cells and at a lower level on a sub-population of T cells (Herre *et al*. 2004).

The health benefits of β-glucans have been studied in many different animals, with most research in fish focusing on their immune-enhancing properties. Selvaraj *et al*. (2005) compared the efficacy of β-glucans administered by three different routes, which were intraperitoneal injection, oral route and bathing, and concluded that intraperitoneal injection of glucan in carp enhanced resistance to *A. hydrophila* infection challenge, and many aspects of non-specific and specific immune response. Meanwhile bathing and oral administration of glucan did not induce any change in the measured parameters. These authors suggested that the advantage in injection method is that glucan can reach the target the organs and enhance the non-specific cellular immune response. According to them, fish probably cannot absorb glucan particles through the bathing route, as they are insoluble in nature. Although glucan particles are supposed to be absorbed through the intestinal wall, when administered orally, only particles less than one micron might be capable of passing through the walls of the intestine and blood capillaries.

On the other hand, several studies has been demonstrated the positive effect of β-glucan oral administration on growth and feed efficiency (Azari *et al*. 2013), disease resistance (Gopalakannan & Arul 2010), improvement of the innate (Ai *et al*. 2007), as well as the acquired immune system (Siwicki *et al*. 2010). Ringo and Song (2016) also show antimicrobial activity and increased activity of antioxidant enzymes.Moreover,

Rufchaie and Hoseinifar (2014) reported a favoring autochthonous and lactic acid bacteria on intestinal microbiota, acting as a prebiotic. It is suggests by the authors that it might occur as a result of the provision of minerals, nucleic acid, B- complex, vitamins and/or amino acids provided by dietary yeast.

Ai *et al*. (2007) verified the performance of yellow croaker *Pseudosciaena crocea* fed two levels of β-glucans, 0.09% (low) and 0.18% (high) and the results suggested that low glucan could enhance growth and innate immunity of large yellow croaker with an 8-week oral administration, but higher supplementation did not influence growth, or further improve immunity of large yellow croaker. The controversial results in the literature regarding the efficacy of β-glucans are probably due to different species, size of the animals tested, study method and product used.

Despite β-glucans present several benefits when used in isolation, several authors have reported the use of conjugated products β-glucans and MOS checking an even greater increase of benefits (Zhu *et al*. 2012; Yu *et al*. 2014; Selim & Reda 2015). In a study with sea cucumbers (*Apostichopus japonicus*), Gu *et al*. (2011) reported the efficacy of β-glucans and mannan oligossaccharides alone, besides give higher survival percentage when challenged by *Vibrio splendidus*. However when combined, β-glucans and MOS provide even greater immunostimulation over a prolonged time. These authors suggest that these immunostimulans together may activate different pathways of sea cucumber immune system, which resulted in high immunostimulating effects compared with single treatments.

Mannanoligosaccharides (MOS): are non digestible carbohydrates that are composed of mannose blocks and can be found in the yeast cell wall in complex formation (Sohn *et al*. 2000; Halas & Nochta 2012). MOS have been assigned to nutricine, which are components of feeds that exert a beneficial effect upon health and metabolism yet are not direct nutrients. The nutricines are those components of feeds and of foods that link health and nutrition (Adams 2000).

Studies carried out *in vitro* showed that on the presence of MOS, enteric pathogenic bacteria attack the own mannan components instead of the epithelium, which reduced colonization. As MOS is non-digestible, it is eliminated with the faeces and carries with them the adhered bacteria (Spring *et al*. 2000). Reduction of harmful bacteria, on the other hand, favors the beneficial bacteria in the occupation of the

surface of the gastrointestinal tract (GT). According Miles (1993), MOS could be related to its ability to promote the growth, as source of energy, for lactic acid bacteria (LAB). Moreover, it was also reported the production of bacteriocins by the LAB, which inhibit growth of certain fish pathogens and thus positively affect the host microflora (Reza *et al*. 2009).

Dimitroglou *et al*. (2009) found that supplementing the diet of juvenile rainbow trout with 0.2 g kg^{-1}MOS modulates intestinal microbial communities, with a significant increase of *Enterococcus* spp. Similarly, Sang and Fotedar (2010) found an increase of aerobic bacteria in the intestine of spiny lobster whose feed was supplemented with 0.4% MOS. According Dillon and Dillon (2004), a higher number of that aerobic microbiota in the gut may increase the host survival, improve digestion efficiency and provide digestive enzymes or vitamins. These results are in accordance with Safari *et al*. (2014), which supplement the narrow clawed crayfish diet for 126 days, with MOS and fructo-oligosaccharide (FOS), alone and combined. The results verified that all of the parameters showed an improvement when the crayfish received MOS, an even greater improvement when products were used in combination. Checking best growth rates and feed utilization, increased activity of amylase, lipase and alkaline protease, additionally, increasing in lactobacillus count. They showed also an improved immune response after challenge to air exposure or bacterial challenge.

The MOS are also related to an improvement in intestinal morphology, with increased microvilli height and number of goblet cells in cobia *Rachycentron canadum* (Salze *et al.* 2008), microvilli density and length in European lobster (*Hamarus gammarus* L.) larvae (Daniels *et al*. 2010), villus length and width and villus height of enterocytes in striped catfish *Pangasionodon hypophthalmus* (Akter *et al*. 2015), increasing absorption surface and mucus production. However, MOS's mechanisms of action are not fully elucidated in fish. Halas and Nochta (2012) under review regarding the use of MOS in pigs, related a series of hypotheses about the mechanisms of action of MOS in intestinal morphology. According to these authors, there is an increased production of short chain fatty acids related to the increase of beneficial bacteria, which positively affect the recovery of the epithelium. Torrecillas *et al*. (2013) reported an increased production of prostaglandins in the posterior gut of European sea bass.These plostaglandins are involved in the gastrointestinal cytoprotection by influencing the

regulation and homeostasis the onset of gastrointestinal inflammation.

It is well known that MOS has beneficial effects on the immune fish system as well as other organisms, such as increased activity of lysozyme (Ahmad *et al*. 2013) and complement pathway (Staykov *et al*. 2007), facilitating phagocytosis (Torrecillas *et al*. 2007), improving the concentration of immunoglobulins (Ta'ati *et al*. 2011), concentration of lymphocytes and other blood cells (Sang & Fotedar 2010; Sang *et al*. 2011; Akrami *et al*. 2012), increase in blood oxidative radicals (Zhou *et al.* 2010; Safari *et al*. 2014), providing greater resistance to disease (Andrews *et al*. 2009) and stress events (Salze *et al*. 2008).

Possible mechanisms of action is based on two assumptions, the first refers to activation and facilitation of antigen processing through mannose binding lectins (MBL) stimulated by liver secretion. MBL is a collectin that shows specificity for mannose, and activate the complement cascade (Staykov *et al*. 2007; Torrecillas *et al*. 2007). The second hypothesis involves the natural production of antimannan antibodies. The antimannan antibodies are direct against on oligosaccharide- based epitope of the viruses and microbes and these carbohydrate- specific antibodies may be produced during a normal immune response against the intestinal microflora (Halas & Nochta 2012; Torrecillas *et al*. 2014).

Chitin: Chitin is a polysaccharide composed of β(1-4)-linked *N*-acetyl-D-glucosamine residues and is synthesized by different crustaceans, mollusks, marine diatoms, insects, algae, fungi and yeast (Kumar 2000). In the fungal cell walls, chitin microfibrils are surround by β-glucan matrix to form alkali-insoluble complexes associated with other polysaccharides and proteins (Klis 1994).

Chitin and its deacetylated product, chitosan, are commercially manufactured from shells of shrimp and crab (Synowiecki & Al-Khateeb 2003). To our knowledge there is no studies carried out on aquaculture with chitin or chitosan from yeast. Nevertheless, zygomycetes fungal contain up to 50% chitosan in its cell wall and because of these, attention has been drawn to fungi for use as an alternative resource of both polysaccharides (chitin and chitosan) (Zamani *et al*. 2010). In yeast, specifically *S. cerevisiae*, chitin shows only 2% of the cell wall (Klis 1994).

Although fungi are practically unused as a source of these polysaccharides; several methods for its utilization in chitin and chitosan production were elaborated

(Synowiecki & Al-Khateeb 1997; Zamani *et al*. 2010). Besides, the utilization of chitin and chitosan from fungal cell walls is advantageous, because they are readily available, high growth rate and inexpensive. Furthermore, the chemical composition of crustacean chitin is the same as yeast chitin (Andrade et al., 2012), additionally, fungi do not contain huge amounts of calcium carbonate and other mineral salts, decreasing the cost of acid treatment during manufacturing (Synowiecki & Al-Khateeb 2003).

Many products with chitin and chitosan, from crustaceans mainly, already are consolided as imunostimulants. Cuesta *et al*. (2003) conducted a study *in vitro* with head-kidney leukocytes of gilthead sea bream (*Sparus aurata*) and concluded that head-kidney leukocytes are able to phagocyte chitin particles(<10 µm size), suggesting that they possess a mannose/fucose receptor.In the same study, chitin of different particle-sizes stimulated leucocyte phagocytosis and natural cytotoxic activity when incubated for 1 or 4 h. Gopalakannan and Arul (2006) also reported improvement of the carp immune system, whose diet was supplemented with chitosan, with improved neutrophil respiratory burst activity and resistance to *A. hydrophila*.

Kumar *et al*. (2006), in a comparative study between purified and natural chitin, perceived drop in growth and survival in *Macrobrachium rosembergii* who received purified chitin. The authors suggest that the highest concentration in the purified product of chitin probably decreases digestibility due to a limitation of hydrolysis ability of the enzymes of this specie. Niu *et al*. (2011) stated that moderate chitosan was benefit to the growth and survival after stress condition (low oxygen levels) of postlarval *L. vannamei* and the optimum level should be between 2.13 and 2.67 g kg^{-1} diet. Chitosan is an active material which is the important component for the growth of aquatic animals, therefore, when it is supplemented in the diet, it could participate in the biosynthesis of the organism at a rapid speed, which could positively affect the growth, enhancing the digestion and absorption of nutrition at moderate levels, as a result, this benefit might create the better growth performance.

3.2.6 Nucleotides (NT)

The forming units of DNA, RNA and ATP (Ringo *et al*. 2012) which are formed of a purine (adenine, hypoxanthine or guanine) or a pyrimidine (uracil, thymine, or cytosine) combined with a ribose or deoxyribose and one or more phosphate (Li & Gatlin 2006). Nucleotides may be synthesized by salvage pathway (recycle from dead

cells), via *de novo* (formation of new nucleotides from amino acid) or be supplemented in the diet (Biswas *et al*. 2012; Zhao *et al*. 2015). As can be produced by the body, they were for long time considered as non-essential nutrients. However, some tissues such as the intestinal mucosa, hematopoietic cells and brain cells have a limited production capacity of NT via *de novo* (Yamauchi *et al*. 2002).

Several studies have reported many benefits of NTs in relation to the immune system, suggesting, for example, the NT restriction acts primarily on T-helper lymphocytes, whose function is cell proliferation, hindering the proliferation of lymphocytes, and as a result, the processing antigen. According Rudolph *et al*. (1994), T lymphocytes depend on extra NT supplementation for their optimal functioning. Furthermore, nucleotides have been related to increased production of antibodies (Welker *et al*. 2011) and increase in immunoglobulins (IgM) levels (Yaghobi *et al*. 2015).

The NTs stimulate the nonspecific immune response, facilitating phagocytosis (Jha *et al*. 2007) and increase in lysozyme activity (Tahmasebi-Kohyani *et al*. 2011).Although the mechanisms of action are not fully understood, supplementing the diet with NTs makes the body to save energy that would be spent on the synthesis *de novo* pathway. The NTs are also related endocrine interactions and modulation of gene expression, particularly in lymphoid organ (Biswas *et al*. 2012).Yousefi *et al*. (2012) noted an increase in the number of hematocrit and hemoglobin of beluga sturgeon *Huso huso*, which may be related to an increase in iron absorption and availability, particularly with regard to inosine and hypoxanthina (Grimble 1996).

The intestinal morphology also is favored by supplementing the diet with NTs with increased villus height and microvilli of enterocytes size of red drum *Sciaenops ocellatus* (Cheng *et al*. 2011) and striped catfish, *Pangasianodon hypophthalmus* (Yaghobi *et al*. 2015). Besides, Safari *et al*. (2014) found that nucleotides favored *Lactobacillus* in crayfish's (*Astacus leptodactylus leptodactylus*) gastrointestinal tract.

Many studies have assessed the efficacy of NTs in growth parameters, feed efficiency, improved liver activity, for fish (Zhao *et al*. 2015), crustaceans (Abedian Kenari & Oujifard 2013) and shellfish (Chaitanawisuti *et al*. 2011).

4. Yeast with aquaculture importance

4.1 Baker's Yeast

S. cerevisiae strains have been selected for many years for their dough-leavening characteristics. Baker's yeast produces the CO_2 that results in dough leavening and contributes to the flavour and crumb structure of bread. Baker's yeast is sold commercially mainly in two forms, compressed yeast and dried yeast. The compressed yeast is sold in blocks format and can be used immediately, but because it is perishable should be kept at low temperatures (0 - 4°C). The dry yeast is presented in two forms, active dry yeast (ADY), which is marketed in airtight packaging and vacuum. Before use, it is necessary that it is rehydrated in water at 35 - 45°C. The second form of dry yeast is instant dry yeast (IDY) which during manufaturament undergoes a quick-drying process, resulting in a product with higher porosity, accelerating the process of rehydration (Beudeker *et al*. 1989).

The species most commonly used in the manufacture of yeast is *S. cerevisiae*. Its predilection is due to the high fermentative power. Specific strains are created for each type of bread and to withstand various processing conditions. Their manufacture is typically done on a molasses substrate, and the production is carried out under strict conditions, so as to ensure genetic purity, consistency, specificity and efficiency of strains (Randez-Gil *et al*. 1999).

One of the major applications of yeast in aquaculture is its use as a food source for rotifers and *Artemia*. Because of their small particle size, high protein content and relatively low production costs, yeast have been considered as an algal substitute for several species of filter-feeders. Rotifers, in general, contain very low levels of vitamins, but when rotifers are cultured with Baker's yeast (rich in complex B vitamins) they will also contain high levels of these vitamins (Moren *et al*. 2011). Rotifers also have low concentrations of trace elements, but it is possible to increase the concentrations by adding the elements, either to the culture or to the enrichment diet (Øie*et al*. 2011), the yeast enrichment with selenium is an example (Penglase *et al*. 2011).

Nevertheless, there are still some restrictions on the use of yeast mainly as regards digestibility, since the cell wall limits access to nutrients. Coutteau *et al*. (1990) found low growth and survival of brine shrimp *Artemia*, in their attempt to replace the

algae *Dunaliella tertiolecta* by baker's yeast. The digestibility of the yeast increases after enzymatic digestion of the cell wall, but treatment with autoclaving without disrupting the wall seems to be sufficient for the provision of nutrients necessary for the growth and survival of *Artemia*. Thus the authors continue to encourage the use of yeast, since if without breaking the cells do not lose their cytoplasmic nutrients to the medium, damaging the filter organisms, nor the quality of water.

In a similar study, Marques *et al*. (2004) found that when *Artemia* had better performance when they received mnn9 yeast mutant, defective in the synthesis of mannoproteins in the outer cell wall. The authors also found that when yeast is harvested in the exponential growth phase, confers to the *Artemia* better performances. In other study, Marques *et al*. (2006) stated that *Artemia* which received yeast cells were more resistant to *Vibrio campbelli* and *V. proteolyticus*.

Baker's yeast can also be used with alternative protein source in partial replacement of fish meal in Koi carp (*Cyprinus carpio*) diets (Korkmaz & Cakirogullari 2011) as well as inducing immune response, promoting greater disease resistance in fry Nile tilapia (*Oreochromis niloticus*) (Abdel-Tawwab *et al*. 2008).

4.2 Brewer's Yeast

According to AAFCO (2008), brewer's dried yeast is the dried, nonfermentative, non-extracted yeast of the botanical classification *Saccharomyces* resulting as a by-product from the brewing of beer and ale.

The brewing industry is, in addition to one of the oldest, one of the most develops over the years. Among the steps of the manufacturing of beer, fermentation, which occur transformation of sugar into alcohol, it is preferably performed by the species of the genus *Saccharomyces*. The choice of species is by properties that make them excellent for industrial use, including the rapid growth, good ability to produce ethanol and stress tolerance. Despite brewer's yeast industries utilize various times (usually 4-6 times), the yeast biomass is a major waste generated, and their use as byproduct becomes a necessity from the point of view of sustainability (Tenge 2009).

In general, the biomass of yeast is inactivated by heat and used as an alternative protein source, received attention as an alternative to replacement of fish meal. Yeast is recognized as a single cell protein (SCP), due to its high nutritional value (Anupama & Ravindra 2000). They are also low in phosphorous, which will lead to less water and

environmental contamination than fish meal and other plant-based alternate protein sources that contain high levels of phosphorous (Cheng *et al*. 2004).The yeast can be sold in liquid form (18-20% dry matter), sprayed dry, or specific strains facing human consumption as food supplement and holistic therapy (Mussatto *et al*. 2006).

Brewer's yeast can replace to approximately 50% of fishmeal protein in sea bass (*Dicentrarchus labrax*) (Oliva-Teles & Gonçalves 2001), cobia (*R.canadum*) (Lunger *et al*. 2006) and Nile tilapia (*O. niloticus*) (Zerai *et al*. 2008). On Thai panga (*P.hypophthalmus* x *Pangasius bocourti*) the replace up to 45% of fishmeal improved growth performance and immune response (Pongpet *et al*. 2015). This study verifies that 60 and 75% of replacement were harmful to growth. The poor utilization of non-starch polysaccharides (NSPs) by the fish should be responsible for the low growth rate of fish fed D60 and D75 diets. NSP is an indigestible carbohydrate containing the derived-plant feed ingredients and brewer's yeast. Moreover, the improvement of the immune response is an outcome of all the nutrient present on brewer's yeast.

Brewer's yeast can be used as an alternative food to use on live food production (Robert & Trintignac 1997). Also may be an excellent immunological promoter from a wide range of organisms, as demonstrated throughout this review, in addition serving as a food source for mass production of *Artemia* (Comabella *et al*. 2004) and free-living nematodes (*Panagrellus redivivus*) (Ricci *et al*. 2003) used during larviculture as live feed for fish and crustaceans.

4.3 Red Yeast

The color of salmonids is a quality criterion and studies have shown that consumer willingness to payincreases with the redness of the salmon (Steine *et al*. 2005). Carotenoid pigment is deposited in the flesh of salmonids or other fishes and in the carapace of crustaceans to give their characteristic color. Among the major carotenoids in this scenario, there is astaxanthin (3,3'-dihydroxy-b,b-carotene-4,4'-dione), naturally obtained from organisms that produce them through via *de novo*, as certain fungi and bacteria, plants and algae, or chemically synthesized (as reviewed by Ambati *et al*. 2014).

Since astaxanthin cannot be synthesized by salmonids and many other animals, it is necessary to include this compound, or a suitable precursor, in the feed to obtain adequate pigmentation. The commercial astaxanthin is mainly from *Phaffia* yeast,

Haematococcus pluvialis (a green microalga, which accumulates high astaxanthin content under stress conditions) and through chemical synthesis (Ambati *et al*. 2014).

Phaffia rhodozyma (perfect state *Xanthophyllomyces dendrorhous*; Golubev, 1995) can accumulate large amounts of astaxanthin in lipid droplets in the cytoplasmatic membranes (Johnson & An 1991), and is used as natural pigment feature that stands out and preference because of the increasingly strict regulations concerning the safety of chemicals as food additives. Ithas been shown to be a good source of nutritionally important substances likeprotein, lipids, vitamins, and minerals which are typical of yeast in general. It may also contain growth factors and feed attractants (Sanderson & Jolly 1994).

Besides the effect of color of the musculature of aquatic organisms, astaxanthin also seems to be related to higher hatching rates of salmonid eggs (Craik 1985), high antioxidant potential (Bell *et al*. 2000; Yang *et al*. 2010), mainly in salmonids whose high concentration of polyunsaturated fatty acids is highly susceptible to oxidation. In shrimp’s lack of carotenoids, especially astaxanthin, is related to the "blue disease", characterized by an exoskeleton appeared blue throughout and blue shrimp fetch prices half to a third (Howell & Matthews 1991). It has also been observed that fishes with a high level of astaxanthin are more resistant to diseases (Scholz *et al*. 1999), probably due to its antibacterial activity (Ushakumari & Ramanujan 2013) and improvement of innate defense (Amar *et al*. 2004).

Despite the synthetic astaxanthin is cheaper and more used (Nguyen 2013), the demand for natural and sustainable products grows every day. Astaxanthin products are available in the form of capsule, soft gel, tablet, powder, biomass, cream, energy drink, oil and extract (Ambati *et al*. 2014).

The studies about the use of red yeast in aquaculture are generally focused on discovering different carbon sources for the culture medium on the production of yeast biomass (Moriel *et al*. 2005; Yang *et al*. 2011) and search for the optimization of production, increasing the astaxanthin yield (Storebakken *et al*. 2004ab; Ni *et al*. 2008). Additionally, many studies have focused on the mutation breeding of more productive *P. rhodozyma* strains (Liu *et al*. 2008; Chi *et al*. 2015).

4.4 Torula Yeast

Definition according to AAFCO (2008),Torula dried yeastor Candida dried yeast (IFN 7-05-534 Yeast torula dehydrated) is the dried, non-fermentative yeast of the botanical classification (torulopsis) *Candida utilis* (formerly *Torulopsis utilis*) which has been separated from the medium in which propagated.

C.utilis has ability to grow on cheap substrates and assimilate various carbon sources such as molasses (Lee & Kim 2001), distiller vinasse (Rodríguez *et al*. 2011), among others. Besides, it has high content of proteins and excellent profile of essential amino acids. The chemical composition of the yeasts may vary according to different factors: substrate, concentration of salts, degree of aeration, number of successive washes to remove impurities and drying technology (Akhtar *et al*. 2008). This process makes it doubly beneficial, since it uses organic residues in the production of alternative protein source.

Øverland *et al*. (2013) showed that *C. utilis* was promising protein sources in diets for Atlantic salmon, capable of replacing 40% of the protein from high-quality fishmeal without adversely affecting growth performance, digestibility or nutrient retention. The limitation of total replacement is due, among other reasons, the deficiency of sulfur amino acids, especially with respect to methionine (Olvera-Novoa *et al*. 2002).

In a study with rainbow trout and coho salmon, Mahnken *et al*. (1980) found that fish meal can be replaced by up to 40% for rainbown trou without causing damage to growth. In relation to coho salmon, the authors tested up to 100% replacement of fishmeal by *Candida* sp., checking that the best substitution rate was 25%. The same authors found that the performance of coho salmon obtained a better growth when it was added methionine to the diets with yeast, however this improvement did not reach the performance of fish that received 100% fishmeal.

In addition to a protein source, it was found that *C. utilis*, combined with *Pseudomonas stutzeri*, plays an important role in the enhancement of the protection of *Artemia* culture against the pathogen *Vibrio alginolyticus*. This result suggests that it is possible to use *C. utilis* as probiotic for the culture of live food(Abdelkarim *et al*. 2010).

5. *Future trends*

The requirement of intensive aquaculture for nutritionally complete feeds or feed ingredients have stimulated considerable research and developmental activities in the field of fish nutrition and feed technology. The envisaged developments involved in aquaculture are that the diet should be formulated with an emphasis on minimizing environmental degradation, enhancing the quality of the produce and larval survival and growth (Gatlin *et al*., 2007).

Feed enzymes help break down anti-nutritional factors (protease inhibitors, lectins, oligosaccharides and phytic acid), and are used to increase the availability of starch, protein, amino acids and minerals. In addition, they can be used to supplement the enzymes produced by young animals where, because of an immature digestive system, enzyme production may be inadequate (Barletta 2010). Yeast cells contain numerous enzymes, including proteases (protein-digesting enzymes) and pectinases (fibre - non-starch polysaccharides - degrading enzyme) (Gainvors *et al*. 1994; Martos *et al*. 2013). Several of these enzymes are already commercially used in the hydrolysis of fruit pulp in the manufacture of juices (Saxena *et al.* 2003). Related studies using isolated enzymes in yeast supplementation of the diet of aquaculture organisms and their potential benefits and commercial production is a field to explore.

Recent studies have sought through genetic engineering promote the production of eicosapentaenoic acid (EPA) by yeast. While *S. cerevisiae* yielded less than 1% of the total fatty acids (TFA) as EPA (Tavares *et al*. 2011), aengineered *Yarrowia lipolytica* strain, Y4305, produces EPA at 15% of the dry cell weight and produces lipids with EPA at 56.6% of the TFA (Xue *et al*. 2013). This study with *Y. lipolytica* is the first example of engineered yeast with a metabolic pathway being used to produce a commercial product and could be the solution for a global overfishing crisis that has arisen due to the growing popularity of the supplement.

The development of strains with varying long-chain polyunsaturated fatty acids (LCPUFA) compositions tailored for specific applications and for developing a versatile platform for the production of other high-value lipid products. By the same way, industrial strains should be improved to face problems related to other bottleneck situations, for example the genes which induce the methionine production, or other amino acids in concern, especially sulfur amino acids, which are found in small amounts in yeast. Other example of genetic manipulation which can be adapted to the

necessities of aquaculture organisms and already has been performed with mammalian gene is the expression of interferon, epidermal growth factor and hemoglobin (Hitzeman *et al*. 1983; Brake et al. 1984; Llano *et al*. 1993).

From what was seen in this review, it is clear that the disruption of the cell walls makes them more accessible nutrients, making the results of growth, immune response, tolerance to stress and disease, among others more evident. However, the autolysis and hydrolysis processes are costly and therefore, knowledge of every kind response regarding the concentration and the administration period is critical. More studies are needed in this area in order to optimize dietary supplementation and supporting it with results that justify the investment. Allied to this, the great diversity of organisms that may be grown and have importance in aquaculture varies in metabolism and physiology. As such, this necessitates that every species be evaluated individually for its optimal nutrient requirements. When these are known, evaluation of diversity within the species for utilization of specific nutrient components can be performed and estimates made.

Table 1: Yeast and derivatives products with importance to aquaculture.

Yeast product	Species	Administration and dose	Study duration	Results	Reference
Probiotics	Grouper (*Epinephelus coiodes*)	0.001, 0.1, 10 g kg^{-1} diet (*Saccharomyces cerevisiae*)	4 weeks	↑ weight, resistance to *Streptococcus* sp. ↑ phagocytic activity, respiratory burst, SOD ↑ serum lysozyme and ACH50 activity	Chiu *et al*. 2010
Probiotics	Giant freshwater pranw (*Macrobrachium rosembergii*)	0.1, 0.2, 0.5, and 1.0% of diet (*S. cerevisiae*)	75 days	↑ protein and lipids carcass contend ↑ weight, SGR, PER, ANPU ↓ FCR	Prasad *et al*. 2013
Fermented yeast	Common carp (*Cyprinus carpio*)	2 g DVAqua kg^{-1} diet	8 weeks	→ FW, WG, FCR, survival, body and flesh index → hepatossomatic and intestinal index ↑ posterior intestinal microvillus lengh → *hsp*70 gene expression on intestine ↓ *hsp*70 gene expression on liver ↑ *hsp*70 gene expression on kidney	Huang *et al*. 2015

Fermented yeast	Rainbow trout (*Oncorhynchus mykiss*)	0.125 g DVAqua kg^{-1} diet	408 days	↓ mortality ↑ FC and growth	Barnes & Durben 2010
Autolyzed yeast; MOS	Rainbow trout (*O. mykiss*)	2.0% of diet	9 weeks	↑ resistance to infectious hematopoietic necrosis virus (IHNV)	Sealey *et al.* 2007
Autolyzed yeast; NT	Spotted babylon (*Babylonia areolata*)	1 and 2% of diet	4 months	↑ FW, WG, SGR, PER ↓ FCR ↑ resistance to *Vibrio alginolyticus*	Chaitanawisuti *et al.* 2011
Hydrolyzed yeast	Pejerrey larvae (*Odontesthes argentinensis*)	*Artemia* enrichment with 1 g L^{-1}	20 days	↑ weight, SGR ↑ CD3 on kidney, spleen and thymus; ↑ CD4 on kidney and thymus; ↑ saline stress tolerance	Führ *et al.* 2016
Selenium yeast	Rainbow trout (*O. mykiss*)	2, 4, and 8 mg kg^{-1} Se from Se-yeast (Selplex®)	10 weeks	→ SGR, FCR or PER ↑ *Se* retention ↑ GSH-Px (after handling stress) → GSH-Px and Trx-R → MDA and TAC → cytotoxic effect on nuclear abnormalities ↓ leukocyte → levels of whole body Fe, Mn and Zn	Rider *et al.* 2009
Selenium yeast	White shrimp (*Litopenaeus*	- 0.3 ppm inorganic Se - 0.3 ppm organic	5 weeks	↑ weight ↑ total haemocytes and	Sritunyalucksana *et al.* 2011

	vannamei)	Se		granular haemocytes ↑ disease resistance after Taura Syndrome Virus challenge	
Yeast Extract	Common carp (*C. carpio*)	5mg fish^{-1}	3days	↑ cytokine genes expression ↑ superoxide anion production ↑ phagocytic activity ↑ resistance to *Aeromonas hydrophila*	Biswas *et al.* 2012
Yeast Extract	Pikeperch (*Sander lioperca*)	20, 40, and 60 g Nupro® kg^{-1} diet	8 weeks	→ growth, proximal composition, hepatosomatic and viscerosomatic index ↓ aspartate and alanine aminotransferase ↑ supranuclear zone (intestinal epithelial cells) ↑ cellular and humoral immunity	Jarmolowicz *et al.* 2012
B- glucan	Kutum white fish (*Rutilis frisii*)	0.5, 1, 1.5 and 2% commercial yeast glucan	60 days	↑ lysozyme activity, serum IgM ↑ autochthonous and lactic acid bacteria ↑ growth, body protein content, survival	Rufchaie & Hoseinifar 2014
B- glucan	Tench	0.5, 1 and 2 g Macrogard® kg^{-}	1 month	↑ respiratory burst and	Siwicki *et al.* 2010

	(*Tinca tinca*)	[1]diet		lysozyme activity, potencial killing activity of spleen phagocytes ↑ lymphocytes proliferation ↑ total serum Ig ↑ resistance to *A. hydrophila*	
MOS	Cobia (*Rachycentron canadum*)	Rotifers and *Artemia* enriched with 0.2% BioMOS®	28 days	→ weight ↑ resistance to hyposaline stress ↑ microvilli height ↓ occurrence and size of supranuclear vacuoles	Salze *et al*. 2008
MOS	White shrimp (*L. vannamei*)	- 3 g MOS kg^{-1} diet - 20 mg serotonin (5-HT) kg^{-1} - MOS + serotonin	75 days	↑ FW, SGR ↓ FCR ↑ number of molting → survival, body composition, morphology of hepatopancreas	Aktas *et al*. 2014
NT	Catla (*Catla catla*)	0.4 and 0.8% yeast RNA	60 days	↑ leukocyte count, total protein, globulin, lysozyma activity, respiratory burst ↑ resistance to *A. hydrophila* ↑ phagocytic activity	Jha *et al*. 2007
NT	Narrow-clawed crayfish	0.5, 1.0, 1.5, 2.0 and 2.5 g	126 days	↑ FW, SGR, FI, survival	Safari *et al*. 2014

	(*Astacus leptodactylus*)	Vannagen® Kg^{-1} diet		↓ FCR ↑ Protein, lipid and energy efficiency ratio, protein, lipid and energy productive value ↑ uricase value ↑ *Lactobacillus* count ↑ phenoloxidase activity (after air exposure)	
Red Yeast	White shrimp (*L. vannamei*)	- 10^8 live cells of *Rhodosporidium paludigenum* g^{-1} diet - 1% dry *R.paludigenum* yeast	6 weeks	↑ WG, SGR, survival → MDA on serum and hepatopancreas ↑ TAC, SOD, GPX, CAT	Yang *et al.* 2010

Symbols represent an increase (↑), decrease (↓) or no change (→) in the specified response

ACH50 (serum alternative complement activity); ANPU (Apparent net protein utilization); CAT (catalase); FBW (Final Body Weight); FC (Feed Conversion); FCR (Feed Conversion Ratio); FW (Final Weight); GPX (Glutathione Peroxidase); GSH-Px (Hepatic Glutathione Peroxidase); PER (Protein Efficiency Ratio); MDA (Hepatic Malondialdehyde); SGR (Specific Growth Rate); SOD (Superoxide dismutase); TAC (Total Antioxidant Capacity); Trx-R (Thioredoxin Reductase);

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CAPÍTULO 2

Artemia enriched with hydrolyzed yeast improves growth and stress resistance of marine pejerrey *Odontesthes argentinensis* larvae

Artigo publicado na revista Aquaculture (2016), **450:** 173-181.

Artemia enriched with hydrolyzed yeast improves growth and stress resistance of marine pejerrey _Odontesthes argentinensis_ larvae

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Artemia enriched with hydrolyzed yeast improves growth and stress resistance of marine pejerrey *Odontesthes argentinensis* larvae

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Abstract

The present study was conducted to evaluate the effects of two commercial yeast products (Y1 and Y2) that were administered via *Artemia* sp. enrichment on the survival, growth, histomorphometry, immunohistochemical and stress tolerance of pejerrey larvae. The larvae received enriched *Artemia* for 20 days. After 20 days post-hatch (dph), a saline stress test was conducted with 10 larvae tank^{-1}. The remaining larvae were kept for 10days (until 30 dph) being fed with dry feed without yeast supplementation. Samples were taken at 0, 10, 20 and 30 dph for weight, length, histological and immunohistochemical analysis. The results showed that the larvae fed with hydrolyzed yeast presented a higher weight and specific growth rate than did those fed *Artemia* without yeast. However, there was no effect on survival or condition factor. Regarding the histological analyses, no difference was observed on intestinal morphology. Hydrolyzed yeast appears to have a positive influence on the area and diameter of the kidney and thymus. The specific label of CD3 for kidney, spleen and thymus revealed that Y1 and Y2 have a significant higher percentage of labeled cells. The kidney and thymus of larvae that received yeast displayed significant more CD4-labeled cells. The control and Y2 groups were significantly more sensitive when subjected to salinity 60‰. Our findings indicate that *Artemia* enriched with yeast may promote growth and immunity and the persistence of these parameters for 10days after the last hydrolyzed yeast supplementation. Furthermore, enhanced stress tolerance of pejerrey larvae was observed.

Keywords: Hydrolyzed yeast, growth performance, cumulative stress index, intestinal morphology, immune response.

ACCEPTED MANUSCRIP

Artemia enriched with hydrolyzed yeast improves growth and stress resistance of marine pejerrey *Odontesthes argentinensis* larvae

1. Introduction

The marine pejerrey *Odontesthes argentinensis* (Valenciennes, 1835) is commonly found in coastal waters from South Brazil to Argentina (Dyer, 1994). *O. argentinensis* is considered a specie for aquaculture (Sampaio, 2006) and has been deemed a good model for ecotoxicological investigations (Rodrigues et al., 2010).

Larvae of *O. argentinensis* hatch with an open mouth and pigmented eyes (Valadares Costa et al., 2009). This means that these larvae are able to feed on the first day after hatching, with *Artemia* sp. because of its large hatching size (between 6 and 8 mm) compared with other marine species, thus dispensing the culture of rotifers *Brachionus plicatilis*, commonly used on the first feeding in marine fish larvae (Dhert et al., 2001). Sampaio and Piedras (2010) found that dry diets are accepted at first feeding, but larval growth is reduced compared with larvae preying on *Artemia* nauplii.

Artemia is an important starter feed used in larviculture; however, it is deficient in certain important nutrients, and enrichment is thus common with nutrients and functional additives such as immunostimulants (Lavens and Sorgeloos, 1996; Lanes et al., 2012; Rodrigues et al. 2012).

Yeast products are becoming widely popular in aquaculture. Many products are being developed with yeast as functional feed additives, probiotic live yeast, autolyzed yeast, yeast fractions (yeast cell walls or yeast extracts) or as a source for more purified products (Ferreira et al., 2010). *Saccharomyces cerevisiae* is a natural product from the baker's or brewer's industry that contains various immunostimulating compounds such as β-glucan, nucleic acids, mannan oligosaccharides and chitin (Gopalakannan and Arul, 2010). *S. cerevisiae* can enhance growth and increase both humoral and cellular immune responses, as well as increase or confer resistance against pathogenic bacteria in various fish species (reviewed by Ringo et al., 2012).

The effects of fermented *S. cerevisiae* were tested in rainbow trout (*Oncorhynchus mykiss*), and the growth performance and non-specific immune parameters were improved (Sheikhzadeh et al., 2012). Similarly, Heidarieh et al. (2013) demonstrated that rainbow trout fed a diet supplemented with fermented *S. cerevisiae*

exhibit improved animal growth, digestive enzyme activities and gastrointestinal morphology.

In addition to *Saccharomyces* sp., other fungi genera have also been used as additives in aquaculture. Torula or Candida yeast refers to a product containing *Candida utilis* (Bekatorou et al., 2006). Torula yeast is promising in aquaculture as a protein source (Overland et al., 2013) and as nutritional additive (Bekatorou et al., 2006).

Among the components of products derived from yeast, the nucleotides represented by nucleic acids are the forming units of RNA, DNA and ATP (Ringo et al., 2012). Although nucleotides had been considered as non-essential nutrients, numerous reports have shown that the dietary supplementation of nucleotides may confer several benefits (Sauer et al., 2011). Nucleotide supplementation can attenuate the costly metabolically process of production by the organisms (Sanderson and He, 1994) and supply the external demand of immune and intestinal cells, which lack the ability to produce nucleotides; moreover, the production of nucleotides may be compromised in stress processes (Barness, 1994). Nucleotides have been successfully used in aquaculture for immune stimulation, either as purified products (Li et al., 2007) or in combination with other yeast components (Welker et al, 2011; Lanes et al., 2012).

Because products of *S. cerevisiae* and *C. utilis* are sources of nucleotides and other compounds known to be immunomodulators, this study aimed to investigate the effect of *Artemia* enrichment with two different hydrolyzed yeast products on growth, stress tolerance, histomorphometry of lymphoid organs and intestinal morphology in pejerrey larvae. Additionally, immunohistochemical analyses of kidney, spleen and thymus marked by CD3 and CD4 antibodies for quantifying the total T lymphocyte and T helper lymphocyte population, respectively, were performed. The persistence of the effect of yeast on growth and immune response 10 days after the last administration was also observed.

2 Materials and Methods

2.1 Yeast products

Commercial products used in this study are Augic 15 (identified by Y1) and Hilyses (identified by Y2). Both products, available as a fine powder, are derived from a commercial product from specific strains of *Saccharomyces cerevisiae* and *Candida utilis* (Y1) and only *S. cerevisiae* (Y2), both are obtained by a process stimulating

breakage of the cells by endogenous and exogenous enzymes promoting the release of nucleosides and nucleotides.

2.2 Experimental procedure

The investigation was previously evaluated and approved by the Ethical Committee of Federal University of Rio Grande — FURG (CEUA, protocol number Pq014/2014). Fertilized eggs of *O. argentinensis* were collected at the Cassino Beach in Rio Grande, Rio Grande do Sul, Brazil (32 ° 12 'S - 52 ° 10' W). Incubation was performed in 300 L tanks; the water temperature was maintained at 23 °C and the salinity at 22 ‰. A few hours after hatching, larvae were individually counted and transferred to 12 fiberglass tanks (50 L), to obtain an initial stock of 500 larvae $tank^{-1}$. Newly hatched *Artemia* nauplii (0.5 nauplii mL^{-1}) were supply at the first and second days post-hatch (dph) and yeast-enriched metanauplii from the 3 dph.

During the experiment, sea water was used, which remained constant (22 ± 0.01 ‰) since the water was maintained in a recirculation system. Three independent recirculating aquaculture systems were used in this experiment, each comprising four fiberglass cylindrical tanks (50 L each), with continuous aeration. Each recirculating system consisted of a 100 µm bag filter, a reservoir with biofilters (40 mm bioball, Aquatic Ecosystems Inc., USA), a foam separator (8 L recirculating chamber, direct current, Plaspiral), submersible bomb (Sarlo Better, 1950 L h^{-1}, Brazil) and a submersible heater (Visi-Therm Deluxe, Italy). The flow rate in the recirculating system was maintained at 12% of tank volume $hour^{-1}$.

Each day, the tank bottoms were siphoned, and 10% of the water per system was renewed. Temperature (23 ± 0.08ºC), dissolved oxygen (6.83 ± 0.1 mg L^{-1}), salinity (22 ± 0.01‰) and pH (8.25 ± 0.03) were measured daily. Salinity was measured with a hand refractometer (ATAGO®, S/Mill-E, Japan), temperature and dissolved oxygen were measured with an oximeter (YSI®, Model 550A, USA), and pH was measured with a digital pHmeter (FE20 – FiveEasyTM, Switzerland). The analysis of ammonia (0.28 ± 0.01 mg L^{-1})and alkalinity (274 ± 4.13 mg L^{-1} of $CaCO_3$) were performed daily according to the methodology described by UNESCO (1983), whereas nitrite (0.35 ± 0.12 mg L^{-1})was measured by the method of Strickland and Parsons (1972). The photoperiod was maintained at 18 light: 6 dark, according to the method of Freitas et al. (2009).

The experiment had three treatments: Control - metanauplii enriched with commercial emulsion (Inve, S.presso, USA) according to the manufacturer's instructions; Y1 – metanauplii enriched with a commercial product associated with hydrolyzed yeast 1; Y2 - metanauplii enriched with a commercial product associated with hydrolyzed yeast 2. Each treatment involved 4 replicates.

On the 20th day of life, the larvae in each tank were counted, and 10 larvae pertank were used for a stress test, which will be detailed later. The experiment was followed by 10 additional days to check for persistence of the yeast effects. For this purpose, larvae were restocked in the same respective tanks (250 larvae $tank^{-1}$) and received dry feed without yeast addition. During this period, the larvae were fed six times daily (08:00, 11:00, 14:00, 17:00, 20:00 and 23:00 h), at a rate of 20% body weight per day (bw d^{-1}).

2.3 Diet composition and proximate analysis

The diet was formulated with purified ingredients to contain 42% of protein and 8.9%lipid (Table 1). The ingredients were mixed with fish oil and warm water was added toproduce stiff dough. The dough was pelleted and dried in an oven at 55 °C for 6 h. The dry pellet was than crushed to a small diameter and stored at -20 °C until used. Diet dry matter (DM) was obtained by keeping the samples at 105°C for 5 h. Ash content was determined after sample incineration at 600°C for 5 h. Lipid content was determined using ether extraction with a Soxhlet extractor. Crude protein content was determined using the Kjeldahl method (N *X* 6.25). All analyses followed the Association of Official Analytical Chemists (AOAC, 1998) standard procedures.

Table 1: Formulation and proximate composition (% dry matter, DM) of experimental diet for pejerrey larvae *Odontesthes argentinensis*

Test ingredients	**(%) DM**
Corn starch	30
Fish meal	5
Casein	36
Gelatin	9
Fish oil	8
Cellulose	11
Vitamin and mineral mix	1
Proximate composition	(% DM)
Crude protein (N*X*6.25)	41.9
Crude lipid	9.6
Ash	2.2

2.4 Live food production

Artemia dry cysts (*Artemia salina* strain Franciscana, INVE® Aquaculture, EUA) were hatched according to the manufacturer's instructions. Nauplii rinsed in salt water and concentrated at a density of 300 larvae L^{-1} were enriched for 24 h with commercial emulsion (S.presso Inve®) alone or combined with Y1 or Y2 (1 g L^{-1}), then filtered and kept in coolers at a temperature of 4 - 9 ºC. Three feeds were offered daily (08:00, 15:00 and 22:00 h). The initial density was 1.0 metanauplii $mL^{-1}$and was gradually increased until 7.0 metanauplii mL^{-1}, ranged according to consumption during the experiment.

2.5 Nucleotide analysis

Artemia, enriched as mentioned above, were collected and washed on a 125 µm mesh. Prior to analysis, samples were freeze-dried. Nucleotides were separated and quantified by reversed-phase HPLC following the description of Werner (1991), and RNA was evaluated by the perchloric acid method (Herbert et al., 1971).Analysis was performed in Laboratory of Biochemistry and Genetics Applied, UFSCAR.

2.6 Sample collection and fixation

At day 0, a sample of 20 larvae was collected,euthanized, weighed and measured for growth analysis and then fixed in Bouin's solution for twohours and subsequently transferred to 70% ethanol for histological analysis. At 10, 20 and 30 dph, 10larvae $tank^{-1}$ were weighed and measured; five of these larvae were fixed in Bouin's solution

for histological analysis, and fivelarvae were fixed in 20% buffered formalin for immunohistochemical analysis.

2.7 Growth measurements

For the wet weight and standard length (SL) determination, 15 larvae samples per tank were collected at 20 and 30 dph. To determine the wet weight, all larvae were euthanized in benzocaine solution (500 ppm). The larvae were measured to the nearest 0.1 mm under a stereoscopic microscope equipped with an ocular micrometer and weighed with a 0.1 mg precision analytical scale (TE214S, Sartorius), after they were blotted dry with absorbing paper. The initial length was equal to 6.78 ± 0.22 mm, and the initial weight was equal to 1.85 ± 0.3 mg.

Survival (S) was calculated as $S = (Nf/Ni) \times 100$, where Nf is the number of larvae at the end of the experiment and Ni is the initial number of larvae.

Specific growth rate (SGR) was calculated as $SGR = [(\ln Wf - \ln Wi)/t] \times 100$, where Wf = final weight (g), Wi = initial weight (g), and t = time (days).

Fulton's condition factor was calculated as $K = Wf/Lf^3$, where Wf is the final weight (g) and Lf is the final length (cm) (Nash et al., 2006).

2.8 Histological and morphometric analysis

Fixed larvae were dehydrated in a graded ethanol series, incorporated with Paraplast (Sigma Aldrich, USA) and cut in longitudinal sections (3 - 5 µm thick). The resultant sections were subsequently stained with hematoxylin and eosin.

Slides were examined under a light microscope (Zeiss Primo Star, Germany) equipped with a digital camera (Zeiss Axiocam ERc5s, Germany). Electronic images were further analyzed using the microscope software AxioVision LE 4.8 (Zeiss) for assessing the dimensions of the thymus, spleen, head kidney and intestinal folds and enterocytes of three fish per tank (12 fish per treatment). The intestinal morphology measurements were performed according to Peng et al. (2013), fold height was measured from the lowest point between two longitudinal folds to the top of the fold (10 measurements per fish). Enterocyte height was measured from the base to the top of the enterocyte (10 measurements per fish). The measurements were performed on the middle and distal intestine. Thymus, head kidney and spleen images were used to manually outline the entire organ to calculate the area. The diameters of the thymus and

spleen were measured according to Romano et al. (1996). Area, diameter and height were manually outlined by the AxioVision LE 4.8 software (Zeiss).

2.9 Immunohistochemistry (IHC)

For IHC, larvae were sampled at 10, 20 and 30 dph (five larvae at each sampling), fixed in 20% buffered formalin, and embedded in Paraplast; sections were stained using the ABC peroxidase method (Vectastain Elite ABC Kit, Canada) as described by Hsu et al. (1981). The sections were incubated with monoclonal anti-CD3 and anti-CD4 antibodies (Sigma®, USA), previously tested for fish in *Xiphophorus helleri* and *Danio rerio* (Romano et al., 2004; Batista et al., 2014). Subsequently, the sections were washed (0.1% diaminobenzidine solution) and dehydrated, and the slides were examined under an optical microscope. The evaluation of CD3 and CD4 receptors was performed by quantitative analysis of the phenotypic percentage by square millimeter (mm^2) of tissue. The expression of these receptors in the thymus, spleen, and head kidney was quantified using Bioscan OPTIMAS 6.1 software according to the method proposed by Weibel (1981) and Romano et al. (1996).

2.10 Salinity stress test

On the 20th dph, 10 larvae from each tank were subjected to a salinity stress test conducted according to the method of Dhert et al. (1992). Larvae from each tank were placed into beakers (1 L) with 60‰ salinity. The 60‰ salinity was prepared with marine salt mix (Coralife, WI, USA). The temperature of the water in the beakers used for the stress test, was maintained at the same temperature water from the tanks in which the larvae were during the experiment (23ºC).

Larvae were monitored every five minutes to check for mortality (opercular beatingstopped) during one hour. Mortality data were used to calculate the sensitivity index, which indicates the larval level of resistance. The sensitivity to salinity stress was expressed as the cumulative stress index (CSI), which was calculated as the sum of the cumulative mortality observed over the test period. A higher numeric value of the index indicated greater mortality of the larvae, which denoted that they were less able to tolerate the stress of the salinity shock.

2.11 Statistical analysis

All statistical analyses were subjected to a one-way analysis of variance (ANOVA) using the software Statistica 7.0. ANOVA assumptions (normality by

Kolmogorov-Smirnov and variance homogeneity by Levene tests) had been previously tested, and differences among the treatment means were tested by the Newman–Keuls post-hoc test. When the ANOVA assumptions were not satisfied, the differences between groups were tested using the nonparametric Kruskal-Wallis test. The level of significance chosen for all analyses was $p<0.05$.

3 Results

3.1 Enriched Artemia analysis

Table 2 lists the nucleotide profiles in the three treatments. The Y2 treatment presented higher ($p<0.05$) RNA levels than did the control and Y1 treatment. Y1 treatment showed the highest level of total nucleotide. Cytidine monophosphate (CMP) was observed only in the control and Y1 *Artemia* groups. Cytosine (C) was detected only in Y1, and adenosine monophosphate (AMP) was found in the control and Y2 groups. The highest concentration of guanosine monophosphate and inosine monophosphate (GMP + IMP) was observed in Y1, whereas the highest concentration of adenosine (A) was observed in Y2.

Table 2: Nucleotide content (% dry weight) in the control group and in bioencapsulated (Y1 and Y2) *Artemia*.

	Control	Y1	Y2
RNA	9.4 ± 0^{a}	8.9 ± 0.4^{a}	10 ± 0.2^{b}
CMP	0.012 ± 0.001^{b}	0.17 ± 0^{c}	0^{a}
C	0	0.08 ± 0.01	0
GMP + IMP	2.21 ± 0.01^{a}	6.75 ± 0.03^{c}	3.48 ± 0.01^{b}
U	0.27 ± 0.01^{a}	4.08 ± 0.005^{c}	0.66 ± 0.01^{b}
AMP*	0.038 ± 0^{ab}	0^{a}	1.54 ± 0.01^{b}
G*	2.85 ± 0.01^{b}	0.93 ± 0.04^{a}	2.76 ± 0^{ab}
A	0.49 ± 0.01^{b}	0.04 ± 0.001^{a}	0.59 ± 0.01^{c}
Total	5.88 ± 0.0^{a}	12.05 ± 0.1^{c}	9.03 ± 0.01^{b}

All values are the mean values of a duplicate dosage.
Quantification in mg of nucleotide *per* gram of material

CMP cytidine monophosphate, GMP guanosine monophosphate, IMP inosine monophosphate, AMP adenosine monophosphate, GMP + IMP guanosine monophosphate and inosine monophosphate (inosine equals uracil), C cytosine, U uracil, G guanine, A adenosine

[a,b,c] Different superscripts in the same row indicate significant differences among treatments ($p<0.05$).

* differences were tested using the nonparametric Kruskal-Wallis test

3.2 Growth measurements

The weight gain is presented in Figure 1. Larvae fed diets with Y1 and Y2 had significantly ($p<0.05$) higher weights than the control group after 10 dph. After 20 dph (dotted vertical line), the larvae began to receive the dry diet without hydrolyzed yeast. At 20 dph, larvae fed with Y1 exhibited a significantly higher weight than the control group did, whereas no differences between Y1 and Y2 neither between Y2 and control group were observed. At 30 dph, 10days after the last supply of yeast, the Y1 group was significant higher in weight than the control group. The Y1 and Y2 groups were not significantly different, and the Y2 and controls did not differ either.

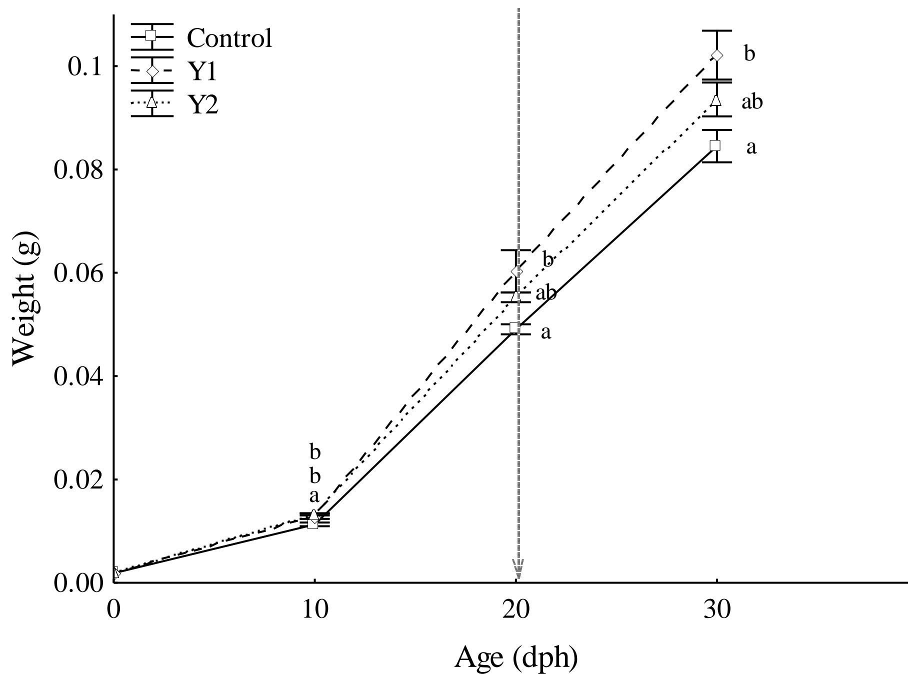


Figure 1. Growth parameters (mean ± SE) of *Odontesthes argentinensis* larvae fed on different live foods (Control, Y1 and Y2). Different letters at the same period indicate significant differences among treatments ($p<0.05$). The dotted vertical line represents

the time of exchanging the larvae diets.

Survival data and growth parameters after 20 and 30 dph are presented in Table 3. The survival and conditional factor was not affected during the entire experimental period. The specific growth rate at the end of 20 dph was higher in the Y1 treatment group than in the control. At 30 dph, 10 days without supplementation with yeast, the result remained, demonstrating a higher specific growth rate in the Y1 group, although no differences were observed between Y1 and Y2 or between Y2 and the controls. After 20 dph, the final weight of the Y1 group was significantly higher than that of the control group. However, neither Y1 and Y2 nor Y2 and the control group differed. This result remained unchanged after 10 days without yeast.

The final length at 20 dph was significantly higher in the Y1 group compared with the control group; however, the Y2 group did not differ from the control and Y1 groups. This result was not sustained after ten days (30 dph) without a yeast supply, when no differences were observed among treatments.

Table 3: Survival (S), Specific Growth Rate (SGR), Final Weight (FW), Final Length (FL) and Condition Factor (CF) of *Odontesthes argentinensis* larvae at 20 and 30 dph.

Parameter	**20 dph**			**30 dph**		
	Control	Y1	Y2	Control	Y1	Y2
S (%)	97.6 ± 3.04	95.9 ± 2.2	95.5 ± 2.01	98.4 ± 0.45	98.6 ± 0.95	98.4 ± 0.98
SGR (%)	16.35± 0.2^{a}	17.31± 0.68^{b}	16.97± 0.18^{ab}	12.6± 0.42^{x}	13.5± 0.23^{y}	13.1± 0.25^{xy}
FW (mg)	49.1± 1.9^{a}	60.3± 8.2^{b}	55.2± 1.9^{ab}	84.5± 6.24^{x}	102.13± 9.5^{y}	93.6± 6.5^{xy}
FL (cm)	2.16± 0.05^{a}	2.28± 0.06^{b}	2.24± 0.04^{ab}	3.2± 0.12	3.36± 0.07	3.31± 0.08
CF (%)	0.5 ± 0.03	0.51 ± 0.04	0.47 ± 0.03	0.26 ± 0.02	0.25 ± 0.01	0.26 ± 0.01

Data are expressed as the mean ± S.D. ^{a,b,x,y} Different superscript letters in the same row indicate significant differences among treatments ($p<0.05$), distinctly for 20, identified with ^{a,b} and 30 dph, identified with ^{x,y}.

3.3 Histological and morphometric analysis

The histological analysis of the present study did not reveal any morphological abnormalities in the analyzed organs. In this experiment, intestine morphometric analyses (Table 4) demonstrated that supplementation with yeast did not increase the middle and distal intestine fold height, at any age (10, 20 and 30 dph). Similarly, no significant difference in enterocyte height was observed at any age, for either the middle or distal intestines.

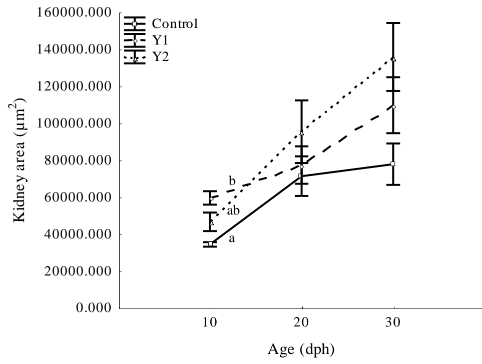
Table 4: Micromorphology of the intestine of pejerrey larvae fed enriched*Artemia* with different nucleotides after 10, 20 and 30 dph. All values are in µm.

Diet		Control	Y1	Y2
Mid- intestine	Dph			
hF	10	83.49 ± 17.7	76.8 ± 12.6	81.39 ± 13.65
	20	106.7 ± 17.4	118.41 ± 11.8	110.93 ± 24.4
	30	142.69 ± 20.99	156.32 ± 18.9	158.08 ± 36.9
hE	10	21.55 ± 1.94	19.43 ± 2.2	20.54 ± 3.3
	20	24.77 ± 3.5	23.44 ± 3.2	25.04 ± 4.1
	30	25.24 ± 2.9	25.8 ± 4.1	26.48 ± 4.6
Distal intestine				
hF	10	89.77 ± 13.7	91.96 ± 10.4	88.3 ± 15.8
	20	121.88 ± 22.9	123.08 ± 18.5	117.87 ± 15.1
	30	150.93 ± 21.6	149.17 ± 20.1	151.39 ± 13.9
hE	10	22.29 ± 3.5	21.07 ± 6.1	20.44 ± 5.5
	20	21.35 ± 2.5	23.59 ± 3.3	22.44 ± 4.3
	30	26.18 ± 6.1	25.29 ± 3.8	27.25 ± 4.2

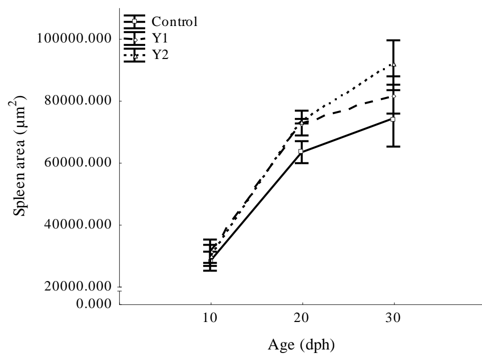
Values are the means of 3 fish from each of 4 replicate groups (10 measurements for each fish). Data are expressed as the mean ± S.D. [a,b] Different superscripts in the same row indicate significant differences among treatments ($p<0.05$).
hF = fold height
hE = enterocyte height.

The histomorphometric analysis of the kidney revealed that, at 10 dph, the Y1 (59436.3 ± 5157.3 μm^2) group presented larvae with a significantly ($p<0.05$) greater area than the control (34287.2 ± 2461.54 μm^2) group. No differences were observed between the control and Y2 groups (46506.4 ± 10050.9 μm^2) or between the Y1 and Y2 groups. At 20 and 30 dph, there was no difference in kidney area (Figure 2a). In addition, the analysis of spleen area and diameter showed no significant difference ($p>0.05$) at any of the observed ages (Figure 2bc).

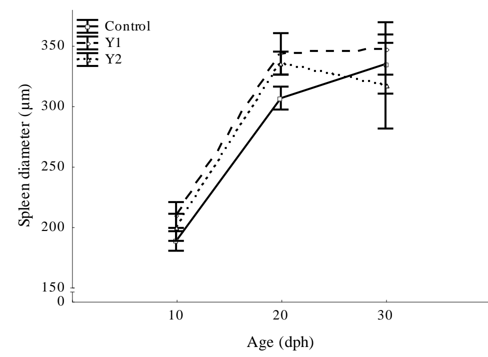
No differences in thymus area at 10 dph were observed. At 20 dph, the control thymus area was significantly smaller (94001.3 ± 8686.6 μm^2) than that for the Y1 (117404.6 ± 10216.9 μm^2) and Y2 (122625.4 ± 12592.84 μm^2) treatments. After 30 dph, when there was no longer yeast supplementation, there was no difference between the thymus areas of the groups. Regarding the thymus diameter, a significant difference was observed at 20 dph, when the control (359.6 ± 20.2 μm) was smaller than Y1 (417.9 ± 25.14 μm) and Y2 (420.15 ± 2.37 μm). No differences were observed at 10 or 30 dph (Figure 2e).



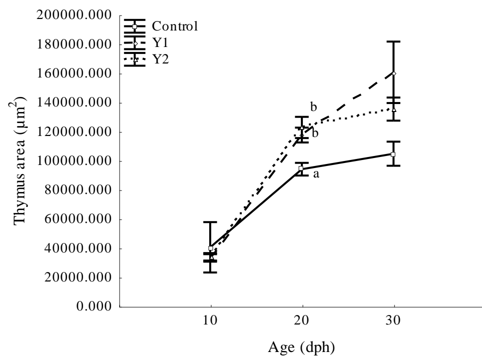
(b)



(c)



(d)



(e)

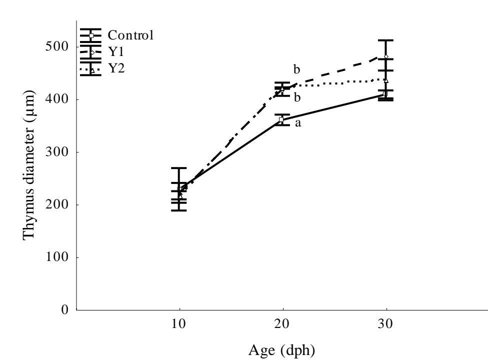


Figure 2. Histomorphometric parameters of pejerrey reared on different live feeds (control, Y1 and Y2), up to 30 days post-hatch (dph). (a) Kidney area (μm^2); (b) Spleen area (μm^2); (c) Spleen diameter (μm); (d) Thymus area (μm^2); (e) Thymus diameter (μm); Data are expressed as the mean ± S.E. Different letters indicate significant differences among treatments ($p<0.05$).

3.4 Immunohistochemistry (IHC)

The primary antibodies anti-CD3 and anti-CD4 showed cross-reactivity in all three treatments. The specific label of CD3 for the kidney revealed significant differences in the percentage of cells labeled with CD3 at 20 dph, where the control presented fewer marked cells (13.9 ± 2.25% CD3/10 mm^2) than did Y1 (20.43 ± 1.9% CD3/10 mm^2) and Y2 (22.63 ± 2.06% CD3/10 mm^2). At 30 dph, there was no longer any difference in labeled cells between the groups (Figure 3a).

Regarding the spleen, only at 10 dph a significant difference in the percentage of cells marked with CD3 was observed, for which the control (4.5 ± 2.1% CD3/10 mm^2) presented fewer cells than did Y2 (7.4 ± 0.5% CD3/10 mm^2), whereas Y1 (6.13 ± 1.3% CD3/10 mm^2) did not differ from the control or from Y2 (Figure 3b).

At 20 dph, the thymus CD3 expression revealed that Y1 (32.33 ± 4.3% CD3/10 mm^2) and Y2 (36.25 ± 5.7% CD3/10 mm^2) were higher than the control (22.95 ± 1.05% CD3/10 mm^2). At 30 dph, Y1 (42 ± 2% CD3/10 mm^2) and Y2 (44.25 ± 4.2% CD3/10 mm^2) continued to have more CD3-labeled cells than the control (34.75 ± 2.98% CD3/10 mm^2) did (Figure 3c).

At 10 dph, the kidney presented significantly more CD4-labeled cells in the Y1 (7.0 ± 2% CD4/10 mm^2) and Y2 (9.33 ± 1.53% CD4/10 mm^2) groups compared with the control group (2.0 ± 0.82% CD4/10 mm^2). However, this difference was not maintained at 20 dph or at 30 dph (Figure 3d).

With respect to cells marked with CD4, no differences were observed in the spleen at any age (Figure 3e).

At 10 dph, the thymus presented significantly more CD4-labeled cells in the Y1 (17.33 ± 4.93% CD4/10 mm^2) and Y2 (14.33 ± 2.08% CD4/10 mm^2) groups compared with the control group (6.0 ± 1% CD4/10 mm^2). At 30 dph, Y1 (46.86 ± 3.18% CD4/10 mm^2) presented more CD4-labeled cells than did the control (35.0 ± 4.24% CD4/10 mm^2) and Y2 (39.67 ± 3.01% CD4/10 mm^2) groups; no difference between the control and Y2 groups was observed (Figure 3f).

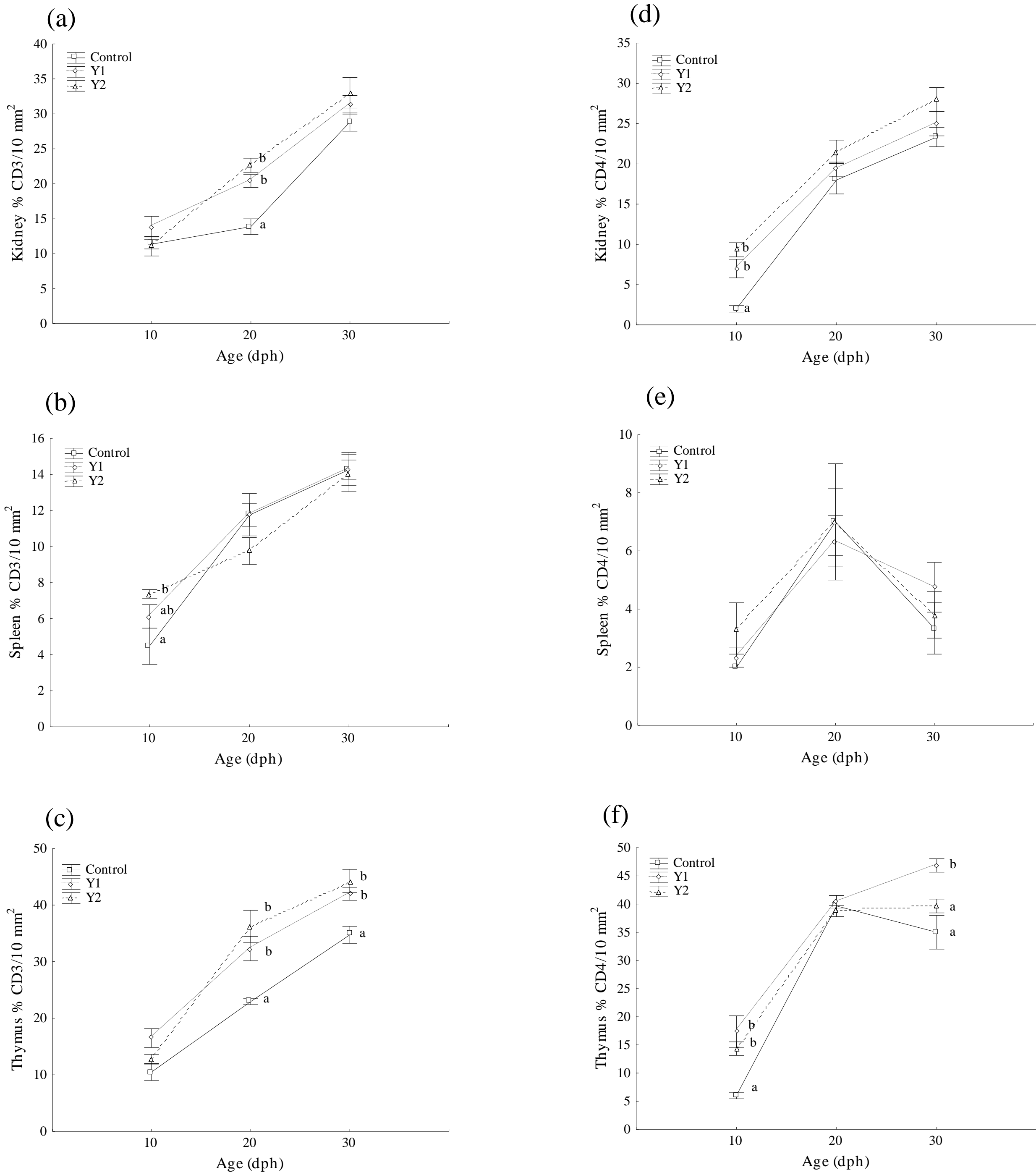


Figure 3. Phenotypic expression of CD3 (a, b, c) and CD4 (d, e, f) receptors in the kidney (a, d), spleen (b, e) and thymus (c, f) of pejerrey reared on different live feeds (control, Y1 and Y2), at 10, 20 and 30 days post-hatch (dph). Data are expressed as the mean ± S.E. Different letters indicate significant differences among treatments ($p<0.05$).

3.5 Salinity stress test

The results of the salinity stress test are displayed in Figure 4, where it can be observed that the cumulative stress index (CSI) was higher for the control group and for treated group Y2, and significantly lower ($p<0.05$) for treated group Y1.

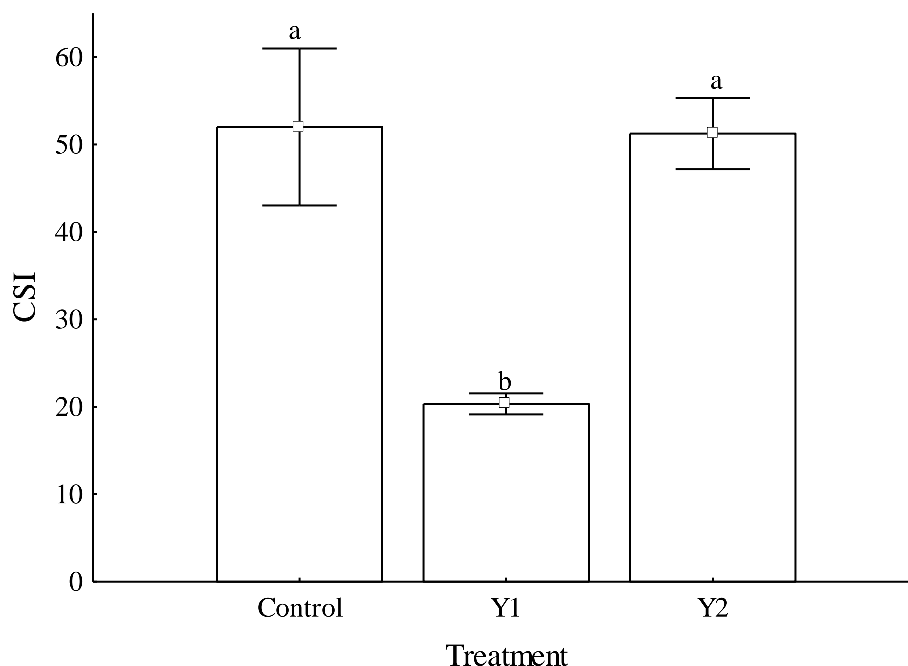


Figure 4. Cumulative Stress Index (CSI) at 20 days after hatching pejerrey larvae fed enriched *Artemia* with different hydrolyzed yeast products. Data are expressed as the mean ± S.E. Different letters indicate significant differences ($p<0.05$).

Discussion

Yeast is widely used in aquaculture feeds; it is mostly used as a protein source but is also used because of the properties of its components (Bekatorou et al., 2006). Previous studies have related increased growth rates, immune response and resistance to disease to yeast compounds consumption, such β-glucans, mannan-oligosaccharides (MOS), chitin, and nucleic acids (Gopalakannan and Arul, 2010). However, one problem of using yeast in the diet is the low digestibility of the yeast cell walls. Digestibility of the yeast can be increased through enzymatic hydrolysis, during which a high volume of nucleic acids is released (Tukmechi and Bandboni, 2014).

The results of this study demonstrate that the enrichment of *Artemia* with hydrolyzed *S. cerevisiae* promotes better growth of *O. argentinensis* larvae. Similarly, Barnes et al. (2006) noted a significant improvement in salmonid growth with the

inclusion of a *S. cerevisiae* based fermented yeast culture in the diet of rainbow trout (*Oncorhynchus mykiss*) during the experimental period. The effect of components derived from yeast, such as nucleotides, was tested by Lanes et al. (2012), whose findings indicate that live food enriched with nucleotides yields rapid growth of Atlantic cod (*Gadus morhua*) larvae.

A better SGR was also observed by Heidarieh et al. (2013) and Sheikhzadeh et al. (2012) in rainbow trout that received fermented yeast. Survival remained greater than 95%, and no significant difference between treatments was observed, similar to the literature (Li et al., 2005).

There are many benefits related to yeasts or their individual components, including improved microbial gut fauna (He et al., 2011; Hoseinifar et al., 2011), an increase in the folds and enterocyte heights (Cheng et al., 2011; Peng et al., 2013; Huang et al., 2015), and an increased number of goblet cells (Heidarieh et al., 2013). Nevertheless, in this study, there were no effects on gut morphology among the treatments. These results are in agreement with those of Heidarieh et al. (2013), who despite finding an increased number of goblet cells, found no significant differences in fold length and thickness between yeast-fed and controlfed rainbow trout.

The immune response in fish is mainly mediated by the following lymphoid organs: head kidney, thymus, spleen and mucosa-associated lymphoid tissue (Press and Evensen, 1999). There are no reports in the literature regarding the influence of fermented yeast on morphometry of the lymphoid organs.

The CD3 complex is a co-receptor that identifies the population of T lymphocytes in fish (Overgard et al., 2009). T lymphocytes are produced, differentiated and matured in the thymus (Patel et al., 2009). The CD4 co-receptor is expressed by a T lymphocyte subclass called helper cells (or T-helper cells). This type of lymphocyte stimulates the secretion of cytokines that produce antibody responses or lead to macrophage activation (Buonocore et al., 2008). Evidence presented here shows that supplementation of a live food for the pejerrey larvae diet with hydrolyzed yeast can exert a consistent effect on lymphocyte production. Immunochemistry confirmed the presence of CD3 and CD4 after 10 dph (first age sampled) in all organs analyzed. Hydrolyzed yeast increased the production of T cells in all organs analyzed, and T–helper lymphocytes in the kidney and thymus, corresponding to an increase of the

humoral immune response and stimulation of T cell differentiation into T helper cells by the kidney and thymus. Similarly, Leonardi et al. (2003) noted an increase in T lymphocytes after the supplementation of a rainbow trout diet with nucleotides.

According to Miceli and Parnes (1993), the increase in the co-receptors of T lymphocytes (CD3 and CD4) can be associated with the speed of development of lymphoid tissue and/or the reactivity of lymphocytes in question.

There are no data in the literature regarding the persistence of yeast effects on the growth of larvae or immune response after the administration is discontinued. In this study, we demonstrated that, 10 days after the last administration of yeast products (30 dph), Y1 still exhibited a difference compared with the control group. Regarding the immune response, the CD3 production in the thymus remained the same, with a higher concentration of CD3 receptors in Y1 and Y2 larvae, as well as a higher concentration of thymus CD4 receptors in Y1 larvae, compared with the control and Y2 groups. This cumulative effect was probably the result of the use of hydrolyzed yeast, as larvae whose diet was supplemented showed better performance than the larvae of the control group, with the former continuing to exhibit a better performance even ten days after the last supplementation.

Natural or artificial stressors associated with routine aquaculture practices have been shown to cause chronically elevated circulating cortisol (Espelid et al., 1996), resulting in immunosuppression, reduced food intake and growth rates and increased vulnerability to disease (Anderson, 1996). Consequently, immunostimulation is indicated mainly in situations that demand high-energy costs, such as metamorphosis, exposure to disease, changes in water quality or vaccination. Stress testing is commonly performed to test the quality of the larvae (Dhert et al., 1992), since the stress has a direct influence on the growth, survival and reproduction.

The product Y2, composed only by *Saccharomyces cerevisiae* strain did not show a positive effect on the stress resistance. These results are in agreement with those of Li et al. (2005) concluded that dietary supplementated diet of juvenile red drum (*Sciaenops ocellatus*) with Brewer's yeast (*Saccharomyces cerevisiae*) did not promote resistance to handling stress. Many studies however, show greater resistance to salt stress (Pooramini et al., 2014) or pathogens challenge (Chiu et al., 2010; Tukmechi and Bandboni, 2014).

In the present study, larvae that fed the product that has two combined strains (*Saccharomyces cerevisiae* and *Candida utilis*) of yeast (Y1) had significant greater resistance to the saline stress. *Candida utilis* (torula) has been widely used in aquaculture feeds mostly as a protein source (Overland et al., 2013), but also for the immunostimulant properties (Abdelkarim et al., 2010; Barman et al., 2013), probably contributing to the better result of salinity stress test in the present study.

Conclusions

Concerning growth parameters, the SGR suggest that the addition of hydrolyzed yeast to the diet benefits marine pejerrey larvae. In addition, there was an increase in lymphocyte production and a higher resistance to stress. There are few studies of live feed enrichment using yeast for larvae immunostimulation, as well as their use in aquatic organisms in general; therefore, this method is promising because it is efficient and not stressful.

Acknowledgements

Fabiane Führ is a graduate student of the Aquaculture Program at FURG and is supported by CAPES. L.A. Romano and M.B. Tesser are research fellows of CNPq.

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CAPÍTULO 3

Evaluation of hydrolyzed yeast as a feed supplement for *Odontesthes argentinensis* juveniles

Artigo submetido para a revista Boletim do Instituto de Pesca.

EVALUATION OF HYDROLYZED YEAST AS A FEED SUPPLEMENT FOR *Odontesthes argentinensis* JUVENILES

AVALIAÇÃO DA LEVEDURA HIDROLISADA COMO SUPLEMENTO PARA A ALIMENTAÇÃO DE JUVENIS DE *Odontesthes argentinensis*

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Abstract

The study was conducted to investigate the effects of hydrolyzed yeast products on growth, intestinal morphology and differential leukocyte count on pejerrey. Juveniles (1.22 ± 0.003 g) were randomly allocated into ninetanks (300 L) at a density of 70 fish per tank. Three diets were tested; a basal control diet (0% yeast), basal diet supplemented with 0.5% hydrolyzed yeast (Y1 and Y2) for 50 days. Results showed that the survival and growth performances were not significantly improved among treatments. Intestinal mucosa morphology analysis indicated that the anterior intestinal villi length in treatment Y1 was significantly increased compared to control ($P<0.05$). Regarding to enterocyte height, no difference was observed. Differential leukocyte parameters were not affected by dietary yeast ($P<0.05$). The results of this study suggest that the inclusion of 0.5% of hydrolyzed yeast in the diet of pejerrey juvenile had no effect on growth or leukocyte count.

Key-Words: Immunostimulant/ growth performance/intestinal villi/ leukocytes

Resumo

O estudo foi realizado para investigar os efeitos de produtos de levedura hidrolisada sobre o crescimento, a morfologia intestinal e contagem diferencial de leucócitos em peixe-rei. Juvenis (1.22 ± 0.003 g) foram distribuídos aleatoriamente em nove tanques (300 L) na densidade de 70 peixes por tanque. Três dietas foram testadas; dieta basal - controle (0% de levedura), dieta basal suplementada com 0.5% de levedura hidrolisada (Y1 e Y2) por 50 dias. Os resultados mostraram que o desempenho zootécnico e sobrevivência não foram afetados pela levedura. Análise da morfologia intestinal indicouaumento da vilosidade do intestino anterior no tratamento Y1 em comparação com o controle ($P<0.05$). Em relação à altura dos enterócitos, não foi observada nenhuma diferença. A contagem diferencial de leucócitos não foi afetada pela levedura na dieta ($P <0,05$). Os resultados deste estudo sugerem que a inclusão de 0,5% de levedura hidrolisado na dieta de juvenis peixe-rei não teve nenhum efeito sobre o crescimento ou a contagem de leucócitos.

Palavras-chave: levedura hidrolisada, desempenho zootécnico, vilosidades intestinais, leucócitos

Introduction

The intensification of aquaculture production tends to generate poor physiological environment and increase susceptibility of fish to infections, which may lead to great losses and decrease production (SAKAI, 1999). In an attempt to prevent losses and minimize the use of antibiotics, whose use might lead to uncontrolled generation of resistant bacteria (NAVARRETE *et al*., 2008; KÜMMERER *et al*., 2009ab), a range of products with immunostimulatory potential has been tested.

Yeast and its derivatives are currently in use in the aquaculture industry and its immunostimulatory potential has been proven for several species (ZHANG *et al*., 2005; NOCEK *et al*., 2011; PRASAD *et al*., 2013; HUANG *et al*., 2015). The advantages of using yeast are the fast production, low cost and can be recycled from other industries (FERREIRA *et al*., 2010).

Among the most commonly used yeast are *Saccharomyces cerevisiae* and *Candida utilis*, whose efficacy has been tested as an alternative protein source (MEDRI *et al*., 2009; OVERLAND *et al*., 2013; GAMBOA-DELGADO*et al.*, 2016), resistance to diseases (CHIU *et al*., 2010; TUKMECHI and BANDBONI, 2014), on stress events (POORAMINI *et al*., 2014; FÜHR *et al.*, 2016) and immune stimulation (ORTUÑO *et al*., 2002; ABDELKARIM *et al*., 2010; BARMAN *et al*., 2013).Studies have confirmed the effectiveness of products derived from yeast such as nucleotides (LANES*et al.*, 2012), β-glucan (GOPALAKANNAN and ARUL, 2010) and mannan oligosaccharides (SALZE *et al*., 2008).

Studies using hydrolyzed yeast have been performed with pejerrey *Odontesthes argentinensis* (Valenciennes 1835) (Teleostei: Atherinopsidae) larvae, and have demonstrated an improvement on growth, immune system and increase resistance to salinity stress test (FÜHR *et al*., 2016). Pejerrey is distributed from São Paulo, Brazil, to the south coast of Argentina (BRIAN and DRYER, 2006) and is an important item in local fisheries, furthermore its introduction has been considered for aquaculture (SAMPAIO, 2006) and for ecotoxicological investigations (RODRIGUES *et al*., 2010; GUSMÃO *et al*., 2012).

The present study was designed to verify the effect of two different dietary

hydrolyzed yeast products on growth, leukocytes quantification and intestinal morphology of *O. argentinensis* juveniles.

Methods

Yeast products

Commercial products used in this study were Augic 15 (identified by Y1) and Hilyses (identified by Y2). Both products are available as a fine powder and are derived from a commercial product from specific strains of *S. cerevisiae* and *C. utilis* (Y1) and only *S. cerevisiae* (Y2).

Fish and experimental conditions

The investigation was previously evaluated and approved by theEthical Committee of Federal University of Rio Grande — FURG (CEUA, protocol number Pq014/2014). Fertilized eggs of *O. argentinensis* were collected at Cassino Beach in Rio Grande, Brazil (32 ° 12 'S - 52 ° 10' W). The eggs were incubated in 300 L tanks, the water temperature was maintained at 23°C and salinity 22‰. Larvae were fed on newly hatched *Artemia* nauplii, according to the protocol of larviculture for this species (SAMPAIO, 2006). The natural feed was gradually replaced by commercial diet at the 20 days post-hatch (dph)(INVE, NRD 500 – 800 µm, EUA), until they reach the desired weight (nearly 100 dph).

Fish (initial weight = 1.22 ± 0.003 g; initial length = 5.57 ± 0.11 cm) were acclimated to a basal diet formulated without yeast (Tabela 1) during one week before start of feeding trial. After acclimation, fish were then randomly allocated in nine 300 L tanks (70 fish per tank). Three independent recirculating aquaculture systems (RAS) were used in this experiment, each comprising three fiberglass cylindrical tanks (300 L each), with continuous aeration. Each RAS consisted a reservoir with biofilters (40 mm bioball, Aquatic Ecosystems Inc., USA), a sand mechanical filter (Sibrape, S-40, Brazil), a skimmer (Plaspiral, Bracket 800, Brazil), a pump (Sibrape, Mod. C63, 1/3 HP, Brazil) and an UVC sterilizer (Sibrape, Pond Clean PC-95 – 95 W, Brazil).

Each day, the tank bottoms were siphoned, and made backwash of the system. Water quality was daily measured. Salinity (30.3 ± 0‰) was measured with a hand refractometer (ATAGO®, S/Mill-E, Japan), temperature (24.2 ± 0.3°C) and dissolved oxygen (6.83 ± 0.05 mg L^{-1})were measured with an oxymeter (YSI®, Model 550A, USA), and pH (7.79 ± 0.07) was measured with a digital pHmeter (FE20 – FiveEasy™,

Switzerland). Ammonia (0.05 ± 0.01 mg L^{-1}) and alkalinity (187.56 ± 3.33 mg L^{-1} of$CaCO_3$) were performed daily according to the methodology described by UNESCO (1983), whereas nitrite (0.004 ± 0.001 mg L^{-1}) was measured by the method of STRICKLAND and PARSONS (1972).

The experiment had three experimental diets: Control – a basal diet without yeast (the same used during the acclimation period), Y1 - basal diet supplemented with 0.5% yeast product (Y1), Y2 - basal diet supplemented with 0.5% yeast product (Y2). Each treatment were carried out with threereplicates. The 0.5% of yeast inclusion was suggested by the manufacturer.

Experimental diets and feeding trial

The feeding trial was conducted during 50 days and fish were fed four times daily (08:30, 11:30, 14:30 and 17:30). All groups of fish were fed their respective diets at the same fixed rate (initially 9% of body weight per day and gradually reduced to 5%).

The diet was formulated with purified ingredients to contain 45% of protein and 11% lipid (Table 1). The ingredients were mixed with fish oil, distilled water at 60°C was added to produce stiff dough. The dough was pelleted in a meat grinder with a die 2mm in diameter and afterwad dried in an oven at 55ºC for 6 h. The dry pellet was than crushed to a small diameter and stored at -20°C until used. Diet dry matter (DM) was obtained by keeping the samples at 105°C for 5 h. Ash content was determined after sample incineration at 600°C for 5 h. Lipid content was determined using ether extraction with a Soxhlet extractor. Crude protein content was determined using the Kjeldahl method (N×6.25). All analyses followed the Association of Official Analytical Chemists (AOAC, 1998) standard procedures.

Table 1 Formulation and proximate composition (% dry matter, DM) of basal experimental diet for pejerrey juveniles *Odontesthes argentinensis*

Test Ingredients		**(%) DM**	
Fish meal		10	
Corn starch		33	
Fish oil		9	
Mineral and Vitamin Premix		1	
Cellulose		2	
Gelatin		9	
Casein		36	
Proximate composition	*Control*	Y1	Y2
Crude protein (N×6.25)	46.5	46.4	47
Crude lipid	13.2	13.3	12.5
Ash	3.5	3.5	3.4

Nucleotide Analyses

Samples of each diet (freeze-dried samples) were submitted to reversed-phase HPLC following the description of WERNER (1991) for separation and quantification of nucleotide and RNA was evaluated by the perchloric acid method (HERBERT *et al.*, 1971).

Growth performance and biometric parameters

To determine the wet weight at the end of the experiment, the fish were euthanized in benzocaine solution (500 ppm). Fish weremeasured, and weighed with a 0.1 mg precision analytical scale (TE214S, Sartorius), after they were blotted dry with absorbing paper.

Survival (S) was calculated as $S = (Nf/Ni) \times 100$, where Nf is the number of fish at the end of the experiment and Ni is the initial number of fish.

Specific growth rate (SGR) was calculated as $SGR = [(\ln Wf - \ln Wi)/t] \times 100$, where Wf = final weight (g), Wi = initial weight (g), and t = time (days).

Fulton's condition factor was calculated as $K = Wf/Lf^3$, where Wf is the final weight (g) and Lf is the final length (cm) (NASH *et al.*, 2006).

Sample collection, fixation and processing

At 0, 20 and 50 days, a sample of five fish from each tank was collected, euthanized with benzocaine hydrochloride solution (500ppm) and blood was collected from the caudal vasculature,through a cut in the tail, for blood smears. At the end of the experiment the viscera were fixed in buffered formalin 20%. Twenty-four hours before sampling, fish were submitted to starvation, as a guaranty that the digestive tract was empty for histological analyzes of the gut. Initial samples were taken for blood and intestine analysis.

Leukocytes quantification

Blood smears, collected at days 0, 20 and 50, were fixed and stained with 20% May Grunwald-Giemsa. Differential leukocyte counts were determined using blood smears under a light microscope. For differential counts were considered as agranulocytic cells, lymphocytes and monocytes, and as granulocytic cells, neutrophils and other granulocytes were grouped into one group called granulocyte. A minimum of 100 cells per slide was counted.

Histological and morphometric analysis

Fixed sample of intestine was dehydrated in a graded ethanol series, incorporated with regular Paraplast (Sigma Aldrich, USA) and cut in transversal sections (3 - 5 µm thick). The resultant sections were subsequently stained with hematoxylin and eosin.

Slides were examined under a light microscope (Zeiss Primo Star, Germany) equipped with a digital camera (Zeiss Axiocam ERc5s, Germany). Electronic images were further analyzed using the microscope software AxioVision LE 4.8 (Zeiss) for assessing the dimensions of the intestinal folds and enterocytes of two fish per tank (six fish per treatment) at each sample day. The intestinal morphology measurements were performed according to PENG *et al*. (2013), fold height was measured from the lowest point between two longitudinal folds to the top of the fold (10 measurements per fish). Enterocyte height was measured from the base to the top of the enterocyte (10 measurements per fish). The measurements were performed on the anterior, middle and distal intestine. The villi height was manually outlined by the AxioVision LE 4.8 software (Zeiss). In all cases, 60measurements per variable in each treatment were made for each sample point. At the beginning of the experiment a sample of five fish was

used to determine the initial measures of the villi and enterocytes.

Statistical analysis

Statistical analyses were subjected to a one-way analysis of variance (ANOVA) using the software Statistica 7.0. ANOVA assumptions (normality by Kolmogorov-Smirnov and variance homogeneity by Levene tests) had been previously tested, and differences among the treatment means were tested by the Newman–Keuls post-hoc test. When the ANOVA assumptions were not satisfied, the differences between groups were tested using the nonparametric Kruskal-Wallis test. In all cases, the significance level was fixed at 5%.

Results

Nucleotide analysis of diets

Table 2 lists the nucleotide profiles in the three treatments. The Y1 treatment presented higher ($p<0.05$) RNA and adenosine (A) levels than did the control and Y2 treatment. For all the others nucleotides there were no significant differences in the three different diets.

Table 2: Nucleotide content (mg of nucleotide per gram of material) in the control and supplemented (Y1 and Y2) diets.

	Control	Y1	Y2
RNA**	0.41 ± 0.06^{a}	0.58 ± 0^{b}	0.41 ± 0.06^{a}
CMP	0.05 ± 0.004	0.049 ± 0.01	0.053 ± 0.005
C*	0.03 ± 0	0.027 ± 0.003	0.029 ± 0.001
GMP + IMP*	0.48 ± 0.045	0.48 ± 0.02	0.51 ± 0.03
U*	0.003 ± 0.001	0.003 ± 0	0.003 ± 0.001
AMP*	0.057 ± 0.01	0.071 ± 0.004	0.052 ± 0.002
G*	0.04 ± 0.003	0.04 ± 0.005	0.05 ± 0.003
A	0.025 ± 0.004^{a}	0.032 ± 0.001^{b}	0.025 ± 0.001^{a}
Total*	0.685 ± 0.03	0.714 ± 0.01	0.71 ± 0.04

Values are the mean of a duplicate dosage. Data are expressed as the mean ± S.D.

[a,b] Different superscripts in the same row indicate significant differences among treatments ($p<0.05$).

CMP cytidine monophosphate, GMP guanosine monophosphate, IMP inosine

monophosphate, AMP adenosine monophosphate, GMP + IMP guanosine monophosphate and inosine monophosphate (inosine equals uracil), C cytosine, U uracil, G guanine, A adenosine.

* Differences were tested using the nonparametric Kruskal-Wallis test

** Quantification in % of dry weight

Survival and growth parameters

Survival and growth parameters at the end of the experiment are presented in Table 3. Survival, specific growth rate, final weight, final length and conditional factor were not affected during the entire experimental period.

Table 3: Survival (S), Specific Growth Rate (SGR), Final Weight (FW), Final Length (FL) and Condition Factor (CF) of *Odontesthes argentinensis* at 50 days of supplemented diet with yeast products.

Parameter	50days			
	Control	Y1	Y2	P values
S (%)*	100 ± 0	100 ± 0	90.5 ± 11.6	0.215
SGR (%)	1.4 ± 0.06	1.32 ± 0.07	1.26 ± 0.1	0.172
FW (g)	2.46 ± 0.08	2.36 ± 0.08	2.29 ± 0.1	0.171
FL (cm)	7.2 ± 0.1	7.12 ± 0.1	7.11 ± 0.1	0.470
CF (%)	0.66 ± 0.02	0.65 ± 0.01	0.64 ± 0.01	0.255

Data are expressed as the mean ± S.D. [a,b] Different superscript letters in the same row indicate significant differences among treatments ($p<0.05$)

* Differences were tested using the nonparametric Kruskal-Wallis test.

Leukocytes quantification

For differential leukocyte counts there were considered monocyte, lymphocyte, neutrofil, and the remaining granulocyte (See Table 4). There was no significant difference between treatments in any of the analyzed periods (0, 20, and 50 days).

Table 4: Differential leukocyte parameters of pejerrey juveniles fed different hydrolyzed yeast for 0, 20 and 50 days. All values are in %.

		Monocyte	Granulocyte	Lymphocyte	Neutrophil
0		2 ± 0.8	2.5 ± 2.4	95.3 ± 2.4	0.25 ± 0.5
20	Control	3.2 ± 1.1	3.1 ± 3.4	93.5 ± 3.6	0.23 ± 0.3
	Y1	1.8 ± 0.9	2.7 ± 1.6	95.5 ± 2.3	0
	Y2	2.1 ± 0.6	3.1 ± 0.5	94.7 ± 1.3	0.27 ± 0.3
50	Control	5.4 ± 2.4	4.4 ± 3.3	90.8 ± 5.9	0.6 ± 0.2
	Y1	3.0 ± 1.4	1.2 ± 0.2	95.5 ± 1.5	0.3 ± 0.2
	Y2	3 ± 0.3	2.1 ± 0.6	94.6 ± 0.9	0.4 ± 0.2

Data are expressed as the mean ± S.D.

Histological analysis

The histological analysis of the present study did not reveal any morphological abnormalities in the intestine. In this experiment, intestine morphometric analyses (Table 5) demonstrated that supplementation with yeast did not increase the middle and distal intestine fold height, at any age (0, 20 and 50 days). At the end of the experiment, the fold height of the proximal intestine of the Y1 group was significantly higher than control group, but no difference was seen between Y2 and Y1, nor between Y2 and control group.

No significant difference in enterocyte height was observed at any age, for either the anterior, middle or distal intestines (See Table 5).

Table 5: Micromorphology of the intestine of pejerrey juveniles fed hydrolyzed yeast products after 50 days experiment. All values are in μm.

			Control	Y1	Y2
*0 days**	*hF*	Proximal-	251.54 ± 15.1		
		Mid-	184.13 ± 9.5		
		Distal-	148.46 ± 44.8		
	He	Proximal-	21.2 ± 1.9		
		Mid-	21.74 ± 2.4		
		Distal-	16.38 ± 2.5		
50 days	*hF*	Proximal-	289.86 ± 49.1[a]	390.57 ± 47.6[b]	359.66 ± 83.4[ab]
		Mid-	273.18 ± 62	291.9 ± 37.1	256.42 ± 54.7
		Distal-	230.9 ± 50.2	243.08 ± 55.4	199.35 ± 18.1
	He	Proximal-	36.56 ± 5.1	39.51 ± 4.7	37.96 ± 6.5
		Mid-	31.21 ± 6.5	41.48 ± 13	33.22 ± 5.9
		Distal-	25.51 ± 4.5	24.33 ± 5.2	19.59 ± 2.2

Values are the means of 2 fish from each of 3 replicate groups (10measurements for each fish). Data are expressed as the mean ± S.D. [a,b]Different superscripts in the same row indicate significant differences among treatments ($p<0.05$).

* Measurements made with 5 fish, 10 measures per fish for each analyzed portion.

hF= fold height.

hE= enterocyte height.

Discussion

Yeast and its derivatives has been increasingly used in fish and shrimp feeds and it is well documented that can be used as a probiotic (LARA-FLORES *et al*., 2003; CHIU *et al*., 2010), as growth promoter (TEWARY and PATRA, 2011; SHEIKHZADEH *et al*., 2012; HEIDARIEH *et al*., 2013), immunomodulator, leading to an increase of the resistance to stress and pathogens (BISWAS *et al*., 2012; FÜHR *et al*., 2016).

The rupture of the yeast cell wall improves its digestibility and energy values (RUMSEY *et al*., 1991). The hydrolysis process involves autolysis induced by addition of chemicals or external enzymes, without necessarily break-down of the cell wall (BABAYAN *et al*., 1981), thus important constituents such as β-glucans and mannan

oligosaccharides (MOS) are maintained. Furthermore, high concentrations of nucleic acids are also found, particularly RNA (SCHULZ and OSLAGE, 1976).

In the present study, the diet supplementation with hydrolyzed yeast products failed to promote growth of pejerrey juveniles. Similar to that observed in this study, HE *et al*. (2009) found that different levels of supplementation with *S. cerevisiae* (0.125, 0.25, 0.5, 1 and 2 g kg^{-1}) had no influence on growth, feed conversion and survival of hybrid tilapia (*Oreochromis niloticus*♀× *O. aureus*♂). The same can be observed in studies with red drum *Sciaenops ocellatus* (LI *et al*., 2005), common carp *Cyprinus carpio* (HUANG *et al*., 2015) and Nile tilapia *O. niloticus* (PEREDO *et al*., 2015) as well as shrimp *Litopenaeus vannamei* (SCHOLZ *et al*., 1999). This stands in some contrast with published studies involving other species, in which enhanced growth occasionally has been associated with dietary supplementation of yeast products and derivatives (SANG *et al*., 2011; YU *et al*., 2014; SELIM and REDA, 2015). Survival remained greater than 90.5%, and no significant difference between treatments was observed, similarly no differences on survival parameters was seen in the literature (HOSEINIFAR *et al*., 2011). The reasons for the differences between species are not clear yet, but might be due to the differences in cultivation and physiological conditions, and the type of basal ingredients in diets.

Hematologic evaluation can be useful in monitoring the health status of fish, as long as interpretation accounts for intrinsic and extrinsic factors that can influence the appearance of cells and the quantitative values obtained (CLAUSS *et al*., 2008). Leucocyte count is considered an indicator of the health status of fish because of its role in non-specific or innate immunity (ROBERTS, 1978). Hematopoietic cells and lymphocytes have limited capacity for "*de novo*" synthesis of nucleotides, and so under conditions such as stress, endogenous production of nucleotides may impair maturation, activation and proliferation of lymphocytes (GIL, 2002). In theory, supplementing the diet with hydrolyzed yeast, a product known to be rich in nucleotides and nucleosides, could increase leukocyte population, especially lymphocytes. However, in the present study, supplementation with 0.5% level hydrolyzed yeast products had no effects on differential leukocyte counts. These results are supported by the findings of TUKMECHI and BANDBONI (2014), who reported that the hydrolyzed powder of yeast alone had no significant effect on the number of leukocytes of the rainbow trout,

Oncorhynchus mykiss. The same authors justified that availability of the cellular constituent in hydrolyzed powder of yeast alone was not sufficient enough to stimulate the immune system. Leukocyte counts and differential leukocyte counts were neither affected by dietary supplementation with commercial inactive brewer's yeast for belugasturgeon*Huso huso* (HOSEINIFAR *et al*., 2011) or whole cells of *S. cerevisiae* inclusion in the diet for common carp *C. carpio* (GOPALAKANNAN and ARUL, 2010).

The improve of gastrointestinal morphology has been associated with the use of whole cell yeast, fermented yeast or some components isolated from whole cell yeast such as MOS or nucleic acids in fish species (GAO *et al*., 2008; SALZE *et al*., 2008; CHENG *et al*., 2011; HEIDARIEH *et al*., 2013).HALAS and NOCHTA (2012) suggested that is an increase on the production of short chain fatty acids related to the increase of beneficial bacteria, which positively affect the recovery of the epithelium. Generally, greater villus and microvillus length and higher villus and microvillus density indicate more mature epithelia and enhanced absorptive function due to increased absorptive area (GAO *et al*., 2008).

The results of this study demonstrate an increase on the fold height on proximal intestine of fish that received hydrolyzed yeast, particularly Y1, after 50 days of supplementation. Regarding to the mid and distal intestine, it was observed no differences in absorptive area. There was also no significant difference in the size of enterocytes in any portion of the intestine. These results are very similar to those of ANGUIANO *et al*. (2013), which supplemented the red drum *S. ocellatus* juveniles diet with 1% Bio-Mos, prebiotic product derived from the outer cell wall of a selected strain of *S. cerevisiae* yeast. Just as in this study, ANGUIANO *et al*. (2013) also observed that MOS influenced the villi of the proximal intestine. These authors suggested that the differences in the effects that dietary supplementation with yeast products has on gastrointestinal tract morphological features and these differences appeared to be species-specific.

In the same way, HEIDARIEH *et al*. (2013) who tested the same concentration of the present study of Hilyses (Y2), have seen that villus arrangements, length and thickness of villus did not change. In a previous study with pejerrey larvae (FÜHR *et al*., 2016) with the same yeast products, the intestinal morphology was also not affected.

Conclusions

Our results failed to show beneficial influences on pejerrey juveniles, whose diet received an inclusion of 0.5% of hydrolyzed yeast, in most of the parameter analyzed.

Acknowledgements

Fabiane Führ is a graduate student of the Aquaculture Program at FURG and is supported by CAPES. L.A. Romano and M.B. Tesser are research fellows of CNPq. R.V. Rodrigues is supported by CAPES. The authors would like to acknowledge teacher Dr. João Sarkis Yunes (Lab. Cianobactérias e Ficotoxinas – FURG) for the lyophilization of samples, and ICC Brazil, for kindly performed the nucleotide analyses.

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DISCUSSÃO GERAL

A utilização de levedura na aquicultura merece destaque devido ao seu alto valor nutricional, relativo baixo valor de mercado, já que existe a possibilidade de utilização de subprodutos de outras indústrias (de bebidas, por exemplo) (Bekatorou et al., 2006) e sua produção pode ser realizada com fontes de carbono alternativas (Lee e Kim, 2001; Rodríguez et al., 2011).

A levedura serve como um veículo para os nutrientes e aditivos de interesse que podem prover além dos nutrientes essenciais, fatores que contribuam para a manutenção da saúde. Já existem vários produtos no mercado, entre eles levedura autolisada, hidrolisada, fermentada, extrato de levedura, além dos seus componentes isolados (β-glucanos, mananoligossacarídeos, nucleotídeos). Entretanto ainda existem muitas lacunas no que diz respeito ao tempo de suplementação, as concentrações utilizadas para cada espécie e fase da vida.

Rumsey et al. (1991) verificaram que a digestibilidade e disponibilidade dos nutrientes presentes na levedura aumentam em produtos cuja parede celular tenha sido rompida. Com isso, pesquisas envolvendo processos de lise da célula de forma que o produto final seja viável economicamente são necessárias. A levedura autolisada, hidrolisada e o extrato de levedura já foram testados e demonstraram resultados satisfatórios no que diz respeito ao aumento da resposta imune celular e humoral e resistência a patógenos em carpas rohu *Labeo rohita* (Andrews et al., 2011), truta arco-íris *Oncorhynchus mykiss* (Sheikhzadeh et al., 2012) e camarão-branco *Litopenaeus vannamei* (Li et al., 2009) e melhora do desempenho zootécnico (Watanabe et al., 2010; Chaitanawisuti et al., 2011; Berto et al., 2015) em pacu *Piaractus mesopotamicus*, gastrópodes *Babylonia areolata* e tilápias *Oreochromis niloticus*.

A levedura hidrolisada, cuja parede celular é quebrada com adição de fatores químicos e enzimáticos, foi utilizada no presente trabalho, na suplementação da dieta de larvas e juvenis do peixe-rei *Odontesthes argentinensis*. Em ambos os experimentos, utilizou-se dois produtos de levedura hidrolisada, o primeiro, denominado Y1, é constituído de cepas de duas leveduras diferentes *Saccharomycescerevisiae* e *Candida utilis* (Torula) e o segundo, Y2, constituído apenas de *S. cerevisiae*.

No processo de autólise e de hidrólise não há desintegração total da parede celular, o conteúdo intracelular é liberado por poros na parede celular e o restante das

reações de lise ocorre no meio extracelular (Babayan et al., 1981; Kollár et al., 1993). Desta maneira produtos autolisados e hidrolisados contam com os constituintes da parede celular que tem importante função imunoestimulante, entre eles os β-glucanos e mananoligossacarídeos, como foi demonstrado para a truta arco-íris *O. mykiss* (Lauridsen e Buchmann, 2010) e o camarão-branco *L. vannamei* (Zhang et al., 2012).

A levedura hidrolisada também possui elevadas quantidades de nucleotídeos, e a sua suplementação externa pode minimizar o gasto energético da produção via *de novo* pelo organismo, principalmente em situações de estresse, além disso, alguns tecidos tem limitada capacidade de produção de nucleotídeos, entre eles, o hematopoiético (Yamauchi et al., 2002). A utilização dos nucleotídeos na aquicultura é relacionada com melhora do sistema imune (Sakai et al., 2001; Siwicki et al., 2010), maior resistência a doenças (Gopalakannan e Arul, 2010; Gu et al., 2011) e condições de estresse (Leonardi et al., 2003), e ganho de peso (Hossain et al., 2016).

No presente trabalho, a levedura hidrolisada (dada a sua constituição rica em fatores capazes de desencadear resposta imune) quando oferecida às larvas de peixe-rei, mostrou-se satisfatória no que diz respeito às taxas de crescimento e resistência ao estresse salino, especialmente quando receberam Y1. Ambos os produtos (Y1 e Y2) também influenciaram positivamente o sistema imune em larvas, com aumento da área dos órgãos linfoides, rim e timo, além da melhora da defesa imune celular com aumento da população de linfócitos T e linfócitos T helper. Além disso, parâmetros imunes e de crescimento permaneceram mais altos que larvas do grupo controle, mesmo 10 dias após cessada a suplementação.

A utilização de produto constituído de duas cepas de leveduras (Y1, *Saccharomyces cerevisiae* e *Candida utilis*) apresentou melhores resultados, especialmente na resistência das larvas, do que aquele que é constituído por apenas uma cepa (Y2, *S. cerevisiae*). O emprego de *C. utilis* ou Torula na aquicultura como fonte de fatores imunoestimulantes ou alternativa à proteína animal (Brown et al., 1996; Øverland et al., 2013) já foi relatado e pode ter contribuído, junto com *S. cerevisiae*, para os melhores resultados em larvas alimentadas com Y1.

Clifford e Story (1976) verificaram em estudo com ratos que altas concentrações de ácidos nucleicos, especialmente purinas (adenina, guanina), podem levar ao acúmulo de ácido úrico (produto final do metabolismo de purinas) no sangue. Oliva-Teles et al.

(2006) verificaram que a adição de ácidos nucleicos na forma de RNA na dieta de pargo europeu (*Sparus aurata*) aumentaram as concentrações de uréia (um dos produtos da quebra do ácido úrico pela ação da uricase) no plasma. Adicionalmente, Rumsey et al. (1992) verificaram que quando trutas arco-íris foram alimentadas com purinas livres (purificadas), a adenina se mostrou ser um potente inibidor de consumo de ração e de crescimento. Apesar de não terem sido examinadas, taxas de consumo de alimento ou os níveis de ácido úrico plasmático nos presentes experimentos, o produto Y2 apresentou níveis de adenina e de RNA (vide tabela 2, artigo 2) mais altos que Y1, o que poderia ter prejudicado os resultados de resistência das larvas.

Os efeitos positivos observados em larvas de peixe-rei não foram observados em juvenis, onde 0,5% de inclusão dos produtos Y1 e Y2 aparentemente não foi suficiente para afetar o crescimento ou a resposta imune. Controverso ao que muitos estudos demonstram, He et al. (2009) verificaram que diferentes taxas de inclusãode *S. cerevisiae* (0,125, 0,25, 0,5, 1 e 2 g kg^{-1}) não tiveram influência no crescimento, conversão alimentar e sobrevivência de tilápia hibrida (*O. niloticus*♀× *O. aureus*♂). Outros estudos também encontraram resultados semelhantes em carpa *Cyprinus carpio* (Huang et al., 2015), tilápia do Nilo *O. niloticus* (Peredo et al., 2015) e até mesmo no camarão branco*L. vannamei* (Scholz et al., 1999). As diferenças encontradas entre as espécies seguem sem uma explicação concreta, mas sugere-se que as diferenças no ambiente de criação, condições fisiológicas e a qualidade dos ingredientes basais utilizados na dieta possam estar relacionados com a ausência de resultado.

Análises hematológicas são importantes para monitorar o estado de saúde. Os leucócitos têm relação direta com o sistema imune não específico (Roberts, 1978) e assim como as demais células que constituem o tecido hematopoiético, tem uma capacidade limitada de produção de nucleotídeos via síntese*de novo*, especialmente em condições de estresse ou patologias, o que prejudica diretamente a produção e maturação de linfócitos (Gil, 2002). A suplementação da dieta com leveduras ou derivados comumente está relacionada com aumento na população leucocitária (Selvaraj et al., 2005; Andrews et al., 2009), entretanto, não teve influência em juvenis de peixe-rei. Resultados semelhantes foram vistos por Tukmechi e Bandboni (2014) em trutas arco-íris *O. mykiss*, que levantaram a hipótese de que os constituintes celulares disponíveis na levedura hidrolisada (1% de inclusão) não teriam sido o suficiente para

estimular o sistema imune. Entretanto, os estudos de Denji et al. (2015), em trutas arco-íris, de Zhu et al. (2012) com bagre do canal (*I. punctatus*) e de Yaghobi et al. (2014) com panga *Pangasianodon hypophthalmus*, que suplementaram as dietas com diferentes concentrações de mananoligossacarídeos, polissacarídeos de levedura e nucleotídeos, respectivamente, também não encontraram resultados na contagem total de leucócitos.

O maior desenvolvimento da superfície absortiva do intestino, com vilosidades e microvilosidades maiores, aumento no número de enterócitos e de células caliciformes, também é relacionado com leveduras e seus constituintes, principalmente os mananoligossacarídeos (Dimitroglou et al., 2009; 2010; Torrecillas et al., 2013). Ambos os produtos utilizados não apresentaram influência na morfologia intestinal de larvas e tampouco de juvenis de peixe-rei (exceto por um aumento nas vilosidades do intestino proximal). Heidarieh et al. (2013) testaram a mesma concentração da levedura hidrolisada utilizada no tratamento Y2, durante o mesmo período (50 dias) em trutas arco-íris e também não obtiveram resultados significativos. A influência de leveduras e seus derivados na morfologia intestinal parece ser espécie específica, além disso, a concentração de 0,5% levedura Y2 por 50 dias não foi eficaz no aumento da superfície de absorção de peixes como o peixe-rei e a truta arco-íris.

Os resultados do presente estudo indicam que a levedura hidrolisada promove crescimento de larvas e desenvolvimento da resposta imune, aumentando sua resistência ao estresse. Por outro lado, a inclusão de 0,5% de levedura na ração de juvenis, não foi eficiente na promoção do crescimento ou da resposta imune. Em ambos os experimentos não foi observada nenhuma influência na morfologia intestinal.

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CONCLUSÕES

- A levedura e seus derivados têm um grande potencial para utilização na aquícultura, seja pelos produtos já existentes no mercado, que por sua vez ainda precisam ser explorados quanto as concentrações utilizadas para cada espécie e o tempo de exposição, ou pelos produtos que ainda estão sendo estudados e cogitados, em função das necessidades nutricionais e imunológicas.
- O enriquecimento de artêmias com 1g L^{-1} de levedura hidrolisada para oferta às larvas de peixe rei promoveu aumento das taxas de crescimento, e melhora da atividade do sistema imune, com aumento da população linfocitária (linfócitos T e T helper), assim como a persistência destes parâmetros mesmo até 10 dias após a cessação da oferta de leveduras. Além disso, as larvas apresentaram maior resistência ao estresse salino quando receberam levedura hidrolisada constituída de *Saccharomyces cerevisiae* e *Candida utilis*.
- A suplementação da dieta de juvenis de peixe rei com 0,5% de inclusão de levedura hidrolisada pelo período de 50 dias não foi capaz de promover crescimento ou a resposta imune, bem como não teve nenhuma influência sobre a morfologia intestinal.